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UNIVERSITY OF ALBERTA

GLUTAMINE: ITS EFFECT ON HEALING OF INTESTINAL INJURY

BY

PAUL E. HARDY, M.D.

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **GLUTAMINE: ITS EFFECT ON HEALING OF INTESTINAL INJURY**, submitted by Paul E. Hardy in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL SURGERY.

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ABSTRACT

This study set out to examine effects of glutamine, an intestinal energy substrate, on absorptive function after intestinal injury.

The injury studied was that produced over a 10-15 cm segment around a surgical anastomosis. *In vivo* fluid absorption was decreased on days zero and one, and was normal on days two through five compared to sham-operated controls. There was no difference in intestinal function on day one between the glutamine and glycine-supplemented groups. There was also no difference in ileal morphometry, DNA content or glutaminase activity. Increased glutaminase activity was noted in jejunal segments of these animals, suggesting that although the glutamine group was affected by its diet, the effect was not noted in ileum where the anastomosis was performed. A followup experiment was performed to determine if, in fact, ileum could be postively affected by oral glutamine. Twenty-five rats underwent an isoperistaltic interposition of 8 cm of ileum to the proximal small bowel. Rats were fasted and then fed either a glutamine or glycine/alanaine-supplemented diet for five days. Jejunal, ileal and native ileal segments were compared with repect to villus height, DNA content and glutaminase activity. Jejunum showed higher glutaminase activity in the glutamine group. Although interposed ileum did show increases in villus height compared to native ileum, there were no changes in any of the parameters mesured between dietary groups.

From these studies we conclude that: 1) Intestinal fluid absorption is transiently impaired over a 10-15 cm segment around a surgical anastomosis. 2) In this model, fluid absortion was unaffected by dietary glutamine probably due to insufficient quantities reaching the ileum. 3) Ileal interposition resulted in increased villus height but no difference between dietary groups, probably because of insufficient glutamine administered.

Further study of enteral glutamine's effect on ileum is required.

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CHAPTER 1

INTRODUCTION

Glutamine is an amino acid which has been shown to be a preferred energy substrate for small intestinal cells [1-4]. Although a special relationship between glutamine and enterocytes was suspected as early as the 1960's [5], extensive basic research on glutamine and the intestine did not take place until the 1970's [1-4]. During this period, metabolism of glutamine in the enterocyte was studied comprehensively. Pathways of glutamine and its breakdown products were elucidated and compared between sources from the blood and the intestinal lumen [6].

Despite this rapid expansion of information regarding glutamine and the gut, it was not until the 1980's, that glutamine's central importance in metabolism of critically ill patients was recognized. During this time period, the gap between basic knowledge of glutamine and clinical medicine was substantially narrowed.

Glutamine has been shown to improve outcome and intestinal morphometrics in several animal models of enterocolitis [11-15]. The focus of these studies was on intestinal morphometric benefit imparted by glutamine in sick animals, with little emphasis on intestinal function. This project has set out, as a priority, to examine functional improvements to the gut from glutamine.

Glutamine is not yet routinely available in total parenteral nutrition (TPN) or enteral feeding preparations [122] due to its low solubility, instability and potential for release of toxic products. Current research is concentrating on incorporation of glutamine into these nutritional formulas for clinical practice. It is likely that critically ill patients are unable to meet their needs in supplying glutamine from muscle synthesis. Therefore exogenous administration may be necessary in selected patients. A relative deficiency of glutamine may have adverse consequences on the intestine and other rapidly dividing cells.

This project begins with a review of glutamine and its effects of the intestine (Chapter 2) as an introduction to the experimental portion. In Chapter 3, the effects of a surgical anastomosis on intestinal function were examined. Impaired fluid absorption around a surgical anastomosis was studied, and the effects of glutamine on this intestinal function were noted. Results of Chapter 3 lead to the experiment in Chapter 4, which looks more closely at the effects of glutamine on various locations in the gastrointestinal tract.

Although much has been learned about glutamine in recent years, there is a necessity to apply this knowledge to clinical medicine. Of significant interest are topics such as TPN, preferred routes of administration and potential side effects.

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CHAPTER 2

GLUTAMINE AND THE INTESTINE*

The amino acid glutamine has become a subject of great interest in recent years. This is related to its vital role in intestinal metabolism. Glutamine has been shown to reduce the effects of enterocolitis in experimental animal models [1-8]. Due to low stability in aqueous solution and relative insolubility, glutamine is not readily available in commercial enteral or parenteral nutrition solution preparations. This paradox of apparent need, yet unavailability of glutamine is the impetus to review glutamine and its effects on healing of intestinal injury.

HISTORY AND BASIC CHEMISTRY OF GLUTAMINE

Glutamine is an amino acid detected in large quantities in many animal tissues. In addition to being a protein constituent, several other functions of glutamine have been elucidated [9]. Glutamine is classified as a non-essential amino acid because of man's ability to synthesize it from smaller constituents.

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The term may be misleading [10], however, in view of the diverse and important roles played by glutamine [11,12].

Its versatility and usefulness are in part due to the amide nitrogen (Fig. II-1) which is donated to several other compounds. These in turn participate in such processes as nucleotide synthesis, energy metabolism, the urea cycle, or acid-base balance.

The hydrolysis of glutamine to glutamate in animal tissues was first reported in 1935 by Krebs [3]. Glutaminase catalyzes the reaction depicted in Figure II-2 where glutamine is converted to glutamic acid and ammonia. Krebs reported this conversion in brain and retina of several species. Metabolic pathways of glutamine have been studied especially in the intestine, liver, muscle, kidney, lung and brain.

Glutamine is an abundant amino acid in animal tissues. In 1944, Hamilton [5] confirmed that an "unstable glutamine-like substance" in the plasma was, in fact glutamine. He measured amounts of glutamine in various tissues and found it to be a prominent constituent of muscle. In dogs, glutamine was shown to have 50-60% of the total free amino acid carboxyl nitrogen of heart muscle. In humans glutamine makes up a large proportion of the amino acid pool in addition to taking part in many important pathways.

Dent and Schilling [15] in 1949 reported the amino acid composition of portal blood in dogs following protein meals. They found that serum levels rose different amounts and concluded that amino acids were metabolized by varying degrees before reaching the liver. Glutamine was shown to rise slowly in the

portal blood following a protein meal, suggesting that this amino acid was partially metabolized prior to its release in portal blood.

