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CHANGES IN HEXOSAMINE SYNTHESIS IN LIVER AND STOMACH DURING STRESS ULCERATION

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CHANGES IN HEXOSAMINE SYNTHESIS IN LIVER

AND STOMACH DURING STRESS ULCERATION

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CHAPTER I

INTRODUCTION

The question "Why doesn't the stomach digest itself during life?" was first addressed by John Hunter in 1772 (1). Although thousands of investigations have been directed toward solving this mystery in the ensuing two hundred years and many facts have been established, at the present time the question remains essentially unanswered. The difficulty lies in the fact that there is no one simple explanation but rather a multiplicity of interacting and overlapping phenomena. The purpose of this investigation was to examine one small facet of this very wide-reaching question, that is, the possible involvement of changes in hexosamine synthesis in the ulcerogenic process.

A Brief History of Ulcer Investigation

Hunter, in a report to the Royal Society, suggested that an unknown "living principle" when possessed by animals or animal parts enabled these tissues to resist gastric digestion (1). Thus until Cruveilhier (2), in 1829 and Beaumont (3), in 1833, described the occurrence of peptic ulceration in living tissue life itself or some property thereof was thought to be the protective agent. Final

reference to the importance of a "living principle" resulted from Claude Bernard's famous frog experiment of 1856 (4). Bernard placed the leg of a living frog in a dog's stomach and observed that after only forty-five minutes considerable digestion had occurred. However, since his own finger was not damaged by inserting it in the dog's stomach, Bernard hypothesized that digestion of the living stomach wall was prevented by the ability of the mucosal epithelium and mucus to block the absorption of pepsin.

This theory was soon challenged by several investigators who cited the presence of uropepsin in the urine (5, 6). Although the gastric origin of this enzyme has been established, it is most likely secreted directly into the blood, not absorbed from the gastric lumen (7). Support for Bernard's hypothesis was provided by Northrup who demonstrated using earthworm, yeast and ameba that, indeed, pepsin was not absorbed by living, uninjured cells (8).

Because Bernard had not considered the function of acid in his system, Frenzel extended the investigation by suspending a frog in such a manner that one leg was immersed in a 0.2% HCl solution and the other in a 0.2% HCl-pepsin solution (9). When, after one and one-half hours only the leg in the HCl-pepsin solution showed signs of digestion, Frenzel suggested that the HCl-pepsin combination led to the death of the tissue and only after death to digestion. Thus, in a similar manner, living gastric tissue should also be digested.

Matthes agreed with Frenzel as to the importance of acid but maintained that the acid merely injured rather than killed the tissue (10). More recently Dragstedt demonstrated that the concentration of the acid was of critical importance (11).

In contrast to Bernard's emphasis on the importance of the surface epithelium in resisting peptic attack, Hartley, in 1860, insisted that it was the mucus which Bernard had also alluded to that was of major importance (12). He demonstrated that gastric tissue from which the mucus coat had been removed was more susceptible to digestive attack than tissue retaining the mucus coat. Subsequent investigations have shown that gastric mucus inhibits peptic digestion (13), slows the rate of hydrogen ion diffusion (14), and possesses some buffering capacity (15).

Shortly before Bernard's report on the importance of the surface epithelium and mucus in the protection of the stomach tissue, Virchow, a German pathologist, suggested that it was vascular derangement that led to ulceration (16). His two part theory was that reduced blood flow limited the extent of neutralization of gastric acid by blood buffers and that vascular occlusion resulting from spasm, thrombosis or embolism led to local necrosis. Pavy gave support to this theory and questioned the importance of the surface epithelium by demonstrating that acid from a normally secreting stomach would not interfere with the rapid healing of a gastric lesion produced by stripping a portion of the gastric mucosa away from the underlying tissues (17). Like Virchow, Pavy attributed the healing to the buffering capacity of the blood. Unknown to these investigators was the fact that interruption of all but a very small fraction of total gastric blood flow can be performed without resulting in autodigestion of the gastric wall (18, 19). This interruption does, however, significantly decrease acid secretion.

Other early theories of gastric protection include the impor-

tance of vagal balance suggested by Rokitansky (20), the absorption of digestive enzymes into the blood before they have the opportunity to attack the cells (21), and the presence in the mucosa of a pepsin inhibitor (22). Of these only the first, vagal balance, is considered to be of importance today.

A rather novel approach to the question of gastric protection was suggested by Becahmp who felt that, indeed, the gastric wall was constantly being digested but that the damage was not noticeable because of the rapid rate of renewal of gastric mucosal cells (23).

The Definition of Ulcer

One of the most frustrating difficulties encountered by the novice delving into the literature on ulcerogenesis is the total lack of a universally accepted system of definitions of commonly employed terminology. In order to illustrate the extent of the confusion, Ivy, in his book Peptic Ulcer, listed fourteen commonly encountered definitions of the word ulcer (24). To simplify the problem he suggested the following definitions:

> 1. An erosion is a circumscribed mucosal defect which does not penetrate the muscularis mucosae.
> 2. An ulcer is a circumscribed defect which extends through the muscularis mucosae. . .
> 3. A peptic ulcer is a benign, nonspecific ulcer located in those portions of the alimentary tract bathed by gastric juice.

Only the first of these three definitions is strictly adhered to.

In most instances ulceration of the upper gastrointestinal tract is divided into two broad categories, peptic ulceration and stress ulceration. Peptic ulceration refers to chronic or potentially chronic,localized, penetrating lesions which may or may not perforate.

Peptic ulcers may occur in either the stomach or the duodenum. The lesions generally occur spontaneously and the presenting symptom is pain. In some instances peptic ulcers are referred to as acute lesions. This term refers not only to the duration of ulceration but also to the lack of extensive connective tissue infiltration. The causes of peptic ulceration are not known but hypersecretion is frequently observed.

Stress ulceration, on the other hand, generally refers to acute, diffuse, non-penetrating and non-perforating lesions which heal rapidly and without scarring. The precipitating factors involved in stress ulceration are usually immediately obvious and include surgical and traumatic stress or the administration of pharmacological agents with known ulcerogenic potential. The presenting symptom of stress ulceration is usually massive hemorrhage. In some instances hemorrhage may occur without the presence of recognizable lesions. Other terminology sometimes used synonymously with stress ulceration included acute gastric ulceration, acute superficial ulceration, hemorrhagic gastritis, stress erosion, massive upper gastrointestinal hemorrhage, acute mucosal ulceration and diffuse acute or diffuse superficial mucosal ulceration.

The difficulty with definitions results in part from the fact that some pharmacological agents may cause either peptic or stress ulceration. Additionally, some authors separate all lesions thought to be induced pharmacologically into a category all of their own and consider stress ulceration to be only those lesions resulting from surgical or traumatic stress.

Were it known that all types of lesions had similar physiological mechanisms, the confusion over the definitions would be unimportant

but unfortunately that is not the case. In fact, in many instances a specific parameter such as gastric secretion may be increased in one instance and decreased in another by the same agent (25).

Unless specifically noted otherwise, all ulceration referred to in the remainder of this investigation report will be stress ulceration. Furthermore, this term shall be used to mean acute, diffuse, hemorrhagic lesions involving only the mucosal layer of the stomach wall. It will include both restraint- and pharmacologically-induced lesions. The lesions will be limited to the gastric fundic mucosa.

The Etiology of Stress Ulceration

The first report of stress ulceration was probably that of Beaumont in 1833 (3). He described the gastric mucosa of his gastric fistulous patient Alexis St. Martin after the latter had consumed excessive amounts of alcohol. Nine years later Curling reported on four cases of duodenal ulcer following extensive burns (26). Since that time the term Curling ulcer has been used to refer to any acute gastric or duodenal lesions occurring in burn patients. The reported incidence of Curling ulcer varies widely (27, 28). This is probably due both to differences in the classification of patients and the severity of their injuries, and to the fact that the lesions may be asymptomatic. Based on studies using dogs, Freisen attributed Curling ulceration to an initial loss of plasma protein followed by hypovolemia, hemoconcentration, vasoconstriction and shock (29). Chernov *et al.* reported sharply decreased serum vitamin A levels in 28 or 35 severely burned patients (28). In a controlled study using burn, trauma and post-operative pateints, parenteral administration of vitamin A in doses

sufficient to return serum levels to normal decreased the incidence of stress ulceration by 50%.

A high incidence of stress ulceration has also been reported to follow various types of neurosurgery, intracranial lesions and head trauma (30-32). Because reports on lesions of this type were first reviewed by Cushing in 1932, the lesions of this type are referred to as Cushing's ulcers (33). The mortality associated with these lesions is high (27).

Many investigators have produced gastric lesions in experimental animals by stimulation or ablation of various brain structures, particularly the hypothalamus (34-36). Leonard et al. found that low frequency stimulation of the anterior hypothalamus caused increased gastric secretion, increased gastric and mesenteric blood flow and decreased systemic blood pressure (34). These changes were vagally mediated. High frequency stimulation led to inhibition of acid secretion presumably by a spread of stimulation to the posterior hypothalamus. In contrast, stimulation of the posterior hypothalamus did not initiate acid secretion but rather led to inhibition of histamine-stimulated secretion, decreased blood flow in the stomach and increased systemic blood pressure. Gastric lesions were produced by anterior hypothalamic stimulation and posterior hypothalamic ablation. Additionally, although stimulation of both regions increased mucus production, the secretion following posterior stimulation was thick with much visible mucus and high sialic acid content while that following anterior stimulation was scant and thin. These changes in mucus production occurred only in vagally innervated pouches. Lucas et al. also demonstrated a relationship between mucus content and stress ulceration (32). Thirteen of

twenty-five injured patients developed bleeding ulcers. PAS staining (for neutral glycoproteins) was decreased in but not around the lesions.

In addition to CNS related surgery, gastrointestinal bleeding has been reported to follow esophageal, gastric, colonic, cardiac, urologic, orthopedic and peripheral vascular surgery (37). Although vagotomy and pyloroplasty is considered the operation of choice if surgical management becomes necessary (38, 39), it is interesting that several cases of stress ulceration have been reported following esophagectomy when the vagi were obviously sectioned (37, 40).

The mortality of stress ulceration following trauma and surgery is probably most closely related to the condition of the patient and the associated illness (39). Since these patients are bad surgical risks conservative treatment including blood transfusions are normally attempted first.

Considering the well-known ulcerogenic potential of adrenocorticoids, the recently reported treatment of stress ulceration using massive doses of dexamethasone seems paradoxical (41). Proudfoot et al. successfully treated eleven of fourteen stress ulcer patients, all bleeding massively, with intravenously administered dexamethasone. These authors suggested that the beneficial effect of dexamethasone was due to increased splanchnic blood flow and prevention of the release of lytic enzymes from lysosomes. Steroids also have beneficial effects on platelet function.

Atik et al. have also suggested the importance of platelet function in stress ulceration (42). In a study of 27 patients with massive gastric hemorrhage following sepsis, burn, surgery or aspirin, platelet aggregation and adhesiveness were significantly reduced.

Although these changes in platelet function would not of themselves cause bleeding, they would contribute to the magnitude and persistence of the bleeding.

A number of pharmacological agents have been shown to cause stress ulceration when administered in therapeutic doses. These include vasopressin, norepinephrine (43), histamine (44), phenylbutazone (45), cortisone (46), reserpine (47), and salicylates (48). Alcohol has also been associated with stress ulceration (49).

The most common drug-induced lesion seen in humans is that caused by aspirin or one of the many salicylate-containing compound drugs. The initiation of bleeding in peptic ulceration is also often attributed to salicylate consumption. According to Croft et al. 80% of the general population are sensitive to aspirin and lose 2-10 ml of blood per day during oral aspirin consumption in therapeutic doses (50). Ten per cent of the population are not sensitive to the drug and the remaining 10% are highly sensitive, losing in excess of 10 ml of blood per day. A number of other investigations have also shown increased gastric bleeding and the appearance of occult blood in the stool following oral salicylate administration (51-53). Intravenous salicylate apparently does not have this effect (54-55).

The form in which the salicylate compounds are consumed is reported to effect not only the rate of absorption and gastric emptying but also the severity of gastric blood loss and the incidence of stress ulceration. Levy and Hayes concluded that administration of whole aspirin tablets increased the severity of mucosal damage and delayed the rate of drug absorption while a solution of the powdered drug in water caused only minimal damage (56). Sodium salicylate and soluble aspirins

were absorbed more rapidly than aspirin and caused significantly less blood loss (51, 57). Buffered (pH 7.0) aspirin solutions were emptied faster and were absorbed more slowly than unbuffered (pH 2.8) solutions (58). This latter fact can be predicted since aspirin, with a pK_a of 3.5, is 91% nonionized and therefore more fat soluble at pH 2.5, but 91% ionized and thus less available for absorption at pH 4.5 (57).

It has been suggested that in addition to enhancing the absorption of aspirin, acid must be present in order for aspirin to induce stress ulceration. Using denervated pouch dogs Davenport demonstrated that irrigation of the pouches with a solution containing 20 mM aspirin and 100 mM HCl caused gastric mucosal damage (59). Sodium and potassium ions entered the lumen and hydrogen ions back diffused into the mucosa. He hypothesized that as a result of aspirin absorption the mucosa became abnormally permeable to water soluble compounds and ions and that histamine was liberated which in turn increased capillary permeability. Thereafter plasma proteins leaked from the interstitial spaces and capillaries and presumably pepsin entered the mucosa. As a final event, hemorrhage occurred. In contrast, if the aspirin was administered in a neutral solution ion fluxes were not altered and bleeding did not occur. A solution containing aspirin and 1 or 10 mmoles HCl altered ionic fluxes but did not cause bleeding (60).

Support for this hypothesis was provided by Johnson who demonstrated that salicylic acid plus 100 mM HCl or acetic acid increased gastric juice histamine content and decreased tissue histamine content in pyloric-ligated rats (61). High acid concentrations have also been shown to increase pepsin secretion in denervated pouch dogs (62). Furthermore, vagotomy and anticholinergics which reduce acid secretion

also reduce the damaging effects of aspirin (63-64). However, five times as much atropine sulfate is required to cause a 50% decrease in ulceration as is needed to cause a 50% decrease in acid output.

Reduced acid secretion commonly seen after aspirin administration could be due to increased acid back-diffusion. However, using a glycine infusion to trap spontaneously secreted HCl Brodie et al. demonstrated that the decreased secretion was not due to hydrogen ion back diffusion (64). Additionally, Narumi et al. effectively blocked aspirin-induced ulceration with thiocyanate despite an increase in acid back-diffusion when both drugs were administered simultaneously. Thiocyanate alone caused acid back-diffusion but not stress ulceration (65). Aspirin has been used to induce ulceration in the jejunum and ileum where there is no acid available for back-diffusion (66).

In addition to acid and histamine effects of aspirin, this drug has been shown to alter the gastric mucosa in several other ways. Geall et al. observed a rapid decrease in mucosal potential difference in men following aspirin administration (67). This decrease, which is indicative of mucosal damage, continued for at least one hour. Johansson et al. demonstrated a decrease in PAS staining (for glycoproteins) of gastric mucosa and an initial increase and subsequent decrease in mucus content of rat gastric juice following aspirin administration (68, 69). The mucus secreted during the period of low mucus output had a low sialic acid content and an increased free acid concentration. This could indicate either increased mucus breakdown or defective mucus synthesis. Menguy et al. made similar observations in rats and dogs except that the initial increase in mucus secretion seen by Johansson et al. did not occur (70).

Max et al. demonstrated that aspirin increased cellular exfoliation in denervated pouch dogs but had no effect on cell renewal (71). These authors suggested that the increased rate of cellular exfoliation might have been due to altered mucopolysaccharides leading to decreased cellular adhesion and a reduced mucus lining.

Although ethanol alone is not thought to cause stress ulceration, it does increase the damaging effects of aspirin (72). That ethanol does damage the mucosal integrity is demonstrated by the fact that mucosal potential difference is decreased following ethanol consumption in men (67). The effect of ethanol on acid back-diffusion is undecided. Neither Schneider et al. nor Smith et al. demonstrated increased acid back-diffusion in humans following ethanol administration but both observed increased K^+ flux into the lumen and Smith et al. measured increased Na^+ fluxes into the lumen (73, 74). In contrast, in denervated pouch dogs increased acid back-diffusion was observed (75). Ethanol also decreased PAS staining in the gastric mucosa of these dogs.

The effects of adrenocorticoids on the gastric mucosa are of dual importance. First, these agents are known to cause stress ulceration when administered therapeutically. Second, adrenocorticoids have been implicated in peptic ulceration.

The mechanism of ulcer production during steroid administration is not clear. The role of acid in this process is particularly complex. ACTH, cortisone, hydrocortisone and prednisone have been observed to increase, decrease and have no effect on acid and pepsin secretion in men and experimental animals (76-81). Mucus secretion is, however, consistently decreased in viscosity and uropepsin secretion is increased in both man and animals (76, 78, 81). The complexity of the acid-

pepsin question is increased by the fact that steroid-induced ulcers will heal completely with antacid treatment without discontinuing steroids (25).