In 1960, Finch and Hird [16] noted that glutamine rapidly disappeared from media which included isolated segments of rat intestine. Of the glutamine that disappeared, 47% was recovered as alanine. This was the beginning of intense studies of glutamine and the intestine. Although glutamine metabolism by the intestine has largely been elucidated, there still remain many questions to be answered. In recent years, glutamine's importance in maintaining a healthy intestinal tract and possible therapeutic implications have been of great interest [17-22].

BIOCHEMISTRY OF GLUTAMINE

The degradation of glutamine to glutamate by glutaminase has been studied in numerous tissues [23] and the enzyme properties are reviewed [9,24]. There are two broad categories of glutaminase. The phosphate-dependent isoenzyme is the predominant form in intestinal tissue and a similar type is active in kidney. It is inhibited by glutamate. The liver glutaminase isoenzyme is characteristically stimulated by glucagon, which may play a role in gluconeogenesis. Most of glutaminase is attached to the inner mitochondrial membrane where its actions take place. Glutamine synthetase is primarily a cytosolic enzyme. Poorly understood transport systems of glutamine to and from mitochondria may influence the rates and directions of various biochemical pathways.

Glutamine absorbed from the intestinal lumen or intestinal arterial blood undergoes a variety of changes. Glutamine metabolism by the intestine is comprehensively reviewed by Windmueller [23,26,25] and others [17-20]. In addition to protein synthesis, glutamine can be broken down in several different ways, depending on the particular tissue and the need. Although labelled carbon and nitrogen from glutamine can appear in a variety of products, there are basically four classes of metabolic yield. See Figure II-3.

1. Energy: Glutamine breakdown products participate in oxidative pathways as substrate for energy production. Glutamine is deamidated to glutamate and further converted to alpha ketoglutarate which is an integral part of the Krebs (tricarboxylic acid) cycle. The intestinal metabolism of glutamine releases labelled CO_2 and lactate as a result of energy production [25,27]. It is this metabolic fate (energy production) which is glutamine's vital importance to the intestine. Not only has energy production from glutamine been demonstrated in the intestine [27,28], but also in lymphocytes [29], fibroblasts [30] and malignant cells [31].

2. Ammonia and nitrogen carriers: In the deamidation of glutamine to glutamate via glutaminase, the byproduct is ammonia. This is released directly into the blood or transferred to complex amino acids such as citrulline for urea synthesis. In acidosis the kidney hydrolyzes glutamine, releasing ammonia in the urine.

3. Simple amino acids and their byproducts: Alanine turns up in large quantities as a metabolic derivative of glutamine [27]. The most direct route of formation would be through transfer of the NH_2 group to pyruvate in the

conversion of glutamate to alpha ketoglutarate. Alanine participates in other processes, including gluconeogenesis.

4. Complex amino acids: carbon labelled in glutamine also is recovered in several amino acids, for example, proline. This is likely through the intermediate glutamate semialdehyde.

Glutamine is an abundant amino acid in mammalian systems. In addition to a protein constituent, it provides energy and acts as a nitrogen carrier. Its versatility is best understood in reviewing glutamine and its predominant role in individual organs.

INTERORGAN RELATIONSHIPS

Glutamine serves particular functions in different organs. It acts as an energy substrate in rapidly dividing cells such as the enterocyte [32] and malignant cells [31,33] and is an important source of ammonium ion for the kidney during acidosis [34,35]. Whether glutamine consumption or production occurs, depends primarily on the organ involved. Glutamine metabolism is also affected by the overall state of the organism. Glutamine levels in both tissue and blood can be assayed with relative simplicity using a colorimetric method [36]. Arteriovenous differences across a particular organ can be measured. The gradient divided by the arterial level is calculated to give the extraction or utilization of glutamine which can be expressed in micromoles per hour per gram of tissue. Interorgan relationships of glutamine are summarized in Figure II-4.

Intestine

The intestine consumes glutamine as an energy substrate both from arterial and luminal sources [37]. The small intestinal enterocyte is responsible for the majority of glutamine utilization. Ammonia is released in portal blood as a result of hydrolysis of glutamine. Glutamine nitrogen is also incorporated into other amino acids, while greater than 50% of glutamine carbon is released in intestinal venous blood as dissolved carbon dioxide [25]. The rate of glutamine utilization by the intestine varies; the influencing factors will be discussed later. Apart from under special conditions *in vitro* [27], intestinal cells do not exhibit net glutamine synthesis. The intestine receives most of its supply of glutamine from synthesis by muscle, and liver to a much smaller and variable degree. Diet is also an important source. The intestine's central role in glutamine metabolism has been reviewed [38-40].

Skeletal Muscle

Glutamine is abundant in skeletal muscle but is released in proportionately higher amounts in perfusates of muscle tissue [41,42]. Glutamine and alanine account for over 50% of the amino acids released by skeletal muscle although they account for less than 15% of the muscle protein content [43]. This suggests a net synthesis of glutamine in the muscle. Glutamine makes up approximately 50% of the free amino acid pool in human muscle. This is glutamine available for export and contrasts with the glutamine in muscle protein, which makes up about 5% [44]. Glutamine release from skeletal muscle and uptake by the splanchnic bed has been documented in non-diabetic [45] and diabetic human subjects [46]. Catabolic states are usually associated with a negative nitrogen

balance. In response to operative stress, it has been shown that glutamine levels fall in whole blood, plasma and skeletal muscle [47]. Under stress, nitrogen is mobilized from skeletal muscle mostly in the form of glutamine and alanine. Glutamine is used by the gut as an energy substrate and alanine is predominantly taken up by the liver for gluconeogenesis. Thus, skeletal muscle usually acts as a glutamine supplier.

Kidney

By rendering rats acidotic, Addae and Lotspeich [34] have shown that urinary ammonia excretion and renal glutamine extraction parallel one another and increase with acidosis. Schrock and Goldstein [35] similarly found that acidotic rats had a net renal utilization of glutamine. The normal rat has an even balance of glutamine metabolism in the kidney. Hence acidosis is a state whereby glutamine can deliver its amide nitrogen to the kidney which is excreted in the urine in the form of ammonium ion. In the normal state the kidney has a balance of glutamine but in acidosis it is a glutamine consumer. Interorgan glutamine metabolism during acidosis involves intricate alterations in several organs, and the subject has recently been reviewed [48].