The reported effects of the adrenal system on mucus content and secretion also vary. Bremen et al. found that cortisone acetate increased the secretion of glycoproteins by denervated antral pouch dogs (82). Comparing sulfate uptake and PAS staining, Lev et al. found increased mucus content in gastric fistula dogs following prednisolone (83). Dickson et al. saw no reproducible effect on secreted mucus from denervated canine antral pouches following ACTH (84). In contrast, Desbaillets et al. and Menguy et al. found decreased mucus secretion and decreased mucosubstance output in denervated antral pouch dogs following ACTH and cortisone acetate, respectively (85, 86). Menguy et al. also demonstrated decreased PAS staining along the rugal folds in rats following cortisone administration. This is the area of most rapid mucus renewal and many steroid ulcers occur along the tops of these folds. Hutcher et al. found that vitamin A which is necessary for intestinal cell replication, and mucus production reduced the incidence of methylprednisolone acetate-induced ulcers (87).

Despite the variable experimental results a relationship between gastric and adrenal function does apparently exist. Studies of Addison's disease patients often reveal achlorhydria or decreased acid and uropepsin secretion which can be reversed by replacement therapy. The gastric mucosas of these patients also appear to be more sensitive to glucocorticoids than normal (88). Kyle found that Cushing's disease patients show an increased acid response to gruel test meals which can be reversed by adrenalectomy (89).

Foley et al. found that cortisone acetate decreased histamine content and mast cell concentration in rats but increased parietal cell concentration (90). The drop in mast cell concentration, however, was not thought to be causally related to the ulceration. This observation was confirmed in restraint-induced ulceration by Guth (91).

In addition to their ulcerogenic potentials, ACTH and cortisone have also been reported to decrease the rate of healing of ulcers induced by thermocautery (92) and local eugenol application (93). This may be due to the fact that although these drugs decrease the rate of cell exfoliation they also decrease the rate of cellular renewal (71).

One of the most common methods of inducing stress ulceration in experimental animals is immobilization. The advantages of this method include simplicity, rapidity, reproducibility and minimal expense. No surgical procedures or drugs are required. However, those procedures and drugs with known beneficial effects on ulceration in humans also modify the incidence and severity of restraint-induced ulceration. Additionally, only one type of lesion occurs and it is always in the acid-secreting portion of the stomach (94).

Rats are most frequently used in restraint experiments. The incidence of ulceration has been shown to be directly proportional to the volume of the restraining container or the degree of immobilization (95, 96). Combination of immobilization with other stresses, such as low ambient temperature, which may or may not be ulcerogenic in themselves, also increases the incidence of ulceration (97).

The daily activity cycle and the season of the year influence the incidence of ulceration, as do the strain of animal used, the origin of the animals, the conditions of rearing, the sex and the

weight of the animals (98, 99). Seasonal effect vary between laboratories but generally ulceration was more prevalent during the winter months (96, 100). Immobilization during the activity phase of the daily activity cycle increased the incidence of ulceration (98, 99). Rearing animals in individual cages and handling them frequently were protective against ulceration (101, 102). Female rats had a higher incidence of ulceration than male rats and small rats a higher incidence than large animals (96). Sines found that incidence of ulceration could be increased in succeeding generations of rats by selectively breeding ulcer-susceptible animals (103).

As in other forms of stress ulceration, the mechanisms of restraint-induced ulceration are not known.

Menguy found decreased acid secretion in pyloric-ligated rats subjected to immobilization (104). Brodie et al., however, suggested that the pyloric ligation distorted the findings. They found that although volume of secretion decreased, the concentration of the acid increased (105). It has been suggested that the decreased secretion seen by some is due to increased acid back-diffusion. For this reason, Gerety et al. compared the rates of back-diffusion during restraint of pyloric-ligated rats to non-restrained controls (106). Despite the differences in ulceration in control and restrained animals, it was found that the rates of acid back-diffusion were similar. The effect of pyloric-ligation on acid back-diffusion was not considered, however.

Regardless of the changes in acid secretion, histamine has been reported to be involved in restraint ulceration. Levine et al. found a significant increase in histidine decarboxylase activity after two hours of immobilization of rats in a cold environment (107). This change could

be blocked by brocresine which inhibits histidine decarboxylase and increased by aminoguanidine which inhibits diamine oxidase. The former agent decreased the incidence of ulceration while the latter increased it. Animals fed on pyridoxal phosphate (a histidine decarboxylase co-factor) deficient diets had a decreased response to restraint (108).

Vascular changes are thought to be very important to restraint-induced ulceration. Guth et al. has shown that blood accumulates in microvessels in the superficial mucosa after only thirty minutes of immobilization (109-112). Although mast cell degranulation accompanies this vascular change in normal rats, adrenalectomy blocks the mast cell change but has no effect on vascular changes. Adrenalectomy also increases the incidence of restraint ulceration. Vagotomy, on the other hand, has no effect on mast cell degranulation but blocks the vascular changes and the ulceration. Further investigation revealed that although gastric mucosal blood flow did change during restraint, no correlation was found between these changes and the development of ulceration. Guth suggested that the engorgement of the mucosa seen during restraint was related to local microcirculatory adjustment such as perhaps mucosal vein constriction (112).

The adrenal glands and the sympathetic and parasympathetic nervous systems have also been implicated in restraint ulceration. Although gastric norepinephrine concentration was not affected in rats subjected to immobilization the turnover rate was increased and the half-life decreased. Norepinephrine has been shown to inhibit cell renewal (113).

Ozdemir et al. confirmed the fact that adrenalectomy increased

the susceptibility of rats to restraint-induced ulceration while ACTH and cortisone decreased this susceptibility (114). They further demonstrated that serotonin augmented the effect of stress (115). In contrast to Ozdemir et al., Dombro et al. demonstrated no protection against restraint-induced ulceration by the anti-serotonin drug methysergide (116). Bonta also found that cortisone, prednisolone and dexamethasone were beneficial in decreasing restraint-induced ulceration but in contrast to Ozdemir et al.,ACTH and corticosterone were found to be detrimental (117).

Many investigators have demonstrated the protection against restraint-induced ulceration afforded by vagotomy and anticholinergics (118-120). After considering the importance of the vagus in vascular function demonstrated by Guth et al. (110) and the fact that much larger doses of anticholinergics are necessary to block stress hemorrhage than to block gastric ulceration, Brodie et al. suggested that the vagus has separate roles in controlling acid secretion and gastric blood flow and, further, that it is possible to suppress these functions individually (120).

The relationship between restraint ulceration and mucus is fairly consistent. Lambert et al. found that restraint decreased radiosulfate incorporation into the non-dialyzable fraction of sulfate in the gastric mucosa of rats (121, 122). This fraction contains sulfated polysaccharides and glycoproteins. Restraint has also been shown to decrease the hexosamine content of the gastric mucosa (123). Additionally, such anti-ulcer drugs as synthetic aluminum silicate, sodium copper chlorophylline, tyrosine, glutamine and gefarnate

increased hexosamine content (118). Threonine, which has no apparent effect on ulceration also increased hexosamine content.

On a cellular basis, restraint has been shown to decrease the number of gastric mucosal cells entering DNA synthesis and mitosis and to depress epithelial cell proliferation. These changes occurred before the appearance of ulceration (124).

The Mechanisms of Stress Ulceration

As can be seen from the preceding discussion the suggested mechanisms of stress ulceration are almost legion. Most likely, there is no one mechanism of ulceration but, rather, many changes which must occur either simultaneously or sequentially. Furthermore, it may be necessary to block only a small percentage of these changes in order to suppress ulceration. This would not necessarily mean, however, that only those changes which were blocked are necessary for ulceration.

The majority of the suggested mechanisms can be divided into four broad categories: 1) vascular and metabolic alterations, 2) acid-pepsin attack, 3) mucus barrier breakdown, and 4) decreased tissue repair and replacement.

Although vascular and acid-pepsin theories have been examined in some depth, the latter categories have been treated only superficially. For instance, there are many studies which indicate that stress ulceration is accompanied by decreased mucus secretion and tissue content but only a very few which attempt to ascertain how these decreases occur. The remainder to this paper will be directed toward that end. Because glycoproteins are the major structural components of mucus,a discussion of these substances seems in order.

Glycoproteins

Structure

Gastric glycoproteins consist of a protein core with multiple carbohydrate side chains O-glycosidically linked through N-acetyl-galactosamine to the threonine and serine residues.

The carbohydrate side chains may contain galactose, glucosamine, galactosamine, N-acetylgalactosamine, N-acetylglucosamine, sialic acid and fucose. The sialic acids and fucose occupy terminal positions on the chains. The sialic acid residues are negatively charged and as such play an important part in maintaining the structural integrity of the mucus. Increased H^+ secretion and decreased pH lead to increased solubility of the mucus due to reduced negative charge and H^+ bonding (126, 127).

Some gastric glycoproteins of rats and dogs are sulfated but according to Lambert et al. the presence of sulfated glycoproteins in collected mucus secretions is indicative of salivary or esophageal contamination. In contrast, however, Glass et al. states that human gastric mucosa secreted small amounts of sulfated glycoproteins (128).

Robert et al. found that in the rat the gastric antrum contained 1.5 mg, the corpus 0.08 mg, the duodenum 0.72 mg, the transverse colon 1.3 mg and non-mucus secreting tissues 0.12-0.37 mg of hexosamine per mg dry weight of tissue (129).

Occurrence

In addition to the many previous references to mucus content and secretion, several other investigations merit mention. Using dogs with total gastric pouches, Wise et al. found that histamine and pentagastrin

increase mucus secretion and the ratio of carbohydrates to protein in that mucus (130, 131). Since increased H^+ and vagotomy both cause an increased CHO/protein ratio these authors suggest that the vagus directly affects mucus makeup.

In contrast, Ley et al. found that histamine and pentagastrin decreased mucus secretion in denervated pouch dogs while muscaran increased it (132).

Glass et al. found that both non-sulfated and sulfated glycoproteins are present in the highest concentration in mucus collected after an overnight fast in humans (128). They also found that histamine decreased the concentration and output of both types of glycoproteins. They suggested that these decreases were related to the accumulation of glycoproteins in the epithelium of the gastric surface and crypts seen by Gerard et al. following histamine stimulation (133).

Synthesis

Since hexosamines are mandatory components of glycoproteins and the pathway of hexosamine synthesis has been studied extensively in several mammalian tissues, this pathway provides a convenient method for examining changes in glycoprotein synthesis.

The hexosamine synthesis pathway is illustrated in Figure 1. The first enzyme in this pathway is L-glutamine: D-fructose-6-phosphate aminotransferase (E. C. 2.6.1.16), hereafter referred to as glucosamine synthetase.

Glucosamine synthetase was first recognized in Neurospora crassa by Leloir and Cardini in 1953 (134). Three years later Pogell reported the presence of a similar enzyme in rat liver extracts (135).

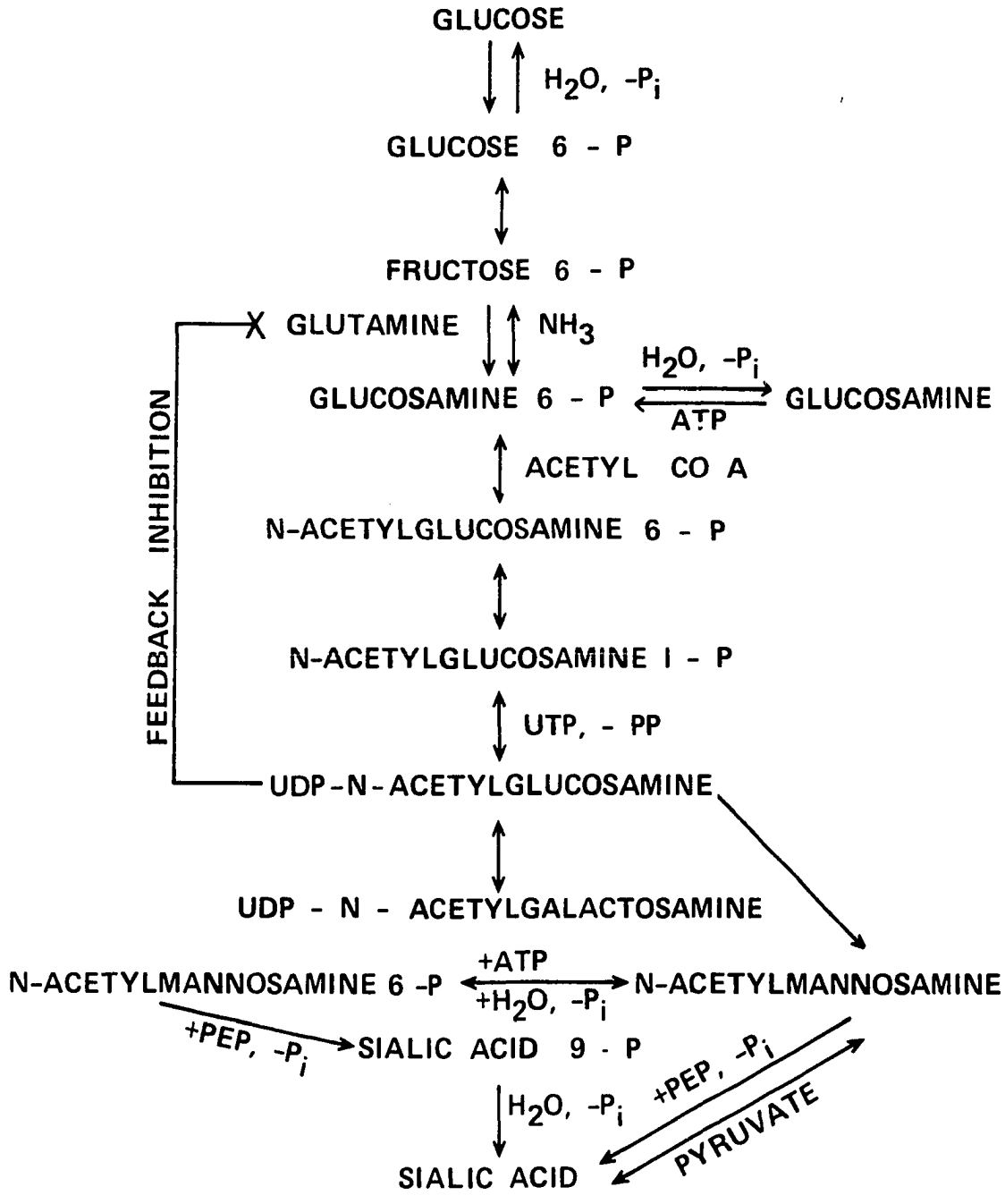


Fig. 1- Pathway of hexosamine synthesis

Pogell et al. determined that this enzyme had an absolute specificity for glutamine but would use either glucose-6-phosphate or fructose-6-phosphate (136). Ghosh eventually established the fact that fructose-6-phosphate was the proper hexose substrate and that a phosphoisomerase in the crude extracts had led to the earlier confusion (137).

Glucosamine synthetase is an irreversible enzyme (137). It is rate-limiting in the hexosamine synthesis pathway (138), and is feedback inhibited by uridine diphospho-N-acetyl-D-glucosamine (UDPAG) both in vitro and in vivo (139).

Winterburn et al. demonstrated that glucose-6-phosphate and AMP potentiated UDPAG inhibition of glucosamine synthetase and UTP behaved as an activator. Furthermore, these substances were found to have an effect only when UDPAG was bound to the enzyme. Glucose-6-phosphate and AMP apparently enhance the binding of UDPAG to the enzyme. ATP inhibits glucosamine synthetase in the absence of the feedback inhibitor (140).

Glucosamine synthetase is located in the cytosol. The liver enzyme has a molecular weight between 360,000 and 400,000 and probably consists of four subunits (141).

Stresses such as laparotomy and partial hepatectomy cause increased glucosamine synthetase specific activities (142). Since this enzyme if normally inhibited by 90%, it would be logical to assume that this increase in specific activity resulted from decreased inhibition (140). However, it was found that the increased enzyme specific activity was due to synthesis of new enzyme (142). Bley et al. suggests that these stresses lead to changes in the concentration of the secondary

effectors resulting in enhanced binding of UDPAG and greater inhibition. Therefore, despite the large capacity for increasing activity by relieving inhibition, new synthesis of enzyme is necessary (143).

A number of studies have been performed indicating a relationship between ulcerogenic potential and hexosamine synthesis. Bollet reported that sodium salicylate inhibited glucosamine synthetase in liver and connective tissue *in vitro*. In contrast, acetylsalicylic acid caused significant inhibition only in connective tissue (144). Perry demonstrated 84% and 61% decreases in enzyme activity in the gastric mucosa of rats following the administration of pharmacological doses of sodium salicylate and phenylbutazone, respectively (145). Zwierz measured the specific activity of this enzyme in ulcerated human gastric tissue obtained during surgical resection but was unable to obtain normal tissues for comparative measurements (146).

The second enzyme in the hexosamine pathway is glucosamine-6-phosphate:N-acetylase (E. C. 2.3.1.4), hereafter referred to as N-acetylase. This enzyme has been studied in yeast (147), sheep brain (148), rabbit liver, kidney, muscle, dog kidney and human liver (149). Kent *et al.* found that this enzyme was inhibited by sodium salicylate *in vitro* in sheep colonic mucosa (150).

Hypothesis

Based on the literature cited, it is our contention that changes in glycoprotein synthesis are causally related to stress ulceration. To demonstrate this relationship we examined the effect of several methods of inducing stress ulceration in rats on two enzymes of the hexosamine synthesis pathway. Since a number of drugs with known ulcerogenic

potential have been observed to alter hexosamine synthesis in tissues other than the gastric mucosa, we suggest that in many instances the changes in hexosamine synthesis are systemic in scope, and, as such can be demonstrated not only in gastric mucosa but also in the liver.

Furthermore, we suggest that changes in the specific activity of the glucosamine synthetase is not mediated by the adrenocorticoids during restraint stress and that changes in UDPAG levels are not responsible for these changes.

CHAPTER II

MATERIALS AND METHODS

Chemicals

Adenosine 5'-triphosphate, disodium salt, Sigma grade, L-glutamine, grade III, D-fructose-6-phosphate, sodium salt, grade I and D-glucose-6-phosphate, disodium salt, hydrate, Sigma grade were obtained from Sigma Chemical Co., St. Louis, Missouri. N-Acetyl-D-glucosamine, A grade, D-glucosamine, HCl, A grade, and coenzyme A, trilithium salt, A grade were purchased from Calbiochem, Los Angeles, California. Sodium salicylate was purchased at Connie's Pharmacy, Oklahoma City, Oklahoma. Atropine sulfate was obtained from Eli Lilly and Co., Indianapolis, Indiana. All other chemicals were reagent grade and were obtained commercially.

Animals

Male rats. 250-300 grams were used in all studies. The animals were housed in cages containing no more than eight rats in the first floor animals facilities of the Hospital Research Building at least one week prior to use. All animals received standard laboratory rat chow and water ad libitum.

Due to circumstances beyond the control of the investigator two different strains of rats, obtained from two different suppliers,were

used. Study I, Group Ia-e and Group II experiments were performed using Stanley-Gumbreck rats (originally derived from the King-Holtzman strain) obtained from The International Foundation For The Study Of Rat Genetics and Rodent Pest Control of Oklahoma City, Oklahoma. Because this strain of rats became unavailable midway through the investigation, all other experiments were performed using Sprague-Dawley rats obtained from the Sprague-Dawley Company., Madison, Wisconsin. The effect of three hours of cold-immobilization (Study I, Group Ia) was repeated using the Sprague-Dawley rats to insure that the two strains of animals reacted similarly.

In order to avoid the possible effects of hourly fluctuations in various hormonal levels on the enzyme specific activities, all stresses were administered at such times that the animals killed immediately after the stress were killed at 12 noon. Those animals killed nine and twenty-one hours after stress were killed at 9 P.M. and 9 A.M. the following day, respectively. Whenever possible, all experiments using a particular agent were performed within several days of one another to avoid possible seasonal variations. Study I involving immobilization required several months.

All animals except those receiving hydrocortisone were fasted 18 hours before the administration of drugs or immobilization. They received water ad libitum except during the time of immobilization or drug action. Control animals were treated similarly. Animals receiving hydrocortisone were fasted only the last 18 hours before death. Those animals which were allowed to recover nine or twenty-one hours after immobilization were maintained without food during this period of recovery. They were allowed free access to water.

Preparation of Crude Enzyme Extract

At the appropriate time after immobilization or drug administration the animals were anesthesized with ether. A ventral midline incision was made, the liver exposed, the portal vein cannulated, the inferior vena cava cut, and the liver perfused with 0.154 M KCl to remove blood. The liver was then removed and placed in the homogenization solution. (See appendix for detailed composition of all solution.) The stomach was removed, opened along the greater curvature and rinsed with cold, running tap water. After visual examination of the mucosa for ulcers, the antral portion of the stomach was cut away and discarded. The stomach was then placed, mucosa up, on a glass plate resting in an ice bath. Two glass microscope slides were used to gently scrape the fundic mucosa free of the underlying serosal tissue. Only the fundic mucosal tissue was used for enzymatic analysis.

Gastric and liver tissues were homogenized separately in 1:2 (w:v) solutions of homogenizing solution containing glucose-6-phosphate to stabilize the glucosamine synthetase. Tissues were homogenized using a motor driven Potter-Elvehjem glass to glass conical homogenizer at 1500 rpm for fifteen seconds with three vertical strokes. The homogenates were then centrifuged for thirty minutes in a refrigerated centrifuge at 37,000 x g. The clear, pink middle layer after centrifugation was withdrawn with a stainless steel needle and syringe and used for enzyme activity and protein measurements. This material will be referred to as the enzyme source.

Procedures of Enzyme Assays

Glucosamine Synthetase

Glucosamine synthetase specific activity was assayed using the method of Kornfeld (151). The incubation media contained 6 mM D-fructose-6-phosphate, 12 mM L-glutamine, 1 mM EDTA and sodium phosphate buffer, pH 7.5, 40 mM for the liver assay and 80 mM for the stomach assay. Total incubation volumes of 1.0 ml for the liver and 0.5 ml for the stomach assays contained 0.5 ml and 0.25 ml of liver and stomach enzyme sources respectively. Liver samples were incubated for one hour and stomach samples for three hours, in a 37°C water bath. Incubation reactions were stopped by boiling samples for one minute.

Glucosamine-6-phosphate, the product formed in the glucosamine synthetase reaction was measured using the Levvy-McAllan modification of the Elson-Morgan reaction (152). After terminating the incubation reaction, the samples were centrifuged for ten minutes at 37,000 x g to remove precipitated protein. Aliquots of 0.6 ml liver and 0.3 ml stomach supernatant were withdrawn for colorimetric determination. Three-tenths ml of water was added to the stomach samples.

One-tenth ml of 1.5% acetic anhydride and 0.5 ml tetraborate buffer solution, pH 9.1, were added to each sample. The tubes were placed in a boiling water bath for three minutes then cooled rapidly under running tap water. Six ml of p-dimethylaminobenzaldehyde reagent were added to each sample and the samples were incubated for twenty minutes in a water bath at 37°C. After cooling to room temperature, optical densities of the samples at 585 mu were read against a blank containing water in place of the supernatant. Product concentrations were determined by comparing the optical densities to a standard curve prepared with glucosamine HCl. All samples were also compared to controls having zero incubation times to eliminate the effect of

endogenous hexosamine intermediates in the homogenate supernatant.

N-Acetylase

This assay was adapted from that of Kent et al. used for sheep colonic mucosal tissue (150). The incubation media for liver samples contained 25 μmoles/ml sodium acetate, 0.12 μmoles/ml CoA, 4 μmoles/ml $MgCl_2$, 1 μmole/ml ATP, and 1.8 μmoles/ml glucosamine-6-phosphate. The incubation media for stomach samples contained 25 umoles/ml sodium acetate, 1 μmole/ml CoA, 4 μmoles/ml $MgCl_2$, 1 μmole/ml ATP, and 1.8 μmoles/ml glucosamine-6-phosphate. Both incubation medias contained 100 μmoles/ml potassium phosphate buffer, pH 7.5, and water necessary to reach final incubation volume. One-tenth ml of gastric enzyme source and 0.06 ml of liver enzyme source per incubation tube were used for a total incubation volume of 0.8 ml in both the stomach and liver assays. Gastric samples were incubated for forty-five minutes and liver samples for ten minutes in a 37°C water bath. The reaction was stopped by boiling for one minute.

Product formation was measured by the Reissig modification of the Morgan-Elson reaction for N-acetylated hexosamines (153). After stopping the incubation reaction, the samples were centrifuged for ten minutes at 37,000 x g to remove precipitated protein. Aliquots of 0.5 ml liver or stomach supernatant were withdrawn for colorimetric measurement. One-tenth ml of tetraborate solution was added to each sample. The samples were placed in a boiling water bath for three minutes then rapidly cooled under running tap water. Three ml of p-dimethylbenzaldehyde reagent were added to each sample and the samples were incubated for twenty minutes in a 37°C water bath. After cooling to room tempera-

ture, optical densities of the samples were read at 585 mu against a blank containing water in place of the tissue sample. Product concentration was determined by comparing the optical densities to a standard curve prepared with N-acetylglucosamine. All samples were also compared to controls having zero incubation times.

Protein

Enzyme specific activities are expressed as nmoles product formed per mg protein per hour. Protein was measured using a Biuret method (154). One-tenth ml of the enzyme source was added to 2.4 ml of 0.9% saline. Two and five-tenths ml of Biuret reagent were added to each sample and optical densities at 540 mu were read exactly 30 minutes later. All optical density measurements were made using a Beckman DB-G Spectrophotometer. Crystalline bovine serum albumin was used as a standard.

Changes in protein concentration following three hours of immobilization were determined by comparing mg protein per gram wet weight tissue in stressed and unstressed animals.

UDP-N-acetylhexosamine (UDPAH) Tissue Levels

UDPAH concentration was determined using the same enzyme source used for enzyme specific activity measurements. Following the modification procedure of Bates et al., one volume of 50% TCA was added to nine volumes of enzyme source. After centrifugation to remove acid-insoluble materials, the supernatant was boiled for ten minutes to release hexosamines from the UDPAH (155). The solution was again centrifuged and an aliquot of the supernatant removed for measurement of hexosamine

content by the Neuhaus and Letzring modification of the Elson-Morgan reaction (156). This method measures all acid-soluble hexosamines but it is assumed, based on the findings of McGarrahan et al. and Molnar et al. that it gives an accurate estimate of the UDPAH pool size (157, 158). These authors found that 90-95% of all acid-soluble hexosamines in the liver are present as UDPAH and the UDPAH pool is an equilibrium mixture of approximately 70% UDP-N-acetylglucosamine and 30% UDP-N-acetylgalactosamine.

Five-tenths of the prepared supernatant, 1.0 ml acetylacetone reagent and 6.5 ml distilled water were placed in screw-top test tubes. The tubes were closed with Teflon-lined caps, thoroughly shaken, and placed in a boiling water bath for 20 minutes. After cooling, 5 ml isoamyl alcohol were added and the tubes again shaken. The layers were separated by low-speed centrifugation and 4 ml of the alcoholic extract transferred to another tube. After adding and thoroughly mixing 1 ml Ehrlich's reagent, the color was allowed to develop for 15 minutes. Optical density at 530 mu was measured against a blank containing water in place of the tissue sample. UDPAH concentrations were determined by comparison of the optical densities to a standard curve prepared with glucosamine HCl. Pool sizes were expressed as nmoles of UDPAH per gram wet weight of tissue.

Determination of the Distribution of Acid-Soluble Hexosamine Intermediates

Four control and four experimental animals were used for this study. Immediately after three hour immobilization stress the control and stressed animals were injected intraperitoneally with 5 μc of glucosamine-

1-C^{14}. The rats were killed by decapitation thirty minutes later. The liver and stomach were removed as rapidly as possible, the fundic mucosa scraped free of the serosa and both tissues homogenized individually in a Waring blender with 5-10 volumes of 50% ethanol. The four liver homogenates and the four stomach homogenates from the control animals were pooled to form a combined liver extract and a combined stomach extract. Material obtained from the stressed animals was similarly pooled. The pooled homogenates were then boiled to denature protein material. Celite (1 gm/20 ml extract) was added to aid filtration and stirred. The homogenates were filtered through Whatman #1 paper using a Buchner funnel under vacuum. The clear extracts were evaporated to dryness under vacuum.

The dried extract was re-suspended in 5 ml of 50% ethanol and applied to a column of Dowex-1-X4-Cl (1 x 24 cm) previously washed alternately with acid and alkali. The column was first eluted with a linear gradient made by mixing 325 ml water with 325 ml 0.04 N HCl. The second elution was with a linear gradient made by mixing 200 ml 0.04 N HCl with an equal amount of 0.4 N HCl. Ten ml fractions were collected at room temperature at a flow rate of 0.7 ml/min. (158).

One ml aliquots of each fraction were mixed with 10 ml of "XDC" scintillation solvent and counted in a Nuclear-Chicago Model 720 counter (159). Data was expressed as counts per minute per fraction x 10^{-3}.

Occurrence of Ulceration

Each mucosa was examined visually for damage and graded according to the extent of involvement of the mucosa in ulceration. The

following four categories were used: 0- normal mucosa, +-reddened areas of mucosa only, ++- pin-point round lesions, +++-elongate lesions less than 2 mm in length, ++++- elongate lesions greater than 3 mm in length. The number of lesions in each category was counted. This grading system refers only to the area of mucosa involved in ulceration, not the severity of the lesions. In no case did the observed lesions penetrate the entire thickness of the stomach wall. In some instances blood was present in the stomach although no lesions were apparent. The scores of animals in a particular group were compared to determine if a relationship existed between the extent of damage and the changes in enzyme specific activity.

Incidence of ulceration was taken to mean the number of rats in each group who had damage of any kind.

Statistics

Data obatined from each group of experimental animals were compared with that from the appropriate controls. The Student's t test was used to determine significance. The 5% probability level was chosen as an indication of significance.

Experimental Design

Study I- Effects of Cold-Immobilization Stress

Although this study is best divided into several sections, the basic immobilization procedure was similar in each instance. All animals were fasted 18 hours before immobilization. On the day of the experiment six to eight rats were placed in individual Fisher plastic holding cages, medium size, and placed in a cold room, 2-4^{o} C. Three hour immobilization period began at 9 A.M. One and one-half hour

immobilization periods began at 10:30 A. M. The cages were placed twelve to fourteen inches apart. An equal number of animals were maintained at room temperature, without food or water, in individual cages large enough to provide complete freedom of movement during the same period of time as the experimental animals were immobilized. These animals were used as controls.

Normal untreated, adrenalectomized, and atropinized rats were used in this study.

Group I- Immobilization of untreated rats. This group was divided into six parts:

(a) Animals were immobilized for three hours and killed immediately after the immobilization period.

(b) Animals were immobilized for three hours and killed nine hours later.

(c) Animals were immobilized for three hours and killed twenty-one hours later.

(d) In order to determine if enzyme activity changed before the appearance of ulceration, animals were immobilized for one and one-half hours and killed immediately after the period of immobilization.

(e) Animals were immobilized for three hours and killed immediately after the period of immobilization. Liver and gastric fundic mucosal tissue levels of UDPAH were determined.

(f) Animals were immobilized for three hours then used to determine the distribution of acid-soluble hexosamine intermediates in liver and gastric tissues.

Group II- Immobilization of adrenalectomized rats. Fasted rats were adreanlectomized bilaterally using a dorsal approach. Ether anesthesia was used. Rats were allowed to recover for three weeks before use. During this time they were maintained on 0.9% saline and normal laboratory rat chow. A second group of rats was sham-operated; a dorsal

incision was made and the adrenal glands were gently moved with forceps; the incision was then closed. These animals were also allowed to recover for three weeks before use.

On the day of the experiment, following an 18 hour fast, half of the adrenalectomized and half of the sham-operated rats were immobilized in a cold environment for one hour. The remaining animals served as unstressed controls.

Group III- Immobilization of atropinized rats. Immediately before the three hour immobilization period the rats were injected subcutaneously with atropine sulfate, 5 mg/kg. Control rats received saline and were also immobilized.

In Study I only glucosamine synthetase specific activity was measured.

Study II- Effects of Hydrocortisone

Hydrocortisone acetate was administered intra-muscularly, 5 mg/day for four days. Control rats received saline injections. Eighteen hours after the last injection the rats were killed and enzyme activity measurements were made.

Study III- Effects of ETOH

Ethanol was administered using a stomach tube, as a 20% (v:v) solution in water, at a dose of 10 mg/kg. Control animals received saline. Rats were killed two hours after ethanol administration.

Study IV- Effects of Sodium Salicylate

Because the pH of the carrier solution has been shown to alter the effect of sodium salicylate, this drug was administered in solutions

buffered at pH 2.5 or 7.5.

Group I- Sodium salicylate, pH 2.5. The drug was administered in a solution containing 25 mg/ml sodium salicylate, citrate buffer, pH 2.5, and 0.1 N HCl at a dose of 10 ml/kg (250 mg/kg). A stomach tube was used. Because salicylic acid precipitates at this pH, the drug was given as a slurry. In order to compensate for possible mechanical damage caused by the drug particles control rats were given a slurry containing Celite, 25 mg/ml, citrate buffer, pH 2.5 and 0.1 N HCl. Animals were killed four hours after drug administration.

In order to determine if changes in enzyme activity occurred before the appearance of ulceration a group of rats were given a one-half strength sodium salicylate solution (12.5 mg/ml) and killed after only two hours.

Group II- Sodium salicylate, pH 7.5. The drug solution which contained 25 mg/ml sodium salicylate in phosphate buffer, pH 7.5, was administered via a stomach tube at a dose of 10 mg/kg (250 mg/kg). Since sodium salicylate is completely soluble at this pH, control rats received only the buffer. The rats were killled four hours after drug administration.

In studies II-IV both glucosamine synthetase and N-acetylase specific activities were measured.