Liver

Hepatic metabolism has been one of the more difficult areas of glutamine to understand. The blood supply to the liver being divided between arterial and portal blood renders studies of arteriovenous gradients less reliable. The liver possesses both glutamine synthetase and glutaminase in considerable activities [9]. To understand glutamine metabolism in the liver, one must consider the possible biochemical transformations and under which circumstances these might

be beneficial. Gluconeogenesis is a vital function performed by the liver which may use breakdown products of glutamine. Glucagon increased hepatic utilization of glutamine in dogs [49], probably by stimulation of hepatic glutaminase. However, in contrast, dexamethasone either stimulated or had no effect on hepatic production of glutamine [50-52]. In the case of glucocorticoids, alanine was used for liver gluconeogenesis and glutamine uptake by the intestine increased. These studies found the liver to be an organ of near balance for glutamine in the resting state. In the fasting conscious dog, Miller et al [53] showed that the liver became a net producer of glutamine after prolonged fasting. Prior to fasting, the dog's liver utilized glutamine to a small degree.

The liver is a scavenger for ammonia from portal blood. Ammonia in portal blood can be derived from two sources [25,32]: 1.) Microflora in the intestine produce ammonia. 2.) The small intestine breaks down glutamine as energy substrate, thus releasing ammonia into the portal blood. One mechanism which the liver uses to handle the ammonia load is through glutamine synthesis. Haussinger [54-56] has reviewed the interaction between the urea cycle and glutamine synthesis in liver. The distribution of hepatic glutamine synthetase is heterogenous as it is abundant in the perivenous sinusoids relative to the periportal sinusoids. The periportal hepatocytes, high in urea synthesizing enzymes, are the first liver cells encountered by portal blood. Two thirds of portal ammonia is converted to urea here, and the remainder encounters the high affinity glutamine synthetase "downstream" in the perivenous hepatocytes. The remaining ammonia is therefore converted to glutamine before this blood enters the systemic circulation. Hepatic glutamine synthetase is up-regulated in acidosis

and inhibited in alkalosis. This is the opposite from kidney glutamine synthetase, which is inhibited in acidosis. The liver, therefore, helps shuttle ammonia to the kidney in the form of glutamine amide nitrogen during acidosis. Net hepatic glutamine production has been demonstrated during acidosis [35], portal ammonium chloride infusion [57], and prolonged fasting [58].

Hepatic balance of glutamine [49,50,53] is probably close to zero in the basal state. Various stimuli can influence the liver to become either a net glutamine consumer or producer, depending on the overall needs of the organism.

Lungs

Recent work has indicated that there is more glutamine release than accounted for by muscle, liver and kidney [59]. The lung was a logical possibility, and patients with pulmonary artery catheters have revealed that glutamine is released into the circulation by the lungs in large quantities during hyperdynamic sepsis [19,60]. The glutamine efflux from the lungs disappeared with the onset of adult respiratory distress syndrome. In the septic patient, the lungs (as long as they are healthy) serve as a glutamine supplier to the circulating glutamine pool.

Brain

Glutamine is a precursor of two important neurotransmitters [9]. 1.) Glutamate is a potent excitatory substance in the CNS and 2.) its metabolite, gamma amino butyric acid (GABA), is an inhibitor. Glutamine is synthesized in large quantities in the brain, however this is likely not significant to the circulating glutamine pool. The synthesis and degradation of glutamine in the brain influence the levels of neurotransmitters. Glutamine synthesis may also

play a role in minimizing brain ammonia levels which would be important in impending hepatic coma. As a supplier or consumer of glutamine, the brain usually has little effect on other organs.

GLUTAMINE AND THE INTESTINE

Intestinal Energy Substrates

In 1965, Neptune [28] reported that glutamine was oxidized in preference to glucose in rabbit ileum. More detailed work on glutamine as an intestinal energy substrate was done in the 1970's by Windmueller and Spaeth [27,32,37], Hanson and Parsons [61,62] and others [63,64]. The contribution of glutamine to respiratory CO_2 in the intestine is high [65], indicating the glutamine's importance as an intestinal fuel. Windmueller suggests that there is little evidence to support a specific role of glutamine in intestinal metabolism other than as an energy substrate [65], although aid to lymphocyte function and nucleotide synthesis may be important. In addition to glutamine, ketone bodies have been shown to act as fuels of the small intestine [66]. Isolated colonocytes [67] metabolize short chain fatty acids and ketone bodies preferentially as energy substrates. These nutrients are primarily derived *in vivo* from fermentation of luminal contents by endogenous microflora. The small intestine uses glutamine as a fuel and the colon shows a preference for short chain fatty acids and ketone bodies (beta hydroxybutyrate, n-butyrate, acetoacetate, propionate) [45,68-70]. There is some overlap in utilization.

By measuring arterial and portal venous levels of various substrates, Souba et al [71,72] studied the intestinal consumption of intravenously

administered fuels. In dogs, there was a significant arterial - portal vein gradient for glutamine and glucose but not beta-hydroxybutyrate in the basal state. During substrate infusion, intestinal glucose consumption remained unchanged whereas that of glutamine and beta-hydroxybutyrate (B-OHB) significantly increased. This supports the hypothesis that glutamine and B-OHB are preferred *in vivo* energy substrates over glucose in the dog intestine.

Electroneutral sodium absorption in piglet jejunum was increased with added glutamine to either the mucosal or serosal side of Ussing chambers [73]. The effect, postulated to be due to the intestinal fuel properties of glutamine, was more pronounced than with the addition of glucose.

Isolated human enterocytes preferentially metabolize glutamine for energy as indicated by oxygen consumption measurements [68]. The oxidation of glutamine in enterocyte mitochondria has been investigated [74]. In contrast, human colonocytes preferentially metabolized short chain fatty acids over glutamine, especially those sampled from the distal colon [75]. Glutamine metabolism was higher in proximal compared to distal colonocytes.