CHAPTER III

RESULTS

Adaptation of Glucosamine Synthetase Assay

Enzyme source concentrations and incubation times were varied using the glucosamine synthetase assay in an attempt to establish that these parameters were linear with product formation under the chosen incubation conditions. Results are shown in Figures 2 and 3. Although three of the curves are approximately linear, the illustrated relationship between product formation and incubation time in the gastric mucosa is not. The depression of product formation seen after three hours of incubation is in accordance with the findings of Perry (145). The lag during the first hour of incubation was not seen by Perry, however. Although we can not explain this lag, it was reproducible.

It was decided to dilute the tissues 1:2 (w:v) for homogenization and to use 0.5 ml liver enzyme source and 0.25 ml gastric enzyme source. A one hour incubation time was chosen in the liver and a three hour incubation time in the stomach. Although a two hour incubation time for gastric tissue, as used by Perry, would have been sufficient in the control animals, the enzyme specific activities were so greatly depressed in the stressed animals that a three hour incubation time was preferrable. Both Figures 2 and 3 illustrate data obtained after pooling tissue homogenates from four animals and diluting as necessary to

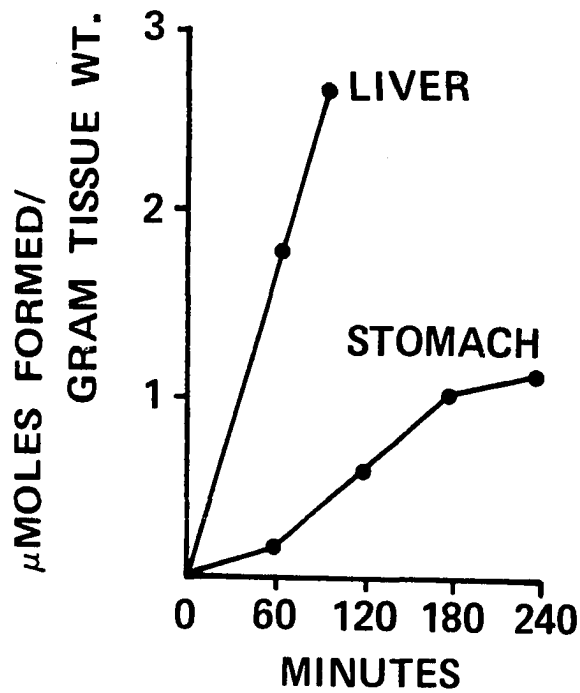


Fig. 2 - Glucosamine synthetase activity vs. incubation time. The standard reaction mixtures were used. Product formed was glucosamine-6-P.

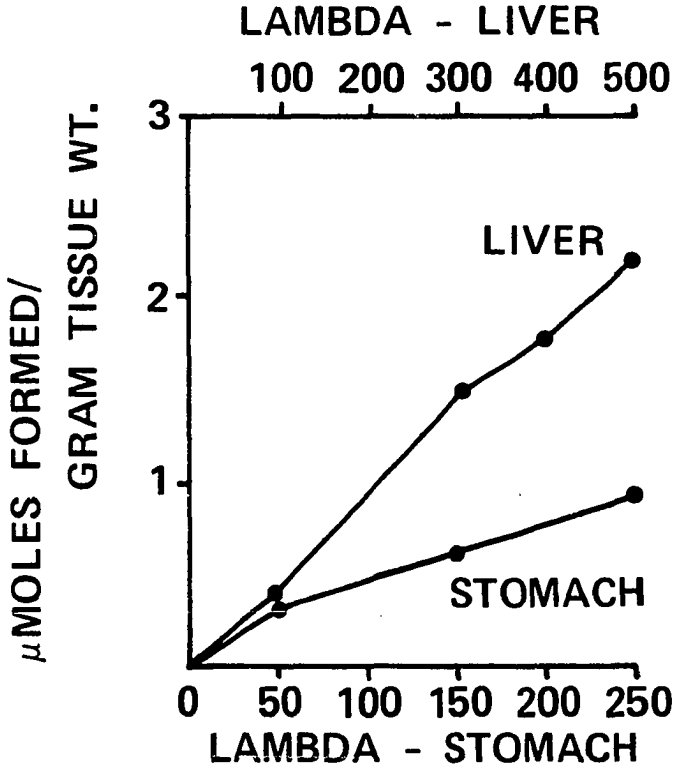


Fig. 3 - Glucosamine synthetase activity vs. volume of enzyme source. Increasing amounts of enzyme source were added to the standard reaction mixture. Homogenizing solution was added to bring reaction mixtures to proper final volumes. Liver samples were incubated one hour, gastric fundic samples three hours. Product formed was glucosamine-6-P.

obtain the various homogenate concentrations.

Adaptation of N-acetylase Assay

The concentrations of the various constituents of the N-acetylase assay were varied individually. Results are shown in Figures 4 and 5. All points on a single curve were determined using a single homogenate in various incubation medias. Optimal concentrations of the various constituents of the liver assay were determined to be 25 μmoles/ml sodium acetate, 0.12 μmoles/ml CoA, 4 μmoles/ml $MgCl_2$, 1 μmole/ml ATP, and 1.8 μmoles/ml glucosamine-6-phosphate. Optimal concentrations in the stomach were the same except that 1 μmole/ml CoA was required. Both incubation medias contained 100 μmoles/ml potassium phosphate buffer, pH 7.5. These concentration are somewhat different than those used by Kent et al. in sheep colonic mucosa (150). Of particular interest is the fact that approximately eight times as much coenzyme A was required in the stomach as in the liver.

The concentrations of enzyme source and incubation times were varied to insure that product formation was linear with these parameters. Results are shown in Figures 6 and 7. Despite repeated attempts linearity was not achieved. Kent et al. was also unable to show linearity with time using this enzyme (150). Concentrations of 0.1 ml gastric enzyme source and 0.06 ml liver enzyme source per 0.8 ml total incubation volume were chosen arbitrarily. Likewise, a forty-five minute incubation period was chosen for gastric tissue and a ten minute incubation time for liver tissue. Both figures illustrate data obtained after pooling tissue homogenates from two animals and diluting as necessary to obtain various homogenate concentrations.

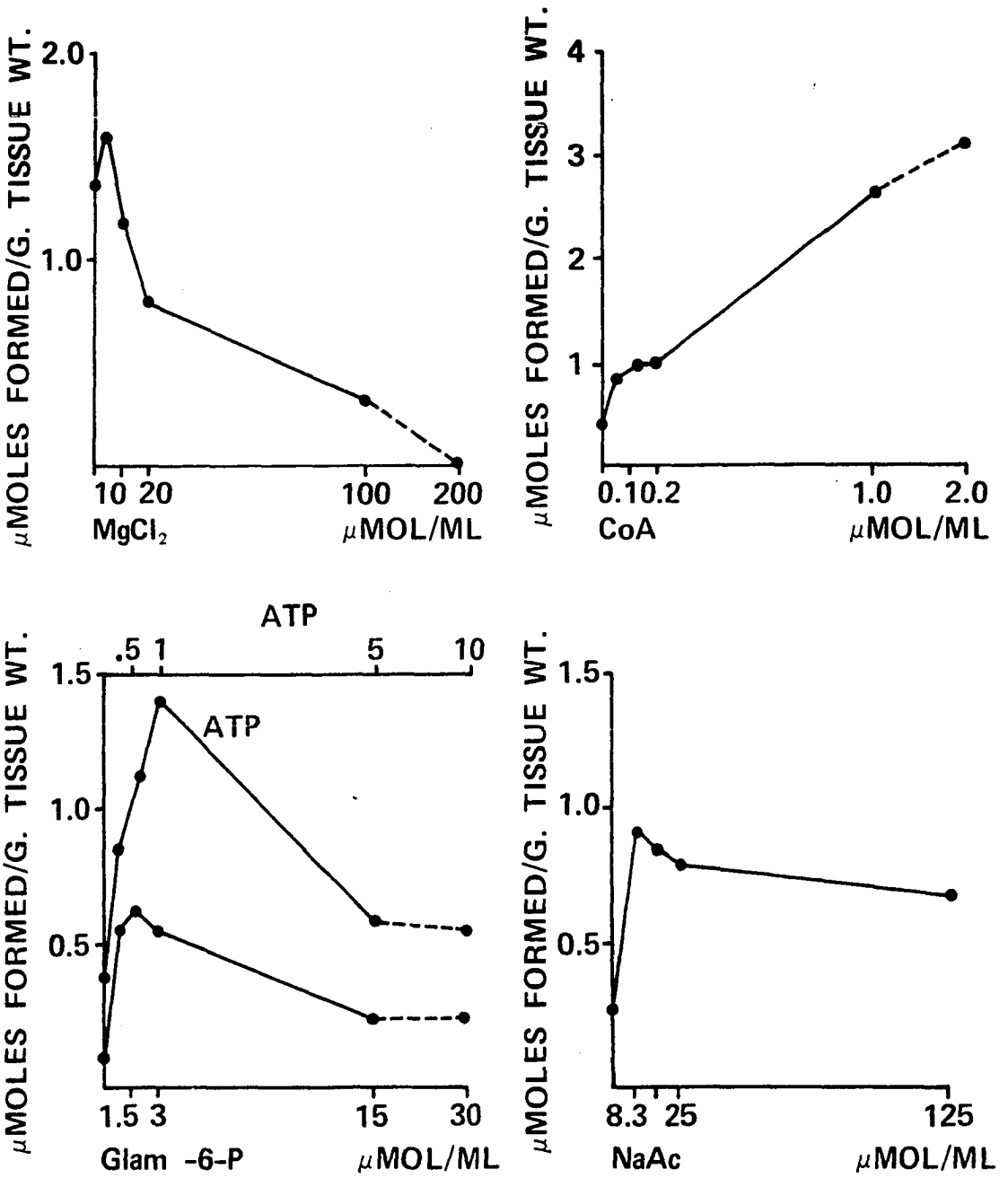


Fig. 4 - Gastric fundic mucosal N-acetylase activity vs. concentration of assay components. Each substance was varied individually in the standard reaction mixture. Product formed was N-acetylglucosamine-6-P.

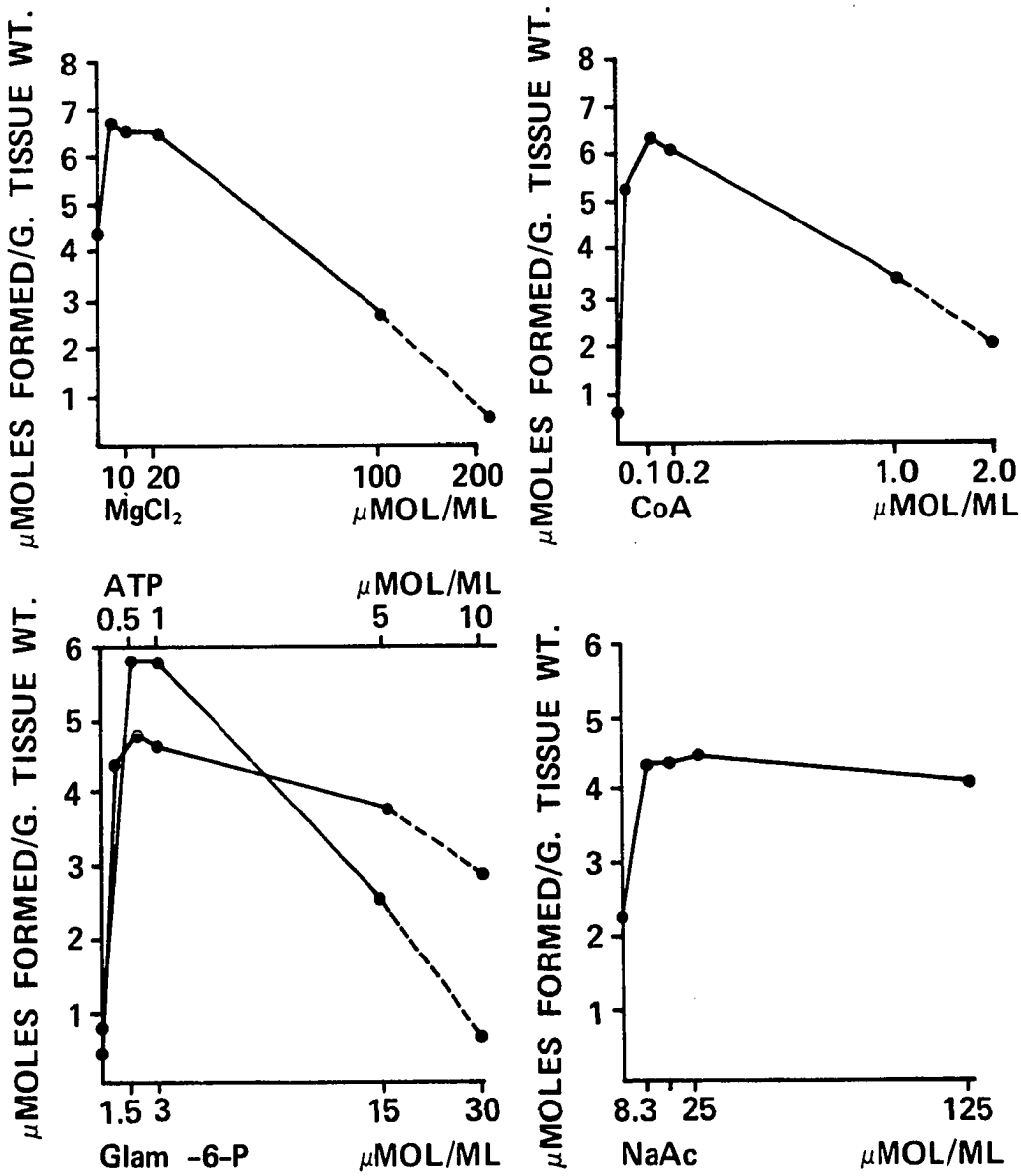


Fig. 5 - Liver N-acetylase activity vs. concentration of assay components. Each substance was varied individually in the standard reaction mixture. Product formed was N-acetylglucosamine-6-P.

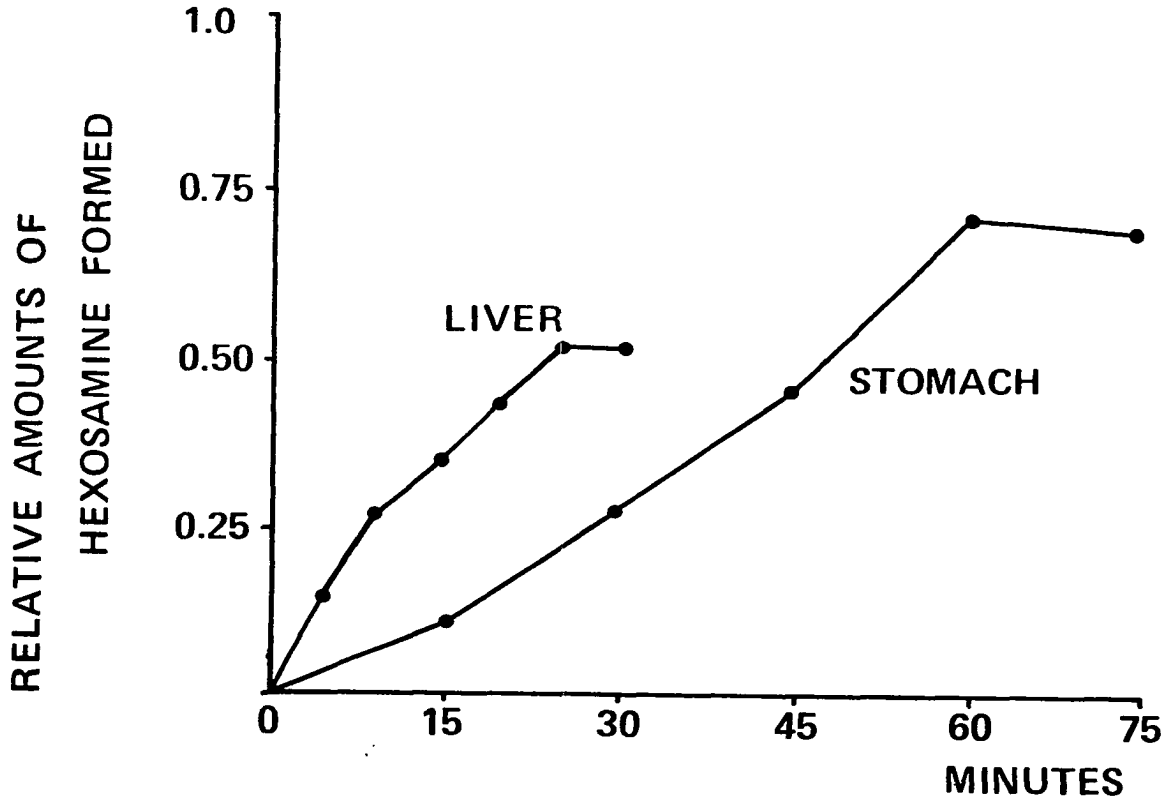


Fig. 6 - N-acetylase activity vs. incubation time. The standard reaction mixtures were used. Product formed was N-acetylglucosamine-6-P.