Utilization

Windmueller and Spaeth [27] used isolated vascularly perfused rat intestine to investigate the metabolic fate of glutamine. With U-14C glutamine, 57% of labelled carbon appeared as carbon dioxide in intestinal venous blood. This was followed by organic acids (predominantly citric and lactic acid - 18%), acid-insoluble material (14%), citrulline (6%) and proline (5%). Most of the end-products were produced via glutamic acid, thus indicating the importance of glutaminase in intestinal metabolism of glutamine. In contrast, glutamine

nitrogen taken up by the intestine was included in citrulline (34%), alanine (33%), ammonia (23%), and proline (10%). Labelled ^{14}C recovered in tissue was in much higher proportion in small intestine compared to colon, indicating the relative importance of small bowel in glutamine metabolism.

They followed these results by studying the *in vivo* metabolism of glutamine by a luminally perfused segment of rat intestine [37]. Labelled CO_2 accounted for 60% of the glutamine carbon recovered. The metabolic products recovered with respect to both glutamine nitrogen and carbon had a strikingly similar profile as the vascularly perfused segments. These results suggest that there is a common glutamine pool within mucosal cells which consists of both luminally and arterially derived glutamine. The experiments achieved similar results using germ-free rats, indicating that intestinal microflora were not responsible for the breakdown of luminal glutamine and ammonia release. Intestinal metabolism of luminally administered glutamine was a function of luminal concentration [37]. At 6mM, only 34% was absorbed intact, while 45mM glutamine increased the proportion to 70%. No ^{14}C glutamate was released in venous blood after glutamine luminal perfusion, indicating that the rate limiting step in intestinal glutamine metabolism is the deamidation of glutamine to glutamate.

In vivo [27], vascularly perfused rat intestine metabolized glutamine to a high degree in a single pass. This was noted predominantly in the small intestine. The glutamine content of mucosal cells was only 3% that of liver and 6% that of muscle, consistent with a high conversion of glutamine in mucosal cells by glutaminase.

Respired CO_2 was compared *in vivo* between an empty rat intestinal lumen and that containing a simulated meal of glucose and amino acids [32]. The carbon dioxide was derived from luminal glutamine in preference to glucose, indicating the importance of glutamine as an intestinal fuel during the absorptive phase. Similarly, arterial glutamine was oxidized in preference to arterial glucose (46% of respired CO_2 originated from arterial glutamine compared to only 10% from glucose).

These results concur with work of Hanson and Parsons [61,62] done on rat intestine *in vitro*. Fasting resulted in unchanged glutamine metabolism and decreased glucose utilization in perfused intestinal segments. Kimura et al [76] also showed that glutamine uptake by rat intestine is unaffected by fasting. Glutamine oxidation in developing intestine is nearly twice the rate of that in adult rat intestine [77].

Weber and Veach [78,79] studied ammonia production in the intestine of dogs. The small intestine released comparable amounts of ammonia into portal blood as did the uncleansed colon. Ammonia from the small intestine was nearly fully accounted for by the breakdown of glutamine. 51% of ammonia released by the colon was due to metabolism of glutamine and urea, the remaining portion presumably being produced by intestinal flora. In luminal perfusion studies [79], glucose was infused to determine if an energy source alternate to glutamine would reduce the amount of ammonia released in portal blood. Instead, luminal infusion of glucose significantly increased mesenteric venous ammonia release and intestinal glutamine utilization. The effect was more pronounced in jejunum than ileum. Hepatic synthesis of glutamine helps detoxify ammonia [55]; the

clinical implications of ammonia production and glutamine have been discussed by Souba [40].

Glutamine uptake by the GI tract in splenectomized dogs nearly doubled by the second postoperative day compared to control animals [80]. This occurred in the face of reduced portal blood flow and lower arterial glutamine levels. Glutamine utilization is probably up-regulated, rather than determined solely by the quantity of glutamine presented to the gut. Conversely, rat intestinal glutamine extraction is decreased in sepsis [81,82], diabetes [83] and soon after massive small bowel resection [84]. Glutamine extraction in the small intestine of peak-lactating rats was increased compared to non-lactating controls [85]. Glutamine utilization was increased *in vivo* and in isolated enterocytes after rats were treated with dexamethasone [86]. Similarly, the gut glutamine uptake doubled in dogs receiving anabolic steroids compared to controls [87].

Glutamine is used by the intestine whether supplied arterially or luminally. The metabolic fate of glutamine is similar for each mode of entry into the mucosal cell. Intestinal glutamine extraction is modified by many factors, probably through modulation of the rate limiting step of glutamine metabolism: deamidation by enterocyte glutaminase.

Glutaminase

Pinkus and Windmueller [23] reported extensively on intestinal glutaminase in 1977. They documented high enzyme levels in bowel mucosal of several species including rat, dog, hamster and monkey. Glutaminase was not abundant in guinea pigs and chickens. Lower blood glutamine levels and intestinal extraction were noted in these two species.

Glutaminase activity can readily be assayed, for both the phosphate-dependent [23] and independent [88] varieties. Intestinal glutaminase is predominantly the phosphate-dependent type [23] and is located in the mitochondria of mucosal epithelial cells. There is an even distribution in enzyme activity from crypt to villus tip. In rats, phosphate-dependent glutaminase was nearly equally distributed between jejunum and ileum, comprising 78% of the total activity in intestinal mucosal scrapings [23]. Colon was 5%, cecum 6%, duodenum 9% and stomach 2%. Luminal chloroketone, a glutamine analogue, irreversibly inhibited metabolism of luminal but not arterial glutamine. Arterial chloroketone inhibited processing of both luminal and arterial glutamine [23]. Intestinal glutaminase had a high substrate affinity which is consistent with the low mucosal cell concentrations of glutamine. Phosphate-independent glutaminase and other enzymes required for glutamine metabolism were of quite low activity.

It has been postulated [8] that stimulation of intestinal glutaminase activity may be necessary for the gut to take full advantage of circulating glutamine. The well-being of the intestine may therefore depend on both the supply of glutamine, and the activity of glutaminase. Glutaminase activity is affected by a number of factors.