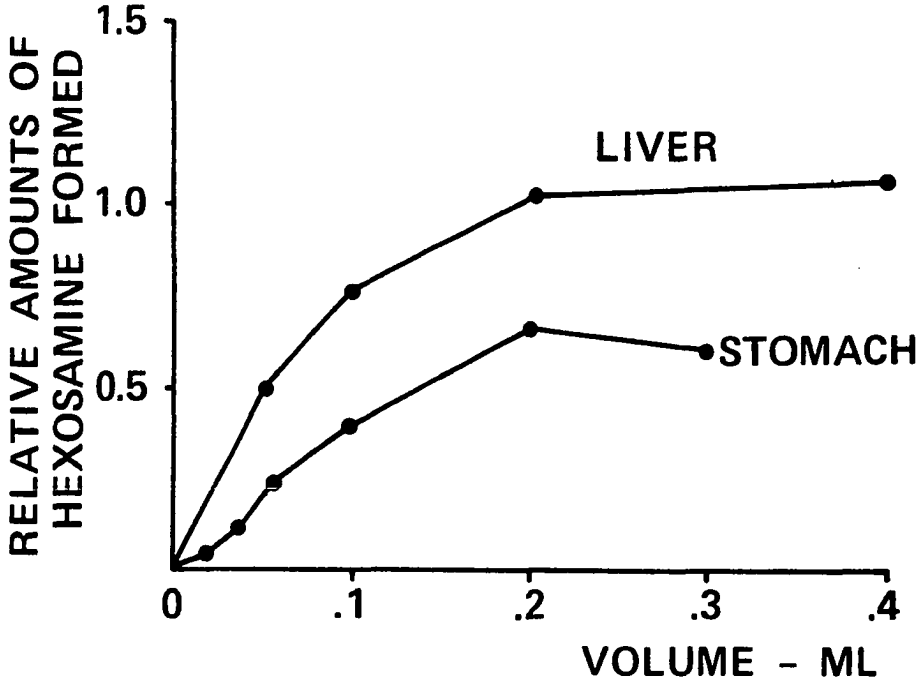


Fig. 7 - N-acetylase activity vs. volume of enzyme source. Increasing amounts of enzyme source were added to the standard reaction mixture. Homogenizing solution was added to bring reaction mixtures to proper final volume. Liver samples were incubated ten minutes, gastric fundic mucosal samples, forty-five minutes.

N-acetylase specific activities varied widely from one day to the next and for this reason data had to be expressed as percentage change from control animals run simultaneously.

Study I- Effects of Cold-Immobilization

Group I- Immobilization of Untreated Rats

The immobilization of rats in a cold environment for three hours resulted in 100% incidence of ulceration. Spontaneous ulceration in control rats occurred in less than 1% of all animals used in this investigation. Although there was no apparent relationship between the extent of ulceration as indicated by the ulcer scoring system and the magnitude of the changes in enzyme activity, there was a drop in glucosamine synthetase activity in the gastric fundic mucosa of all animals with ulceration. There was also a dramatic drop in glucosamine synthetase activity in the liver after three hours of immobilization. These changes are listed in Table 1. Gastric fundic mucosal and liver glucosamine synthetase activities after stress were 70.07±5.89% and 25.17±5.23% of controls, respectively.

Because the data were initially expressed as umoles product formed/mg protein/hr it was important to establish whether protein concentrations changed as a result of immobilization stress. As can be seen in Table 1, the 8% drop in protein concentration seen in the liver was insignificant. The 13% drop in the stomach was significant, but would tend to minimize rather than maximize the observed changes in glucosamine synthetase specific activity.

Although the fluctuations in enzyme activity levels in control and stressed animals did not fluctuate remarkably between animals used

TABLE I

THE EFFECT OF THREE HOUR COLD-IMMOBILIZATION
ON GLUCOSAMINE SYNTHETASE SPECIFIC ACTIVITY

Group	Protein mg/g[a]	% Change	Specific Activity nmoles/mg/hr.[a]	% Change
Liver				
Control n=26	81.7±3.7		15.29±0.92	
Stressed n=31	74.9±3.9		3.85±0.80	
	p> 0.200	-8.32	p< 0.001	-74.83
Stomach				
Control n=21	45.0±2.2		33.75±2.41	
Stressed n=17	39.1±1.2		23.65±1.99	
	p < 0.025	-13.11	p < 0.001	-29.93

a= mean ± s.e.m.

on one particular day, the variations in levels from one day to the next were sometimes quite dramatic. For this reason all data gathered in this investigation have been expressed as the percentage changes from controls run simultaneously with the stressed or drugged animals.

Because of the necessity of switching from the Stanley-Gumbreck strain of rats to the Sprague-Dawley strain, the three hour immobilization study was repeated on a small number of Sprague-Dawley rats. In these animals the glucosamine synthetase specific activity dropped to 26.41±7.13% of controls in the liver and 40.23±9.58% of controls in the stomach. Although these drops are similar to those seen in the Stanley-Gumbreck animals, the enzyme specific activities expressed as nmoles hexosamine produced/mg protein/hr were quite different. As can be seen in Table 2, the control value in the liver tissue of Stanley-Gumbreck animals was 15.29±0.92 nmoles/mg/hr while that in Sprague-Dawley rats was 31.28±3.16 nmoles/mg/hr. In the stomach tissue, glucosamine synthetase specific activity in Stanley-Gumbreck control animals was 33.75±2.41 nmoles/mg/hr while that in Sprague-Dawley animals was 76.43±8.13 nmoles/mg/hr. Two facts must be considered in evaluating these differences between strains of animals. First, the Stanley-Gumbreck animals were used during the winter months, from November, 1972, through January, 1973, when Sprague-Dawley rats were not available. The Sprague-Dawley rats were used during August, 1973, when Stanley-Gumbreck rats were no longer available. It is conceivable that seasonal differences in control levels of this enzyme might exist. However, a second and more likely explanation for the differences related to the preparation of the tissues for enzymatic specific activity measurements.

TABLE 2

THE EFFECT OF THREE HOUR COLD-IMMOBILIZATION STRESS ON GLUCOSAMINE SYNTHETASE SPECIFIC ACTIVITY IN STANLEY-GUMBRECK AND SPRAGUE-DAWLEY RATS

Group	Stanley-Gumbreck Specific Activity nmoles/mg/hr[a]	% of Control	Sprague-Dawley Specific Activity nmoles/mg/hr[a]	% of Control
Liver				
Control	15.29 ± 0.92 n=26		31.28 ± 3.16 n=6	
Stressed	3.85 ± 0.80 n=31		8.26 ± 2.23 n=8	
	$p < 0.001$	25.17	$p < 0.001$	26.41
Stomach				
Control	33.75 ± 2.41 n=21		76.43 ± 8.13 n=8	
Stressed	23.65 ± 1.99 n=17		30.75 ± 7.32 n=8	
	$p < 0.001$	70.07	$p < 0.001$	40.23

a= mean ± s.e.m.

When using the Stanley-Gumbreck rats the tissues were homogenized using a Polytron homogenizer. Although this method seemed to work quite well for a number of months, we were eventually faced with the apparent inactivation of the enzyme during homogenization. For this reason the remainder of the studies were carried out using tissue which had been homogenized using the Potter-Elvehjem technique as described earlier. All glucosamine synthetase control values measured using this method of homogenization approximated those given for the Sprague-Dawley animals. Unfortunately, the change in strain of rats occurred simultaneously with the change in homogenization method and season. Under the circumstances, the changes were unavoidable.

In order to ascertain if changes in glucosamine synthetase specific activity occurred before or after the appearance of gastric lesions, a group of animals was stressed for one and one-half hours in a cold environment. The results of this experiment are shown in Table 3. The incidence of ulceration after this period of stress was 62.5%. However, the drop in glucosamine synthetase activity in the stomach to 21.03±9.84% of controls was greater than after three hours of stress. Of the three animals in this group which did not have ulceration, two exhibited decreases in glucosamine synthetase activity. Since the incidence of ulceration after three hours of stress was 100% one can assume that these animals would have developed visible lesions had the period of stress been extended. In contrast, the drop in enzyme activity in the liver to 51.26±12.32% of controls was somewhat less than that observed after three hours of stress.

In order to investigate the time course of the recovery of the enzyme specific activity to control levels, this activity was measured

TABLE 3

THE EFFECT OF 1½ HOURS COLD-IMMOBILIZATION STRESS ON GLUCOSAMINE SYNTHETASE SPECIFIC ACTIVITY

Group	Specific Activity nmoles/mg/hr		Condition of Gastric Fundic Mucosa
	Liver	Stomach	
Control	10.91	29.04	normal
	9.29	23.43	normal
	8.31	20.39	normal
mean ± s.e.m.	9.50±0.71	24.48±2.58	
Stressed	9.76	20.39	normal
	2.87	1.90	normal
	2.44	0.39	normal
	2.72	2.53	+++2
	9.16	1.69	++6
	8.39	2.01	++1
	1.39	2.31	++1
	2.23	10.14	++1
mean ± s.e.m.	4.87±1.17	5.17±2.39	
	$p < 0.010$	$p < 0.005$	

in animals allowed to recover from a three hour immobilization period for nine and twenty-one hours. In the stomach, recovery of enzyme specific activity was complete after nine hours. Lesions were still visible in the gastric fundic mucosa at this time, as they were at twenty-one hours after stress. In the liver, recovery at nine hours had proceeded to only 72.05±7.73% of the control levels. At twenty-one hours after stress recovery was complete in the liver also.

The effect of stress and recovery on glucosamine synthetase specific activity in untreated rats is shown in Figures 8 and 9.

One possible mechanism of decreasing glucosamine synthetase specific activity would be an increase in UDP-N-acetylglucosamine, a feedback inhibitor of this enzyme. As the data in Table 4 indicate, the tissue concentrations of this substance were not affected in either the liver or the stomach by three hours of immobilization. The tissue concentrations measured in the liver agree well with those found in the literature (160). UDPAH concentrations have not been reported for the stomach previously.

Figures 10, 11, and 12 illustrate the distribution of radioactive intermediates in the livers and gastric fundic mucosas of normal and three hour immobilization stressed rats. Figure 10 shows the distribution obtained when the extract from control livers was divided in half and subjected to duplicate chromatography. The two runs were quite reproducible and agree well with the literature (158).

Figure 11 shows the distribution in the liver after stress. The total radioactivity incorporation was approximately one-third of the normal amount. This was probably due to a lowered metabolic rate. Rats subjected to three hours of immobilization in a cold environment

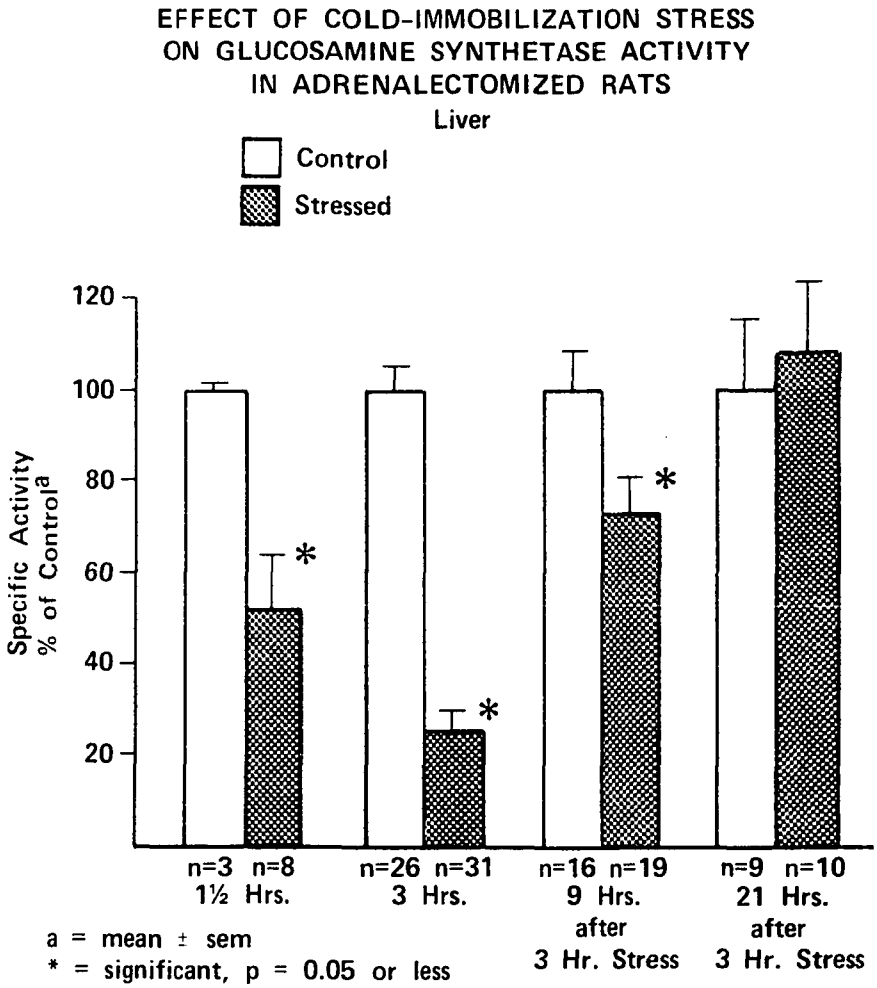
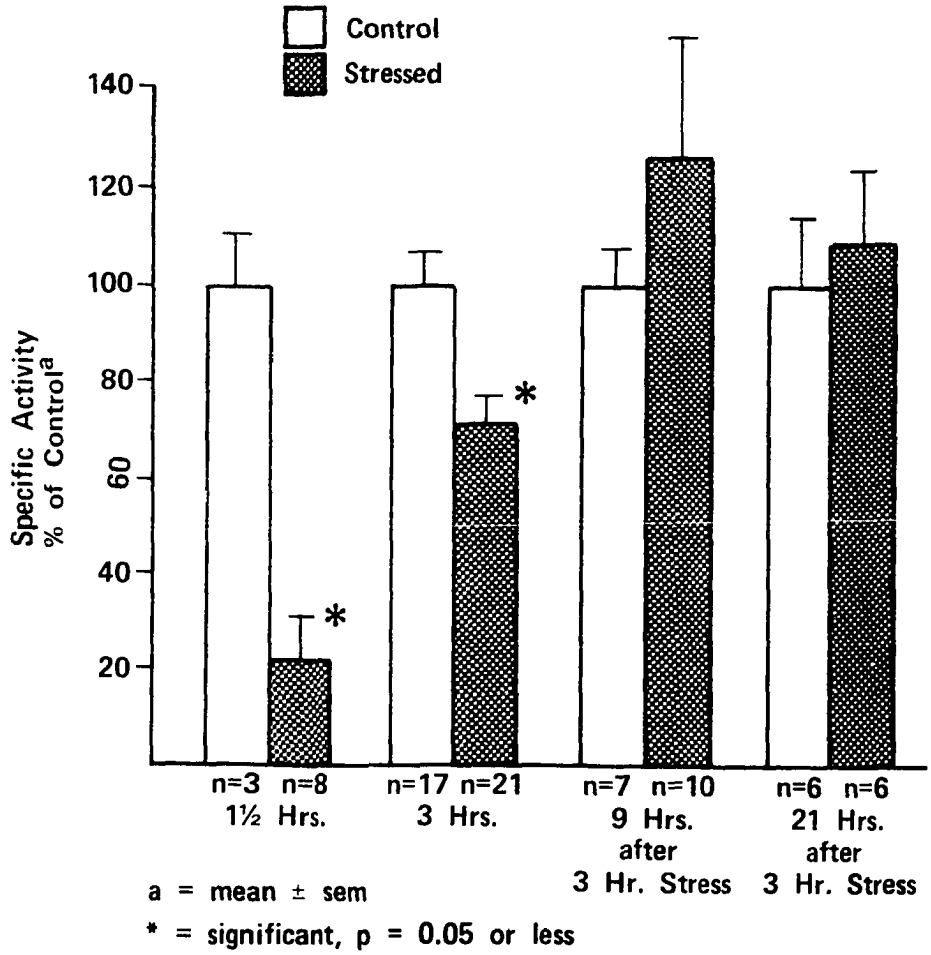

EFFECT OF COLD-IMMOBILIZATION STRESS
ON GLUCOSAMINE SYNTHETASE ACTIVITY
IN ADRENALECTOMIZED RATS
Liver
Control
Stressed
Specific Activity
% of Control[a]
120
100
80
60
40
20
*
*
*
n=3 n=8
1½ Hrs.
n=26 n=31
3 Hrs.
n=16 n=19
9 Hrs.
after
3 Hr. Stress
n=9 n=10
21 Hrs.
after
3 Hr. Stress
a = mean ± sem
* = significant, p = 0.05 or less

Fig. 8

EFFECT OF COLD-IMMOBILIZATION STRESS
ON GLUCOSAMINE SYNTHETASE ACTIVITY
Gastric Fundic Mucosa



a = mean ± sem

* = significant, p = 0.05 or less

Fig. 9

TABLE 4

THE EFFECT OF THREE HOUR COLD-IMMOBILIZATION STRESS ON UDPAH TISSUE LEVELS

Group	UDPAH nmoles/gm tissue[a]	
	Liver	Stomach[b]
Control	280.93 ± 14.49 n=11	190.02 ± 8.18 n=5
Stressed	253.48 ± 14.83 n=11	176.71 ±16.81 n=5
	p> 0.1	p> 0.4

a= mean ± s.e.m.
b= each n represents a pooled sample, 2 rats/sample

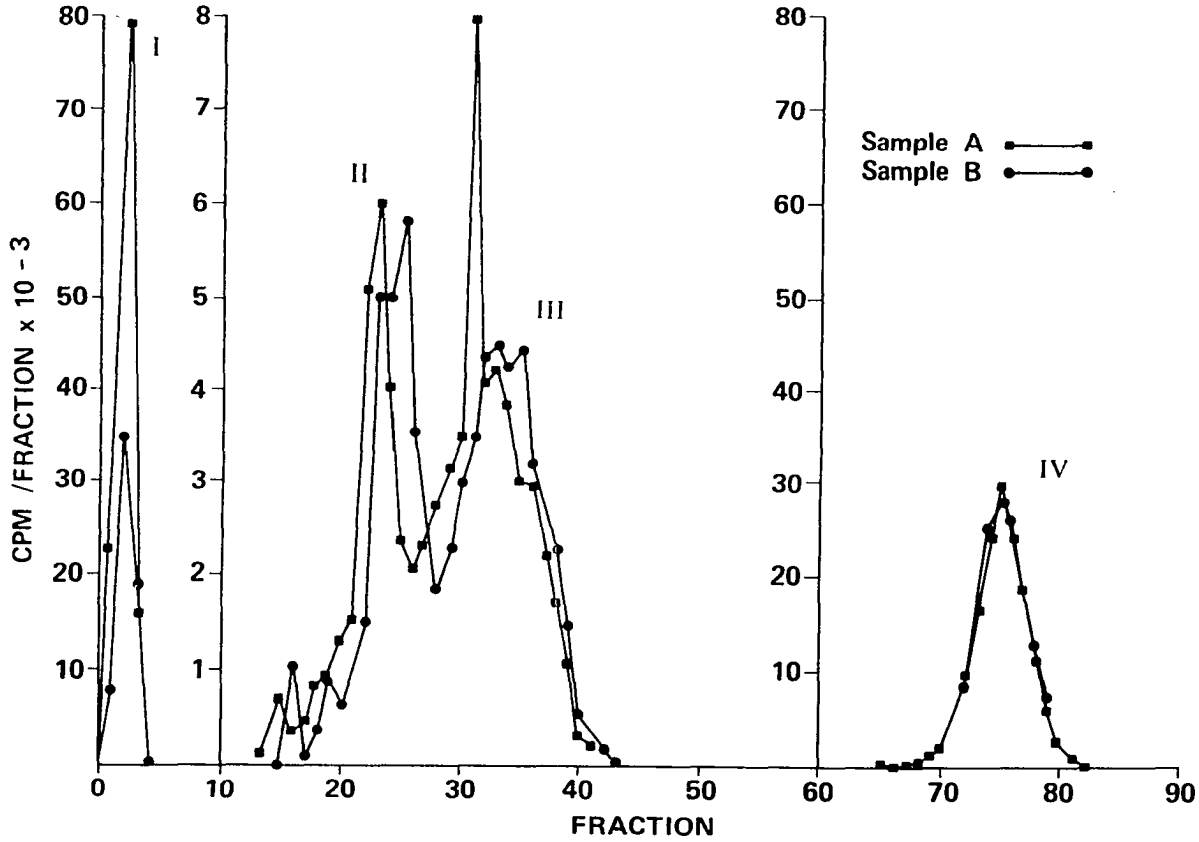


Fig. 10 - Distribution of glucosamine-1-C^{14} in the acid-soluble hexosamines of liver tissue of control rats. Combined liver extract was divided in half and subjected to duplicate chromatography.

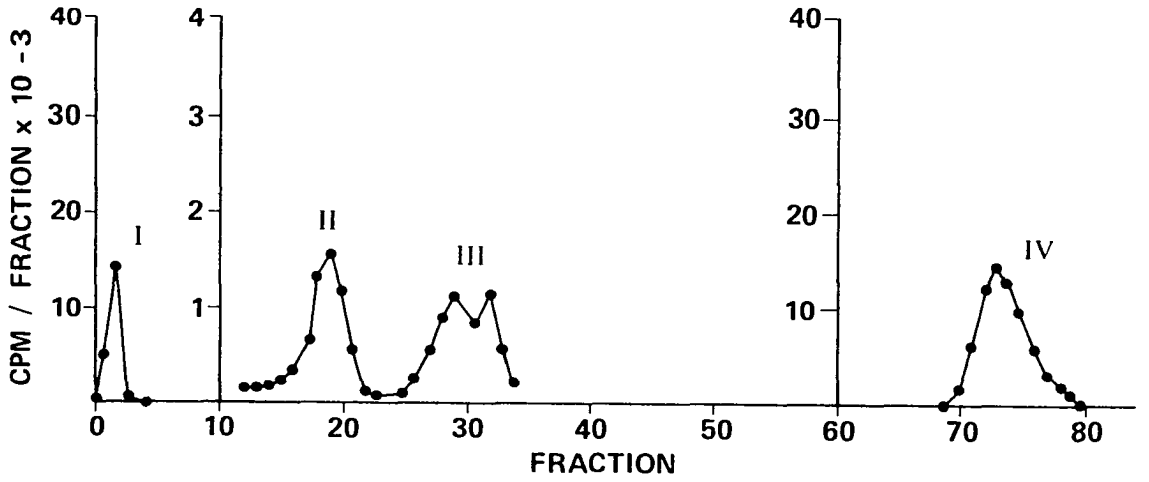


Fig. 11 - Distribution of glucosamine-1-C^{14} in the acid-soluble hexosamine intermediates of liver tissue from 3 hour cold-immobilization stressed rats.

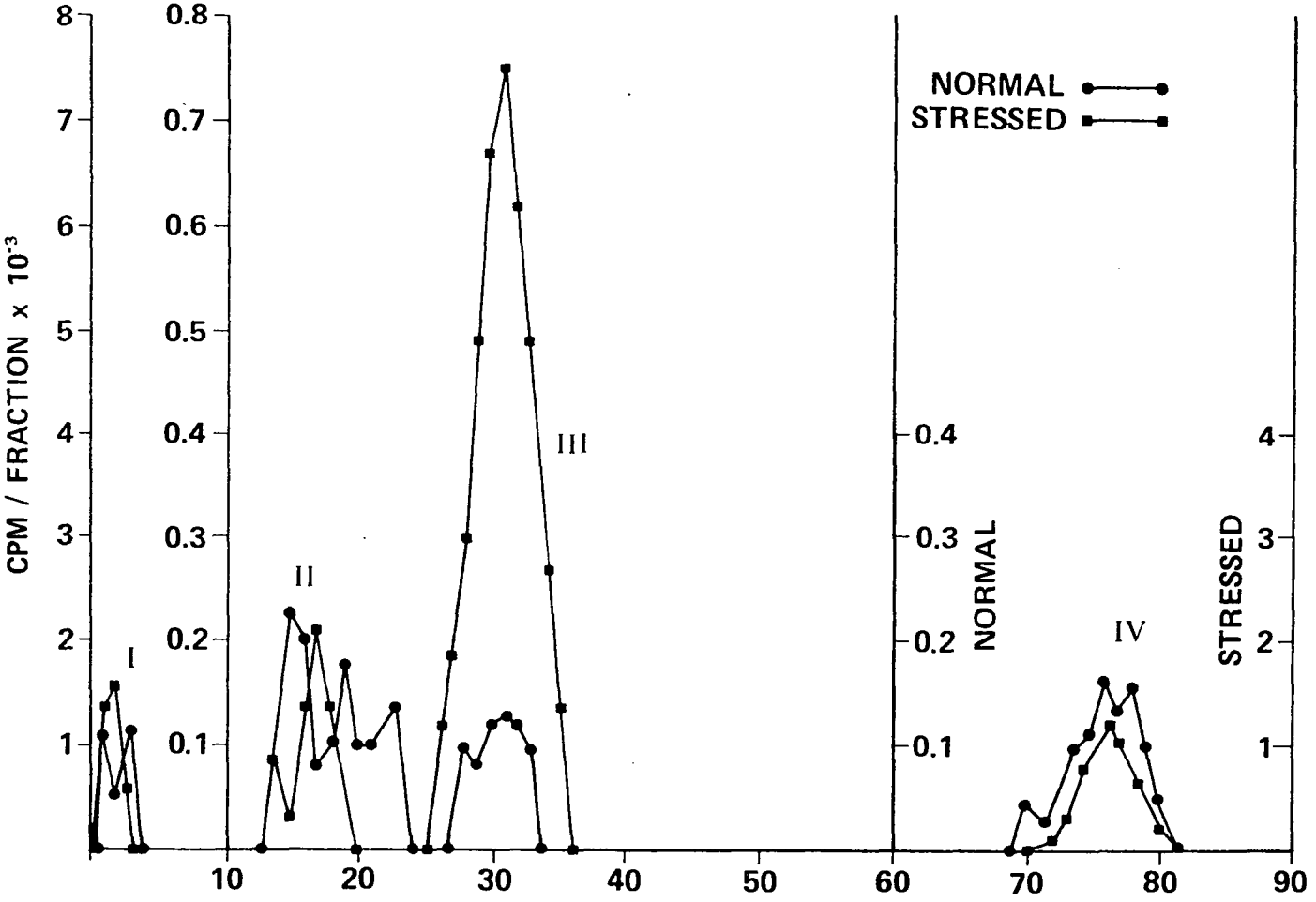


Fig. 12 - Distribution of glucosamine-1-C^{14} in the acid-soluble hexosamine intermediates of gastric fundic mucosal tissue. Normal rats were unstressed controls. Stressed rats underwent 3 hours of cold-immobilization.

frequently have a lowered body temperature and react very sluggishly. In some instances, central nervous system depression is evidenced by the temporary loss of the righting reflex. In other instances, however, the animals seem quite normal in outward appearance and actions except that they are obviously cold.

Despite the change in total incorporation the distribution of the radioactivity was not markedly altered by the stress. The slight shift to the left of peaks II and III was probably due to a change in the elution gradient. According to Molnar et al. Peak I consists of free glucosamine, N-acylhexosamines, glucosamine-6-phosphate and glycogen (158). Peak II is primarily sialic acid; Peak III a mixture of N-acetylhexosamine-1 and -6-phosphates and Peak IV, the largest component, a mixture of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine in a ratio of two to one. Note that Peaks II and III are plotted on a ten-fold smaller scale than Peaks I and IV.

Figure 12 shows the distribution obtained in the gastric fundic mucosa of normal and stressed animals. The scales used here are ten-fold less than those used for the liver. The same four fractions appear to exist in the stomach as in the liver. Lukie et al. found four similar fractions in the small intestine. Identification of the sugars in each peak found by these authors yielded the same distribution as seen by Molnar et al. except that instead of a distinct peak containing sialic acids in position two, two small peaks were seen, one containing glucosamine-6-phosphate and the other containing sialic acid (161).

In the liver, the majority of the radioactivity was present in Peak IV, while in the stomachs of the control animals only 18% of the

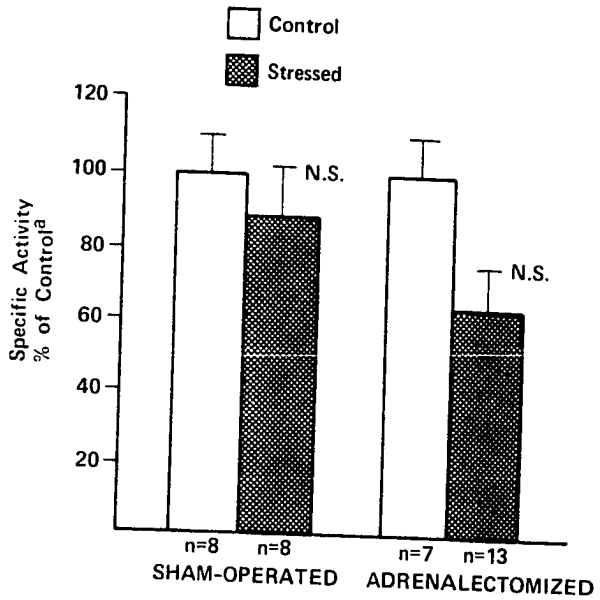
radioactivity was in this fraction. These percentage distributions of radioactivity agree relatively well with those in the literature for liver and small intestine (158, 161). This is the first report on hexosamine distribution in the stomach using glucosamine.

Although the data are not expressed on a per gram tissue basis the extracts were approximately equal in this respect and it therefore appeared that the stomachs of the stressed animals contained considerably more radioactivity than those of the controls. Peak III was also markedly higher in the stressed animals. This might indicate a block in the UDP-N-acetylglucosamine-1-phosphate $\xrightarrow{UTP}$UDPAG reaction, perhaps due to a limited supply of UTP. There was also a much larger percentage of the total radioactivity represented in Fraction IV in the stressed animals.

Group II- Immobilization of Adrenalectomized Rats

The involvement of the adrenal glands in the ulcerogenic process presents a paradoxical situation. As discussed earlier, although adrenocorticoids are ulcerogenic in some cases, they may be protective in others. Figures 13 and 14 illustrate the effect of adrenalectomy on the changes in glucosamine synthetase specific activity after cold-immobilization stress. Because of the reduced capability of adrenalectomized animals to withstand stress, these animals were immobilized for only one hour. This may in part explain why the decrease in glucosamine synthetase activity in the liver was reduced. As discussed earlier, immobilization of normal rats for one and one-half hours caused a 49% drop in enzyme activity in the liver. In this study immobilization of sham-operated rats for one hour caused only a 10.83% decrease.

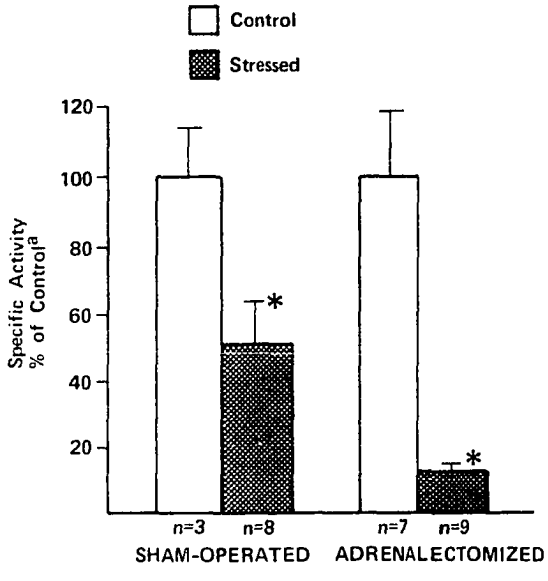
EFFECT OF COLD-IMMOBILIZATION STRESS
ON GLUCOSAMINE SYNTHETASE ACTIVITY
IN ADRENALECTOMIZED RATS
Liver



a = mean ± sem
N.S. = not significant at p = 0.05

Fig. 13

EFFECT OF COLD-IMMOBILIZATION STRESS
ON GLUCOSAMINE SYNTHETASE ACTIVITY
IN ADRENALECTOMIZED RATS
Gastric Fundic Mucosa



a = mean ± sem
* = significant, p = 0.05 or less

Fig. 14

Adrenalectomy tended to increase the effect of the stress. The decrease in specific activity in the livers of adrenalectomized animals was 38.48%. Both of these reductions were insignificant.

In the gastric fundic mucosa immobilization of sham-operated animals led to a 48.27% reduction in glucosamine synthetase activity. The incidence of ulceration was 62.5% and in only one of the eight animals were the lesions of the third type (+++).

Adrenalectomy increased the effect of immobilization. The incidence of ulceration was 84.62% and six of the eleven animals had lesions of the third type (+++). The decrease in enzyme activity was 88.41%.

Inexplicably, despite the fact that the sham-operations were performed three weeks prior to the immobilization experiment, five of eight sham-operated control animals exhibited damage to the gastric fundic mucosa which was accompanied by reduced enzyme activity levels. Four of these animals had + lesions, the fifth had one ++ lesion. These five animals were not included as sham-operated controls.

Group III- Immobilization of Atropinized Rats

Atropine, when given in very large doses, decreases the incidence of immobilization (63-64). In this study atropinization decreased the incidence of ulceration following three hours of immobilization from 100% in controls to 50% in atropinized animals. Glucosamine synthetase specific activity was not affected by the atropine in either the liver or the gastric fundic mucosa. (Figure 15). In the liver glucosamine synthetase specific activity after three hours of immobilization of rats given saline in place of atropine was reduced to 26.41±7.13%

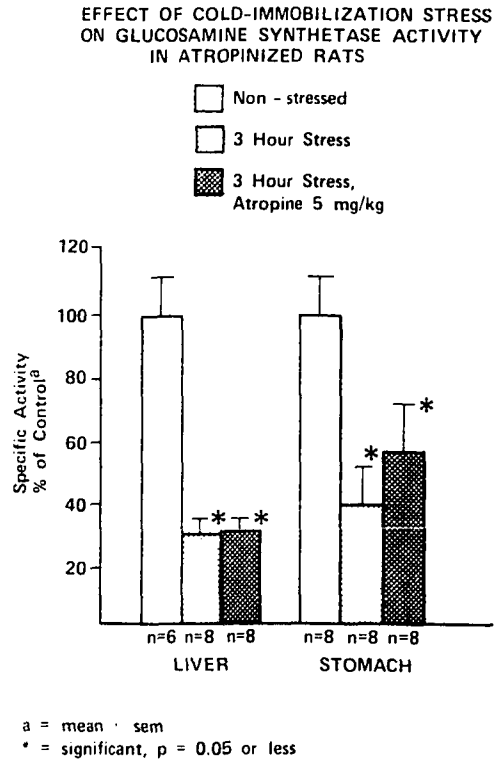


Fig. 15- Significance indicates differences from control levels.