Intestinal glutaminase activity was shown to decrease after 48 and 72 hours of starvation in rats [69,89,90]. Supplementation of drinking water of young adult rats with 275 mM glutamine for up to 7 days, resulted in unaltered intestinal mucosal glutaminase activity. However, in another study, a lesser concentration of glutamine (0.39 g/60cc), after a period of fasting did result in

increased jejunal glutaminase activity over glycine supplemented controls [91]. Dexamethasone [92] increased glutaminase specific activity in both jejunum and colon of rats, two days after administration. The enzyme was unchanged by acidosis [23,89,93] or potassium deficiency [89]. A conflicting result showed a decrease in small intestine glutaminase in chemically induced acidosis [94], with no change in the colonic enzyme. The effects of acidosis on intestinal glutaminase are clearly different from that of kidney, where acidosis up-regulates renal glutaminase in order to promote urinary ammonia excretion [23].

Septic rats have demonstrated decreased utilization of glutamine along with reduced glutaminase activity in small intestine mucosal scrapings [82]. Less important enzymes in glutamine metabolism were also decreased. Starvation has been shown to reduce an alternate step in glutamine processing, that catalyzed by transglutaminase enzyme [95].

Streptozotocin-diabetic rats [94] had decreased glutaminase activity in both colonic mucosal scrapings and the whole colon. A substantial increase was observed in small intestine glutaminase activity [93,94] for the whole organ but not for mucosal scrapings. The increased enzyme activity paralleled the increase in intestine size which normally accompanies induced diabetes in rats. The higher total enzyme activity was accompanied by complete suppression of glutamine extraction as measured by arteriovenous differences [93]. This example in diabetic rats is an exception to the hypothesis that glutamine utilization is predominantly a function of glutaminase activity.

Glutamine Transport

Until recently, transport of glutamine across the brush-border membrane of the intestine was not well understood. Transport across the plasma membrane in hepatocytes is a sodium-dependent process distinct from other amino acids and is termed System N. Glutamine transport in hepatocytes and across mitochondrial membranes has been reviewed [96].

Recent studies [97,98] using a brush border membrane vesicle technique in rat intestine have characterized enterocyte glutamine transport. Brush-border membrane glutamine transport occurs by two mechanisms. The primary route is via a sodium-dependent system which resembles System N in liver. Glutamine is also transported by a sodium-independent carrier-mediated system. Both are saturable and inhibited by other amino acids. Glutamine transport occurred against a chemical gradient as evidenced by an "overshoot" phenomenon prior to equilibration. Similar glutamine transport was documented in human brush-border membrane vesicles [99].

Basolateral membrane transport of glutamine, that which would occur from arterial blood during fasting, has also been characterized in rats [100-102]. Glutamine transport was carrier-mediated, resembled System A, and probably involved an exchange of extracellular glutamine for intracellular alanine. Alanine, a metabolite of glutamine [25], is a logical substance to exchange: as a non-energy byproduct of glutamine metabolism, it is available to the liver for gluconeogenesis as more glutamine enters the enterocyte for processing. Similar transport systems were reported for rat colonocytes [103] and human small intestine basolateral membrane vesicles [104].

Brush-border membrane (BBM) transport of glutamine was increased in diabetic rats [105]. This is in contrast to decreased endogenous glutamine intestinal cell uptake [106], implying that the diabetes favors dietary, rather than endogenous, glutamine use by the enterocyte. The enhanced transport [105] was due to an increased Vmax and not Km (carrier affinity). The effect was reversed by insulin.

Other conditions have been shown to affect intestinal glutamine transport. It is altered in burn patients [107]. Another study reported the effect of dietary glutamine supplementation for four days after an equal period of fasting in rats [97]. This resulted in a 75% increase in glutamine BBM uptake over controls. The clinical relevance of enhanced intestinal glutamine transport is not yet clear, although an impact on gut metabolism, structure and function is speculated [97].

Intestinal Injury Models and Glutamine

There are several reasons to suggest that glutamine may protect the health of the intestine. The high levels of glutaminase specific to intestinal mucosal and glutamine's role as an energy substrate has been explained. Baskerville [108] administered parenteral glutaminase to monkeys and other mammals in order to investigate its properties as an anti-tumor agent. All but the lowest doses were fatal within ten days. All animals had vomiting, dysentery, and diarrhea. Pathological features included lesions in liver, kidney and intestine, the most prominent being an acute necrotizing colitis. The colonic lesions were histologically similar to human ulcerative colitis, and a mechanism suggested, is that of glutamine deficiency induced by the glutaminase enzyme [108]. Healthy

rats [109] receiving parenteral nutrition had increased jejunal mucosal weight, DNA content and villus height when glutamine was added to the solution. Recovery including weight gain and intestinal adaptation after 60% bowel resection in rats was enhanced [110]. In 1957, Shive [111] reported on glutamine as a beneficial treatment for peptic ulcer disease in humans. These observations support glutamine as an agent which promotes intestinal well-being. Animal injury models provide even more convincing evidence [112,113].

Glutamine has been shown to be beneficial in the treatment of intestinal injury induced in rats by 5-fluorouracil [1,2], methotrexate [3-5] and radiation [6-8].

Rats with intraperitoneal 5-fluorouracil (5-FU) induced enterocolitis [1] were fed total parenteral nutrition (TPN) with or without glutamine. After four days, glutamine supplemented rats had increased small bowel mucosal DNA content and villus height. There was a trend towards increased survival in glutamine-fed rats. Similar results were obtained with and without 5 days of prefeeding of glutamine-supplemented TPN. Significantly higher arterial glutamine levels were noted in glutamine supplemented animals who were not treated with 5-FU. The same group looked at the effects of enteral feeding [2] glutamine in 5-FU-induced enterocolitis in rats. In this study which included 5 days of prefeeding, increased protein and DNA content were observed in colon but not small bowel.

Methotrexate-induced enterocolitis in glutamine-treated rats has also been studied [4,5]. In these experiments, an enteral diet was supplemented with glutamine for the treatment group and glycine for controls. A four day period of

prefeeding was employed. Groups treated with glutamine had less protein and DNA loss in the colon and jejunum. Animals fed supplemental glutamine had improved survival curves and a significantly lower incidence of positive blood cultures. Mechanisms suggested were glutamine's role in cell differentiation and DNA synthesis. Glutamine may have also reduced the injury by improving the metabolism and excretion of methotrexate.

Glutamine has also been shown to be beneficial in radiation enteritis models in rats [6-8]. Oral glutamine and glucose supplementation in drinking water were the only nutrients provided to treatment group rats following abdominal radiation [7,8]. Radiated controls received isonitrogenous supplementation of glycine and glucose. In this model with a nutritionally incomplete diet, bacterial translocation as indicated by culture-positive mesenteric lymph nodes was significantly decreased in the glutamine radiated group [7]. Mucosal mass was maintained and weight loss was less with glutamine treatment. Reasons postulated include protection of the gut mucosal barrier from glutamine's role as an energy substrate and as a nitrogen donor for nucleotide synthesis. Glutamine may also have been enhancing lymphocyte function to decrease bacterial translocation. Glutamine improved survival, reduced bloody diarrhea, and improved small bowel morphometrics [8] following whole abdominal radiation. Arterial glutamine levels, intestinal glutamine extraction and intestinal glutaminase levels were all higher in the glutamine-fed irradiated animals. Gut glutamine extraction was 35%, compared to 12% in the glycine radiated controls. Arterial glutamine [2,7,8] levels and extraction [8] are significantly increased only when dietary supplementation is combined with intestinal injury.

Glutaminase levels, however, have increased with dietary glutamine alone after an initial period of fasting [91]. Glutaminase may help the animal take advantage of circulating glutamine from endogenous sources [8]. Similar benefit to the gut was demonstrated with a nutritionally complete elemental diet supplemented with glutamine a four day prefeeding period [6].

Of the injury models discussed (5-FU, methotrexate and radiation), glutamine was administered through the gastrointestinal tract in all but one study [1]. All employed isonitrogenous, isovolumic diets between treatment and control groups. All except three experiments [1,7,8] had a period of prefeeding, where treatment animals received glutamine and controls did not for at least four days prior to injury. Two studies in which prefeeding was not included [7,8] used nutritionally incomplete diets where glucose and one amino acid were the only sources of caloric intake. The injury models examined glutamine's effect predominantly on intestinal morphometrics, although bacterial translocation [5-7] and survival [1,5,8] were also considered. Although these findings cannot be directly extrapolated to humans, they are suggestive of many possible clinical applications, and glutamine's role in supporting gut mucosal structure and function.

GLUTAMINE AND THE INTESTINE: CLINICAL ASPECTS

Most of the data on glutamine and the intestine are obtained from animal experiments, however, glutamine's importance is clear. Problems seem likely to arise when glutamine shortage is combined with stress to an individual.

Glutamine's absence from enteral and parenteral solutions renders deficient those who probably need it most.

In addition to the injury models discussed, there is indirect evidence that further consideration of glutamine and human inflammatory bowel disease may be important. Ulcerative colitis as an energy substrate deficiency has been suggested [114], while glutamine may be important in the biosynthesis of mucus glycoproteins formed in pseudomembranous colitis [115].

Effects of conditions such as diabetes, acidosis and glucocorticoids on glutamine metabolism have been discussed. Clinical significance of glutamine is more substantial in the catabolic patient.

Starvation

Significant alterations of glutamine metabolism occur during starvation, and the topic has recently been reviewed [116]. During the postabsorptive state or early fasting, the intestine obtains most of its glutamine from the arterial blood. After four days, the intestine increased its uptake of glutamine from the circulation by 80%. This was accompanied by the liver switching to an organ of glutamine release. The intestinal uptake of glutamine is increased in starvation despite lower mucosal glutaminase levels and therefore is likely a reflection of increased supply from other endogenous sources. The kidney also increased its consumption of glutamine during starvation.

Surgical Trauma

Trauma and surgical stress result in nitrogen loss from muscle catabolism. Elective cholecystectomy in man results in decreased plasma amino acid levels, including glutamine, in the early postoperative period [117]. The negative

nitrogen balance associated with surgery was significantly reduced in six patients undergoing elective resection of colonic carcinoma [118] compared to controls. The treatment group received glutamine-containing parenteral fluid. Similar benefit to postoperative nitrogen balance has been observed as a result of intravenous infusion of glutamine in humans [119,120].

Laparotomy and insertion of vascular catheters in dogs resulted in negative nitrogen balance, lowered intracelluar glutamine in skeletal muscle and lower arterial glutamine levels by the third postoperative day [47]. The effect was more pronounced than with fasting. The gut uses substantially more glutamine after operative stress [22] and this is probably partially mediated through glucocorticoids [51] and glucagon [49].

Souba et al [121] studied the effects of enterectomy on glutamine interorgan exchange in dogs. Animals underwent laparotomy, splenectomy and 60% small bowel resection. Controls had a splenectomy and ileal-jejunal bypass. Arteriovenous differences were measured six hours after surgery. Arterial glutamine levels were significantly higher in enterectomy animals compared to controls. After bowel resection, the intestine became an organ of near glutamine balance, compared to that of an avid glutamine consumer in controls. The lack of intestinal utilization of glutamine after bowel resection could account for the higher arterial levels. Alanine was released from the intestine at a significantly lower rate in the resection group. This experiment illustrates the intestine's action of consuming glutamine in the postoperative state, by demonstrating reduced glutamine utilization by the intestine in the partially resected animals.

Alanine and glutamine are released from muscle in large quantities [41] in healthy and critically ill [122] patients. Alanine and glutamine were studied [122] in healthy volunteers and critically ill patients who underwent laparotomy. These amino acids were significantly decreased in arterial blood in the patient groups. A group of two patients who underwent greater than 80% bowel resection had a markedly decreased extremity glutamine efflux. The healthy volunteers and those who underwent laparotomy without enterectomy both had normal glutamine mobilization. The authors suggest a mechanism whereby the intestine provides feedback to peripheral tissues to modulate glutamine efflux.

Surgical stress resulting in catabolism and negative nitrogen balance is characterized by increased intestinal uptake of glutamine. Alanine and ammonia are released in portal blood which are converted to glucose and urea by the liver. Muscle provides glutamine to the gut, however, if no exogenous glutamine is available to the stressed patient, then the circulating pool becomes depleted.

Sepsis

As with trauma, the septic patient displays increased mobilization of glutamine with an associated negative nitrogen balance. However, in sepsis, intestinal utilization of glutamine is decreased in rats and humans [82,81]. At the expense of intestinal uptake, lymphocyte and hepatic utilization of glutamine both increase in sepsis [123]. Additional glutamine is supplied to the circulating pool by the lungs [60].