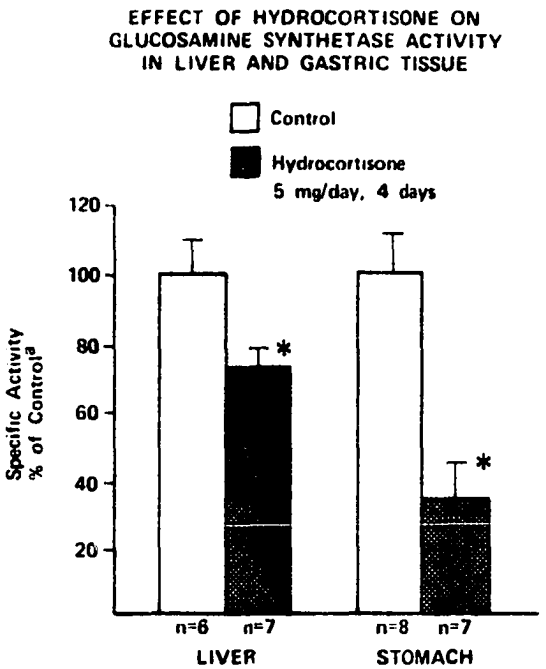
of unstressed controls. The reduction in atropinized rats was 26.79± 6.17% of the unstressed controls. The two reduced values differ significantly from the unstressed controls but not from each other. In the gastric fundic mucosa three hours of immobilization of saline-injected and atropinized animals led to reductions in glucosamine synthetase specific activity to 40.23±9.58% and 58.46±12.40% of the unstressed control level, respectively. Again, these values were significantly different from the unstressed control value but not from one another.

Study II- Effects of Hydrocortisone

Hydrocortisone acetate, administered daily for four days in pharmacological doses, caused a 100% incidence of gastric ulceration. There were multiple type ++ ulcers in each animal. Control animals had no ulcers. Figures 16 and 17 illustrate the effect of this drug on glucosamine synthetase and N-acetylase specific activities. In the liver glucosamine synthetase specific activity was decreased to 70.62 ± 6.59% of the saline-injected controls while N-acetylase, the second enzyme in the pathway, was not altered. In the gastric fundic mucosa glucosamine synthetase activity was reduced to 34.48±9.82% of the control level. As in the liver, there was no alteration in N-acetylase specific activity.

Study III- Effects of Ethanol

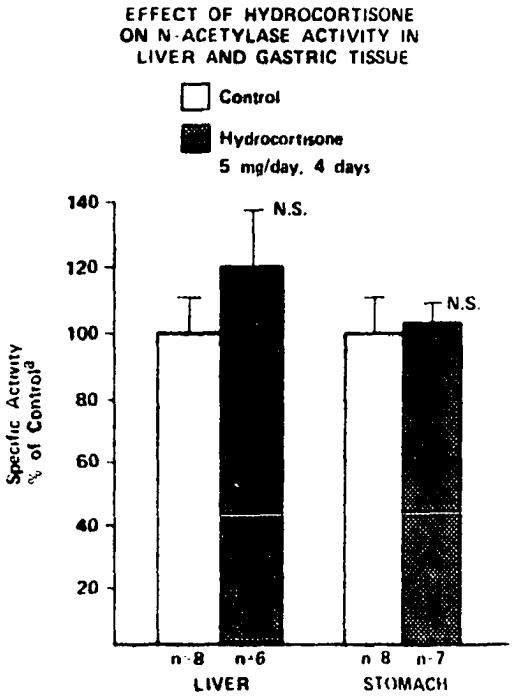
Although ethanol did not cause visible damage in the mucosas of any untreated animals, it did lead to significant reductions in the activity of glucosamine synthetase in the gastric fundic mucosa. The



a = mean ± sem

* = significant, p ≤ 0.05 or less

Fig. 16



a mean ± sem
N.S. = not significant at p = 0.05

Fig. 17

effect of this drug is illustrated in Figures 18 and 19. Glucosamine synthetase specific activity was reduced to 51.94±9.90% of the saline-injected control level in the gastric fundic mucosa. The slight decrease in specific activity in the liver was not significant. N-acetylase activity was not altered in either tissue.

Study IV- Effects of Sodium Salicylate

Group I- Sodium Salicylate, pH 2.5

Because this drug was delivered in the form of a slurry, it is questionable whether any of the animals actually received the prescribed dose. The drug had a tendency to stick to the sides of the syringe and stomach tube.

In three of the eight rats given buffer, acid and Celite to test the effect of the drug particles on the mucosa, abnormalities were seen. In two of the animals there was a single gash in the mucosa; in the third animal there were two gashes. The mucosas gave the appearance of having been cut with a knife blade. However, the glucosamine synthetase specific activities in both the liver and the stomach were similar to those seen in other control animals. Table 5 compares the control levels in both the stomachs and the livers of the buffer, acid and Celite treated animals with the untreated, unstressed controls of the atropine study.

Sodium salicylate, 250 mg/kg for four hours caused a 100% incidence of ulceration. Overall, the extent of ulceration was greater in this group than seen after any other ulcerogenic agent used in this investigation. All rats had multiple lesions. The accompanying reduction in glucosamine synthetase activity was of 50.05±12.84% of the

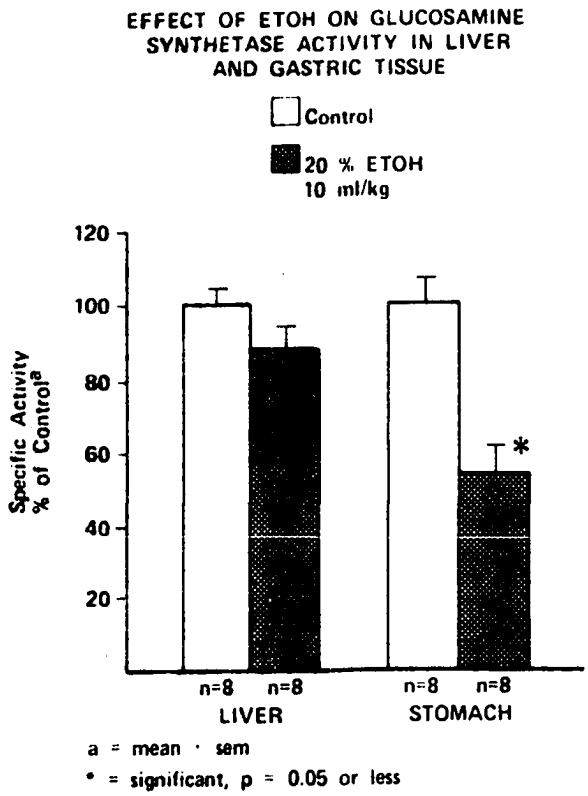

EFFECT OF ETOH ON GLUCOSAMINE SYNTHETASE ACTIVITY IN LIVER AND GASTRIC TISSUE
Control
20 % ETOH
10 ml/kg
Specific Activity
% of Control[a]
120
100
80
60
40
20
*
n=8
n=8
n=8
n=8
LIVER
STOMACH
a = mean · sem
* = significant, p = 0.05 or less

Fig. 18

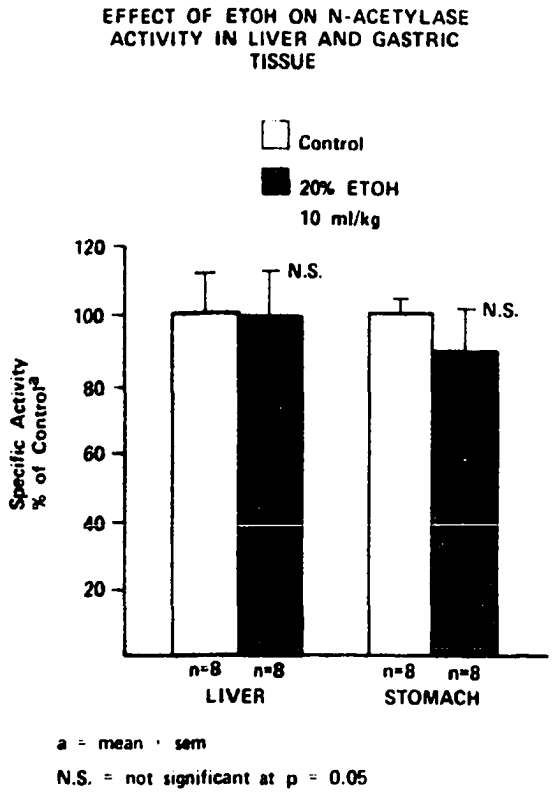

EFFECT OF ETOH ON N-ACETYLASE ACTIVITY IN LIVER AND GASTRIC TISSUE
Control
20% ETOH
10 ml/kg
Specific Activity
% of Control[a]
120
100
80
60
40
20
N.S.
N.S.
n=8
n=8
n=8
n=8
LIVER
STOMACH
a = mean · sem
N.S. = not significant at p = 0.05

Fig. 19

TABLE 5

THE EFFECT OF CELITE, ACID AND BUFFER, pH 2.5
ON GLUCOSAMINE SYNTHETASE SPECIFIC ACTIVITY

Group	Specific Activity nmoles/mg/hr[a]	
	Stomach	Liver
Untreated Controls (from Study I, group III)	76.43 ± 8.13 n=8	31.28 ± 3.16 n=6
Celite, Acid and Buffer, pH 2.5	74.38 ± 7.58 n=8	30.96 ± 2.01 n=7

a= mean ± s.e.m.

control level.

Sodium salicylate, 125 mg/kg for two hours caused a 50% incidence of ulceration. These lesions were not as severe as those seen in the previous group. However, the reduction in glucosamine synthetase specific activity to 28.86±3.85% of controls was greater. Although these data do illustrate that the enzyme activity was decreased before the appearance of ulceration, it can not be concluded that by the end of four hours the enzyme is in the recovery phase because the two reductions in activity are not significantly different.

N-acetylase specific activity was not altered by either dose of sodium salicylate at this pH.

The data are shown in Figures 20 and 21.

In the liver the situation was somewhat different. Sodium salicylate at a dosage of 250 mg/kg for four hours did not significantly alter glucosamine synthetase activity while a dosage of 125 mg/kg for two hours did reduce the activity significantly to 30.62±6.46% of the control level. These reductions were significantly different and suggest that by the end of four hours the enzyme was recovering from the stress caused by the drug.

In contrast to the situation in the stomach, N-acetylase specific activity in the liver was decreased by 250 mg/kg sodium salicylate for four hours to a level of 44.49±10.72% of the controls.

These data are illustrated in Figures 22 and 23.

Group II- Sodium Salicylate, Ph 7.5

These experiments were done over the course of two weeks and the fluctuations in control levels from day to day made it necessary

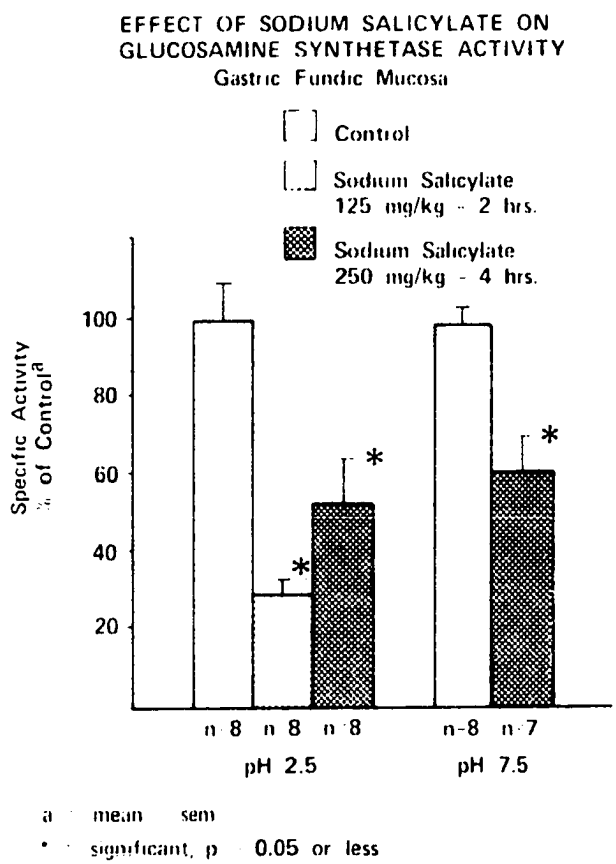


Fig. 20 - Significance indicates difference from controls.

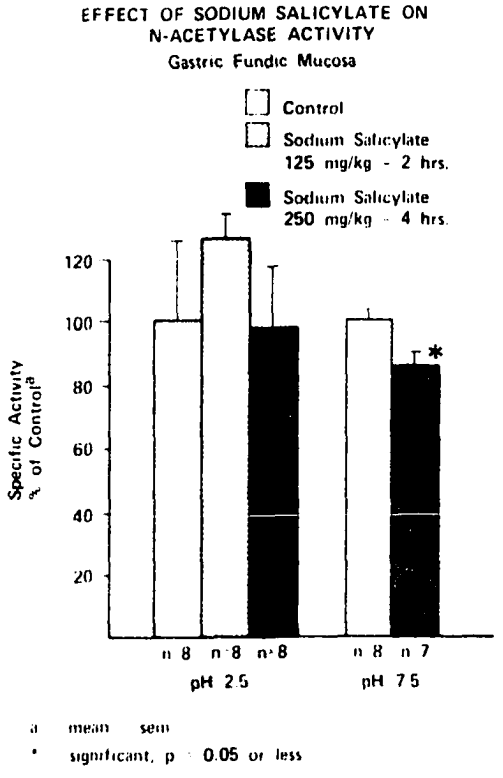

EFFECT OF SODIUM SALICYLATE ON
N-ACETYLASE ACTIVITY
Gastric Fundic Mucosa
Control
Sodium Salicylate
125 mg/kg - 2 hrs.
Sodium Salicylate
250 mg/kg - 4 hrs.
Specific Activity
% of Control[a]
120
100
80
60
40
20
*
n 8 n 8 n 8
n 8 n 7
pH 2.5
pH 7.5
a mean sem
* significant, p = 0.05 or less

Fig. 21

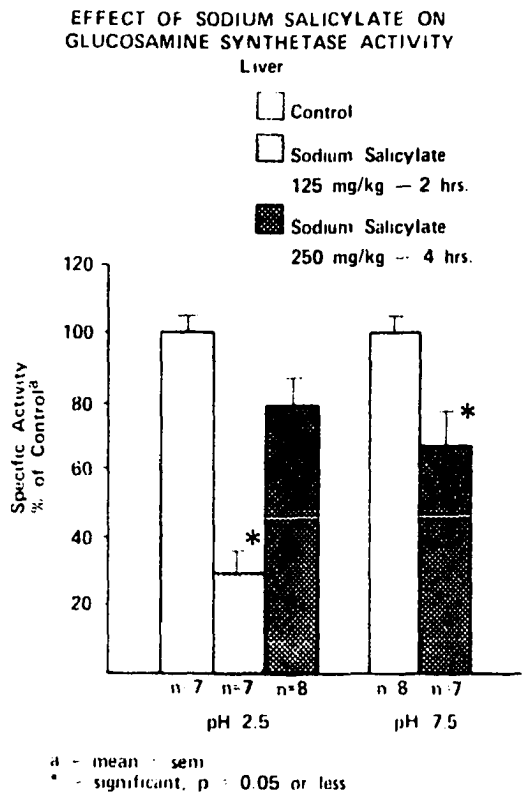

EFFECT OF SODIUM SALICYLATE ON
GLUCOSAMINE SYNTHETASE ACTIVITY
Liver
Control
Sodium Salicylate
125 mg/kg — 2 hrs.
Sodium Salicylate
250 mg/kg — 4 hrs.
Specific Activity
% of Control[a]
120
100
80
60
40
20
*
*
n=7
n=7
n=8
n=8
n=7
pH 2.5
pH 7.5
a - mean ± sem
* - significant, p ≤ 0.05 or less

Fig. 22

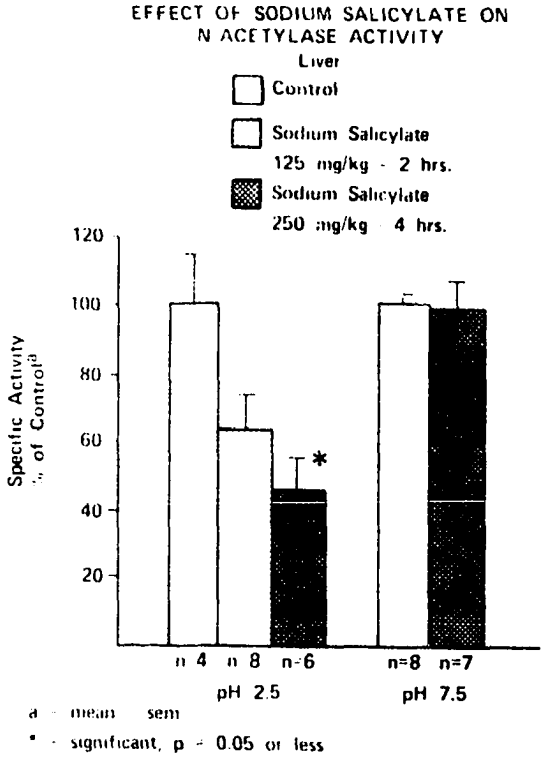

EFFECT OF SODIUM SALICYLATE ON
N ACETYLASE ACTIVITY
Liver
Control
Sodium Salicylate
125 mg/kg - 2 hrs.
Sodium Salicylate
250 mg/kg - 4 hrs.
Specific Activity
% of Control[a]
120
100
80
60
40
20
*
n 4
n 8
n-6
n=8
n=7
pH 2.5
pH 7.5
a - mean sem
* - significant, p = 0.05 or less

Fig. 23

necessary to compare the values only in terms of percentage difference from controls run simultaneously.