A detailed study of a multiple trauma patient revealed a steady decrease in splanchnic uptake of both glutamine and alanine over time [124-126]. Both reached zero as the patient became systemically septic and began deteriorating on

day 10. Although this does not distinguish intestinal and hepatic utilization, the patient's poor condition was likely coincidental with a decreased intestinal utilization of glutamine (for gut energy substrate) and reduced hepatic uptake of alanine (for gluconeogenesis). In the basal state, glutamine is utilized by the intestine as an energy substrate.

Patients with Malignancy

Glutamine is the principal amino acid consumed by malignant cells [31,33] and therefore consideration must be paid to both the tumor and the intestine in the cancer patient. Tumor cells may compete with the intestine for use of the circulating glutamine pool as suggested by an experiment using tumor-bearing rats [127]. Glutamine's benefit to the intestine in the radiation enteritis model [6-8] may also benefit a malignant tumor treated by radiation. Therefore, indiscriminate administration of glutamine as an intestinal protectant to the patient with a malignancy would not be prudent. Preliminary research on glutamine's effect on tumor relative to healthy tissue has been performed. Glutamine has been shown to support muscle without stimulating tumor growth in rats [128]. It afforded more benefit to the intestine than lymphocytes from 5-FU toxicity [1]. Until a better understanding of glutamine's role in malignant disease is obtained, caution regarding glutamine supplementation is urged [129].

Glutamine and Nutritional Preparations

Changes in the gastrointestinal tract that occur as a result of parenteral feeding have been well documented [130,131]. Morphological and functional alterations including intestinal mucosal hypoplasia, decreased carbohydrate transport and reduced enzyme activities all occur. These changes are

predominantly reversed by enteral feeding. It is suggested that enteral glutamine is especially important in maintaining healthy intestinal mucosa [131,132]. Glutamine supplementation of an elemental enteral diet for rats resulted in increased jejunal mass and crypt mitotic rate over controls [133]. Hepatic mass and protein content increased while hepatic fat content was reduced in those rats fed enteral solution with glutamine. A glutamine-supplemented enteral diet prevented pancreatic atrophy and fatty liver in rats after a small bowel resection [134]. A similar protective effect on the pancreas was demonstrated with parenteral glutamine [135]. Glutamine and fiber were added to diets of separate groups of rats and neither reduced bacterial translocation, although benefits to jejunal mucosal architecture were noted [136].

Glutamine is not presently available in parenteral solution due to its low solubility, instability and potential release of toxic products (ammonia and glutamate). Much work has recently been completed on incorporation of glutamine into TPN.

Parenteral nutrition supplemented with glutamine resulted in increased jejunal weight, protein, DNA, mucosal thickness and villus height in rats [109]. A dose response experiment [137] revealed 2 mg of glutamine per 100 ml as a necessary strength. Improved jejunal DNA content correlated with glutamine but not with total nitrogen intake. Similar benefit was demonstrated in colon and stomach in addition to enhanced jejunal disaccharidase activity [138].

Glutamine has been suggested beneficial to the immune system through support of gut-associated lymphatic tissue [139]. Supplementation of TPN with glutamine significantly reduced positive bacterial cultures of mesenteric nodes in

rats [140]. This may have been due to improved immune function as secretory IgA levels in bile were observed to be significantly higher than in those receiving glutamine-deficient TPN.

Hepatic steatosis, normally associated with TPN, was not present in rats on TPN with added glutamine [141]. Avoidance of fatty infiltration compared to controls was probably through modulation of portal insulin/glucagon ratio and promotion of hepatic lipid export. Intravenous glutamine prevented atrophy of jejunal smooth muscle in rats [142]. This effect was noted only when other amino acids were not administered. Beneficial effects of parenteral glutamine on intestinal morphology were enhanced by the addition of epidermal growth factor [143], supporting the general hypothesis that specific nutrients in combination with hormonal factors can provide maximal growth of tissue.

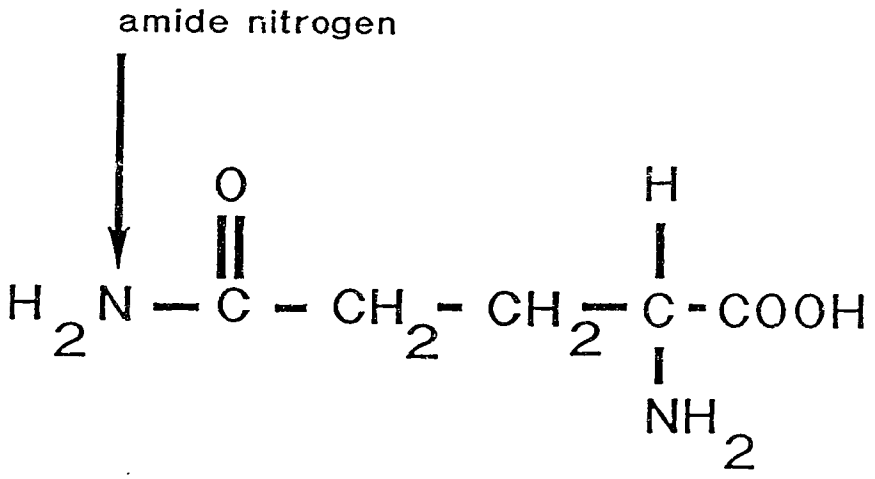
The safety of intravenous glutamine has been studied in healthy volunteers [144,145]. There were no untoward side effects noted, including signs of CNS toxicity. Plasma levels of ammonia and glutamate, two potential toxic metabolites of glutamine, were not significantly elevated. Amino acid levels, cortisol, insulin, glucagon, growth hormone values and routine chemistry analyses were all unchanged. Intravenous glutamine did increase insulin levels slightly in another study [146]. There was a significantly higher rise in arterial glutamine from intravenous compared to oral glutamine, suggesting splanchnic uptake of glutamine in the orally administered group. Glutamine has also been given in an intravenous parenteral solution over four weeks to patients receiving bone marrow transplants [145]. This was well tolerated with no untoward effects.