Although sodium salicylate at this pH caused no lesion of any kind in any of the animals, all the gastric mucosas were very pale and the stomachs were filled with an opaque, foamy fluid.

In both the liver and the stomach glucosamine synthetase specific activity was reduced, to 65.24±12.66% and 60.06±8.21% of controls, respectively. These reductions were not significantly different from those seen when the drug was administered at a pH of 2.5. This would indicate that while a reduction in enzyme specific activity alone is not sufficient to initiate ulceration, it may result in a lowering of the resistance of the mucosa to the final ulcerogenic insult.

In contrast to the effects of sodium salicylate at pH 2.5, at pH 7.5 N-acetylase activity in the stomach was decreased to 85.03±5.40% of control but was not altered in the liver.

These data are also illustrated in Figures 20, 21, 22 and 23.

CHAPTER IV

DISCUSSION

The validity of any conclusions drawn from this investigation depends heavily on the reproducibility of the assays used. This reproducibility was a major concern throughout this study.

Difficulties with reproducibility are thought to result from two unrelated factors:

(1) Glucosamine synthetase is an extremely labile enzyme. The mammalian enzyme has a relatively large molecular weight and consists of four subunits, all of which are necessary for activity (141).

(2) Gastric mucosal tissues are notoriuosly difficult to homogenize completely. The abundant mucus secretions tend to act as lubricants for the homogenization equipment.

Frequently, those homogenization processes which are harsh enough to completely disrupt the tissue integrity also inactivate the enzyme.

It was surprising to us to discover that the solution to these difficulties was to only minimally homogenize the gastric tissue. The homogenates used in the latter part of this study (those resulting from homogenization with the Potter-Elvehjem apparatus) were very

chunky. However, as the data in Table 2 indicate, the specific activities measured using this technique were much greater than those following Polytron homogenization of the tissues. The Polytron yielded a smooth homogenate. Studies with both methods of homogenization demonstrated that a definite relationship between measurable specific activity and duration of homogenization exists. The optimal conditions for homogenization determined were those described in the methods section of this report.

The specific activity of liver glucosamine synthetase measured in this study compares favorably with those reported by other investigators (160, 151). This comparison can not be made for gastric tissue because of differences in tissue preparation used by various investigators (145, 146). However, as Table 5 illustrates, the control levels from various studies were quite similar.

Restraint, hydrocortisone, ethanol and sodium salicylate all led to decreased glucosamine synthetase specific activity.

The restraint and sodium salicylate, pH 2.5 studies demonstrate that the enzyme activity drops before the appearance of ulceration. Before it can be said that a particular physiological change may cause ulceration it must be shown to occur before the appearance of lesions. However, using ethanol or sodium salicylate, pH 7.5, the enzyme activity decreased but ulceration did not occur. This seems to suggest that while a decrease in hexosamine can not alone cause ulceration, it may well be a necessary prerequisite. Conceivably, the decreased hexosamine synthesis may result in a lessened resistance to some other factor involved in the ulcerogenic process, perhaps acid.

One of the many confusing factors related to the mucus question is the fact that studies in human ulcer patients are conflicting (162, 163) In many instances no abnormality relating to secreted mucus or tissue mucus are detected. The rapid recovery of normal glucosamine synthetase specific activity seen in the restraint and sodium salicylate, pH 2.5, studies suggest that decreases in hexosamine synthesis may be necessary only as initiating events. If this is the case, alterations in tissues which have been ulcerated for extended periods of time would not necessarily be expected.

The confusion surrounding the influence of adrenocortical hormones on ulcerogenesis has not been in the least clarified by this study. The fact that adrenalectomy previous to restraint stress and hydrocortisone both decrease glucosamine synthetase specific activity suggest that perhaps adrenomedullary hormones exert a protective effect. The literature, however, does not support this suggestion (43). A more plausible hypothesis is that offered by Bonta (117). Based on his studies comparing the effects of ACTH and various glucocorticoids on restraint ulceration, Bonta suggested that the effect of glucocorticoids on the gastric mucosa is related to a balance between the anti-stress and ulcerogenic effects of the compounds. Further speculation might relate to the state of health of the individual. Proudfoot states that while dexamethasone is beneficial in the treatment of stress ulceration it should not be used in the case of peptic ulceration (41). It is interesting that this study indicated tendencies in the liver similar to those seen in the gastric mucosa. Decreases in the liver were insignificant, however.

Simultaneous measurement of glucosamine synthetase specific activity decreases in the liver during ulcerogenesis suggest that stress ulceration may, indeed, be a manifestation of systemic difficulties. Whether these systemic alterations are causally related to ulceration is entirely another question and was beyond the scope of this investigation.

The inhibitory effect of sodium salicylate on N-acetylase specific activity merely strengthens the case in favor of the possible involvement of hexosamine synthesis in ulcerogenesis. Kent et al. and Lukie et al. also demonstrated an inhibitory effect of sodium salicylate on N-acetylation (150, 161).

Further support for the importance of glycoprotein synthesis in ulcerogenesis is provided by studies on the drug carbenoxolone sodium. This liquorice derivative has been used quite successfully in Great Britain for the treatment of ulceration. It has been suggested but not demonstrated that one of the beneficial actions of this drug is induction of glycoprotein synthesizing enzymes.

Having ascertained that ulcerogenic procedures do in fact decrease hexosamine synthesis, the question then becomes "How does this occur and what effect does it have on the gastric mucosa?"

The decrease in glucosamine synthetase could be attributed to three things: (1) increased destruction of the synthesized product, (2) increased degradation of enzyme, and (3) increased inhibition of enzyme.

The first possibility, increased destruction of synthesized product, was ruled out experimentally. Incubation of glucosamine-6-phosphate with the enzyme source for three hours did not lead to a

measurable loss of product in either the liver or stomach (unpublished observation by author).

Although an increased degradation of product can not be overlooked, an increased inhibition of enzyme activity is most likely. As Table 4 indicates, UDPAH concentrations did not change remarkably in either the liver or gastric tissues after restraint. However, Winterburn and Phelps have shown that it is the concentration of secondary effectors such as glucose-6-phosphate, ADP and UTP, which influence the binding of UDPAH to the enzyme, that are of major importance (140). Glucose-6-phosphate and ADP increase UDPAH binding and therefore increase inhibition. UTP, on the other hand, functions as an activator. As alsready suggested, based on the studies involving the distribution of radioactivity in acid-soluble hexosamine intermediates, UTP may be limited in the gastric mucosa of stressed rats. Although a similar limitation of UTP was not indicated in the liver, only actual measurement of the concentrations of these secondary effectors can answer this question.

Lastly, the significance of an inhibition of hexosamine synthesis on the gastric mucosa must be considered. Although many studies have shown quantitative and qualitative changes in secreted mucus and tissue mucus content which accompany ulceration, it is difficult to understand how these changes could account for ulceration (25). While mucus does have buffering capabilities (15), does slow H^+ diffusion (14), and can inhibit pepsin activity (13), the magnitude of these capabilities is so small as to render them almost unimportant. However, hexosamines of the gastric mucosa are not limited to the mucus. Glycoproteins, of

which hexosamines are mandatory components, have been shown to be cell membrane components (165). The function of these constituents is not entirely understood but the glycoproteins have been suggested to be involved in antigen-antibody reactions and cell recognition. Nonethe-less, it seems logical to assume that decreased synthesis of glycoproteins or synthesis of abnormal glycoproteins might result in altered cell membrane structure and function. This could be of particular importance in a tissue such as the gastric mucosa with a very high rate of cell turnover.

CHAPTER V

SUMMARY

Two enzymes in the hexosamine synthesis pathway, L-glutamine: D-fructose-6-phosphate aminotransferase (E.C. 2.6.1.16) and glucosamine-6-phosphate: N-acetylase (E.C.2.3.1.4),were studied under various conditions of induced stress ulceration in rats.

Three hour cold-immobilization stress, hydrocortisone (5mg/day for four days, i.m.) and sodium salicylate (250 mg/kg and 0.1 N HCl buffered to pH 2.5, orally) all induced ulceration and caused significant decreases in glucosamine synthetase specific activity in both gastric fundic mucosal and liver tissues. Ethanol (20% in water, orally) and sodium salicylate (250 mg/kg in phosphate buffer, pH 7.5, orally) also led to decreased glucosamine synthetase activity but did not cause stress ulceration.

Glucosamine-6-phosphate: N-acetylase specific activity was reduced only by sodium salicylate.

Immobilization of rats for one and one-half hours or oral administration of lower doses of sodium salicylate (125 mg/kg and 0.1 N HCl, buffered to pH 7.5) resulted in decreased glucosamine synthetase specific activity but did not cause ulceration in all animals.

Atropinization of rats immediately prior to three hours immobilization reduced the incidence of ulceration but did not alter the

decrease in glucosamine synthetase specific activity. Bilateral adrenalectomy of rats three weeks prior to one hour immobilization increased both the incidence of ulceration and the degree of glucosamine synthetase activity reduction.

These findings demonstrate that although decreases in hexosamine synthesis alone do not initiate stress ulceration they may indeed be a prerequisite for this occurrence. Glycoproteins of the gastric mucosa are components not only of secreted mucus, but also of the cell membrane. The decreased synthesis of hexosamines, which are mandatory constituents of glycoproteins, may result in decreased mucosal resistance to attack by other agents involved in the ulcerogenic process, such as acid.

Furthermore, changes demonstrated in the liver indicate that stress ulceration may be a manifestation of a systemic disorder.

Studies utilizing atropinization, whcih blocks acid secretion, and adrenalectomy indicate that the observed decreases in glucosamine synthetase activity after immobilization were not caused by acid or adrenocorticoid hormones. The fact that hydrocortisone induced ulceration and led to decreased glucosamine synthetase activity suggests that a balance between anti-stress and the ulcerogenic actions of glucocorticoids may be important.

Measurement of UDP-N-acetylhexosamine concentration indicated that the observed decreases in glucosamine synthetase specific activity can not be attributed to increased concentrations of this feedback inhibitor. It was suggested that changes in the concentration of secondary effectors such as glucose-6-phosphate ADP, and UTP, which

influence binding of the inhibitor to the enzyme, may be involved.

Studies of the distribution of radioactive glucosamine in the acid-soluble intermediates of the hexosamine synthesis pathway indicated that in the liver immobilization did not alter the distribution of these intermediates although metabolism was depressed. In contrast, in the gastric fundic mucosa an increase in the fraction containing N-acetyl-glucosamine-1 and -6-phosphates after stress suggested a block in the synthesis pathway perhaps related to a limited supply of UTP.

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APPENDIX I

PREPARATION OF REAGENTS

Homogenizing Solution

This solution was prepared by dissolving 6.9 gm KCl, 202 mg EDTA, disodium salt, and 2.383 gm glucose-6-P, disodium salt in 500 ml glass distilled water. The pH was adjusted to 7.5 with 1 N KOH and glass distilled water added to final volume.

Glucosamine Synthetase Assay

The follwoing stock solutions were prepared: (a) D-fructose-6-phosphate, disodium salt, 0.2256 gm/10 ml,(b) L-glutamine, 0.1752 gm/10 ml, (c) EDTA, disodium salt, 0.336 gm/ 100 ml, (d), buffer, pH 7.5, 2.76 gm $NaH_2PO_4 \cdot H_2O$ and 5.36 gm $Na_2HPO_4 \cdot 7H_2O$ / 100 ml, and (e) buffer, pH 7.5, 2.76 gm $NaH_2PO_4 \cdot H_2O$ and 5.36 gm $Na_2HPO_4 \cdot 7H_2O$/50 ml.

The standard reaction mixture used in liver tissue contained 0.1 ml each of stock solutions (a), (b), and (c), 0.2 ml of stock solution (d), and 0.5 ml enzyme source.

The standard reaction mixture for gastric fundic tissue contained 0.05 ml each of stock solutions (a), (b), and (c), 0.1 ml stock solution (e) and 0.25 ml enzyme source.

The stock standard solution contained glucosamine HCl, 1 uM/ml.

N-Acetylase Assay

The following stock solutions were prepared: (a) buffer, pH 7.5, 2.1774 gm/20 ml, potassium phosphate, (b) ATP, 0.2205 gm/10 ml, (c) $MgCl_2$ 0.8132 gm/10 ml, (d) sodium acetate, 0.3402 gm/7.5 ml, (e) glucosamine-6-phosphate, 0.0819 gm/ 10 ml, (f) coenzyme A, trilithium salt, 0.433 gm/ 6 ml (for stomach assay) and (g) coenzyme A, trilithium salt, 0.433 gm/ 30 ml (for liver assay).

The standard reaction mixture for use in the stomach consisted of 0.1 ml buffer, 0.1 ml ATP, 0.0008 ml $MgCl_2$, 0.06 ml NaAc, 0.06 ml glucosamine-6-phosphate, 0.1 ml of the appropriate coA stock solutions, 0.1 ml enzyme source and glass distilled water to a final volume of 0.8

ml.

The standard reaction mixture for use in the liver assay contained 0.1 ml buffer, 0.1 ml ATP, 0.008 ml $MgCl_2$, 0.06 ml NaAc, 0.06 ml glucosamine-6-phosphate, 0.06 ml CoA stock solutions, 0.06 ml enzyme source and glass distilled water to a final volume of 0.8 ml.

The stock standard solution contained N-acetylglucosamine, 1 umole/ml.

Levvy-McAllan and Reissig Assays

Acetic anhydride- 1.5%. Mix 0.15 ml acetic anhydride in 9.85 ml acetone. This solution was prepared immediately before use to prevent precipitate formation.

Borate buffer, pH 9.1. For the Levvy-McAllan assay 5.35 gm potassium borate were dissolved in 100 ml water. For the Reissig assay 5.35 gm potassium borate were dissolved in 50 ml water.

PDMAD solution- Dissolve 10 gm p-dimethylaminobenzaldehyde in 100 ml glacial acetic acid containing 12.5% (v/v) 10 N HCl. This solution can be stored up to one month refrigerated. Shortly before use dilute with nine volumes of glacial acetic acid.

Biuret Assay

Dissolve 45 gm sodium potassium tartarate and 5 gm $CuSO_4 \cdot 5H_2O$ in 200 ml of 0.2 N NaOH. Add 5 gm potassium iodide then bring final volume to 1 liter with 0.2 N NaOH.

The standard solution contained 10 mg/ml albumin.

Glucosamine-1-C^{14} Distribution

The "XDC" scintillation solvent contained 129 ml xylene, 429 ml dioxane, 429 ml methyl cellosolve, 80 gm naphthalene, 10 gm POP and 0.5 gm POPOP. Other reagents used were 50% ETOH, 2N NaOH, 2N HCl, 0.4 N HCl, 0.04 N HCl, Dowex-1-X4-Cl, Celite, and glucosamine-1-C-14- 10 uc/ml.

Neuhaus and Letzring Assay

Sodium carbonate buffer- dissolve 3.0 gm sodium bicarbonate and 17.2 gm anhydrous sodium carbonate in 100 ml water.

Acetylacetone- Add 1.2 ml acetylacetone to 20 ml carbonate buffer. This reagent was prepared immediately before use.

Ehrlich's reagent- Dissolve 0.6 gm pDMAB in 2.25 ml of concentrated HCl and 20.25 ml isoamyl alcohol.

APPENDIX II

ABBREVIATIONS USED

Glam-6-P: glucosamine-6-phosphate

Glucosamine synthetase: L-glutamine: D-fructose-6-phosphate aminotransferase

NaAc: Sodium acetate

N-acetylase: Glucosamine-6-phosphate: N-acetylase

UDPAG: Uridine diphosphate N-acetylglucosamine

UDPAH: Uridine diphosphate N-acetylhexosamine

nmoles: Nanamoles

umoles: Micromoles

uc: Microcuries