To circumvent the potential difficulties of glutamine as a parenteral agent, the use of dipeptides with glutamine and an additional amino acid have been considered [147]. Three dipeptides containing glutamine were compared in conscious dogs [148]. Alanyl and glycyl glutamine dipeptides were hydrolyzed *in vivo* rapidly, resulting in prompt elevation of arterial glutamine. In humans, L-alanyl-L-glutamine was hydrolyzed more rapidly than L-glycyl-L- glutamine [149]. The mechanism suggested reflects both plasma and extracellular cell membrane hydrolysis. Intravenous use of N-acetyl-L-glutamine was studied in fourteen healthy humans [150], however the kinetics of utilization of this dipeptide render it less than ideal for clinical use. Clinical applications of glutamine dipeptides have been reviewed [151,152]. L-alanyl-L-glutamine seems to be the most practical glutamine precursor for use in humans. Its properties have been studied in dogs [153], rats [154] and humans [155]. L-alanyl-L-glutamine is sixteen times more soluble than glutamine alone and it is stable under conditions of heat-sterilization and storage [156]. No side effects were noted in clinical trials [156], and the benefits of L-alanyl-L-glutamine have been noted in catabolic patients [157,118]. Glutamine dipeptides have been given intravenously to septic patients with no impairment of peptide metabolism [147], however more study is required if it is to be used in patients with either hepatic or renal failure.

SUMMARY

Glutamine is an amino acid which is the principle energy substrate for small intestinal cells. It also acts as a nitrogen carrier, through its amide nitrogen.

Arterial glutamine is supported by net synthesis in skeletal muscle. Glutamine is rapidly metabolized by the intestine whether it is supplied from the lumen or the arterial circulation. Intestinal uptake of glutamine increases after trauma and operative stress. The utilization of glutamine by the gut may in large part be dependent on mucosal glutaminase activity. Glutaminase is located on the inner mitochondrial membrane and its activity can be affected by a number of conditions. Enterocyte transport may also play a role in intestinal uptake of glutamine. Glutamine has been shown to improve gut morphology and outcome in animal models of enterocolitis. It may play a similar role in aiding repair of human intestinal injury compared to those who are glutamine deficient. Glutamine may have a positive effect on the immune function of the intestine. Glutamine is not presently available in nutritional preparations for clinical use, yet it has recently been shown of benefit to humans in maintaining nitrogen balance. Due to instability and low solubility of glutamine, the use of dipeptides has been studied. L-alanyl-L-glutamine seems to be the most promising glutamine precursor for parenteral use in humans as it is safe and is rapidly hydrolyzed *in vivo* to release free glutamine. The exact role of glutamine as a therapeutic agent to promote intestinal well-being is yet to be determined. Preliminary evidence suggests glutamine will be helpful in a variety of clinical scenarios.



GLUTAMINE

Figure 1: Glutamine. The amide group is an important nitrogen donor.

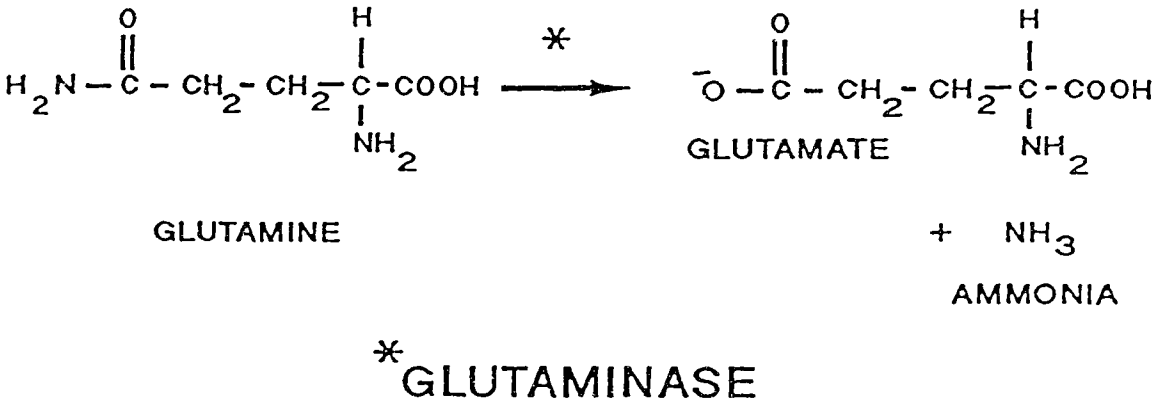


Figure 2: The enzymatic degradation of glutamine to glutamate and ammonia.

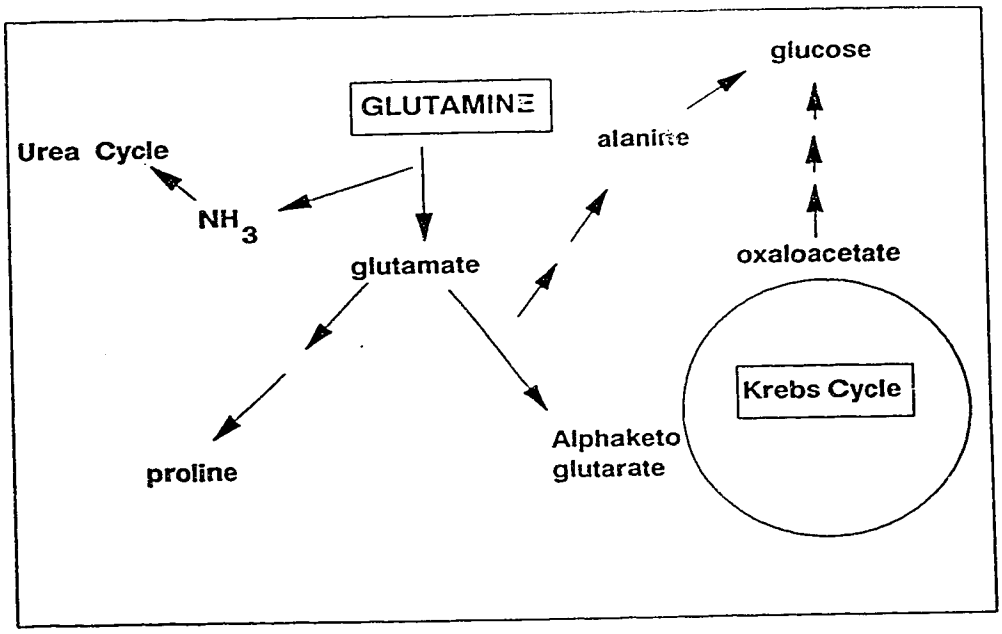


Figure 3: Pathways of glutamine metabolism in rat intestinal mucosa. (Adapted from Windmueller [26]).

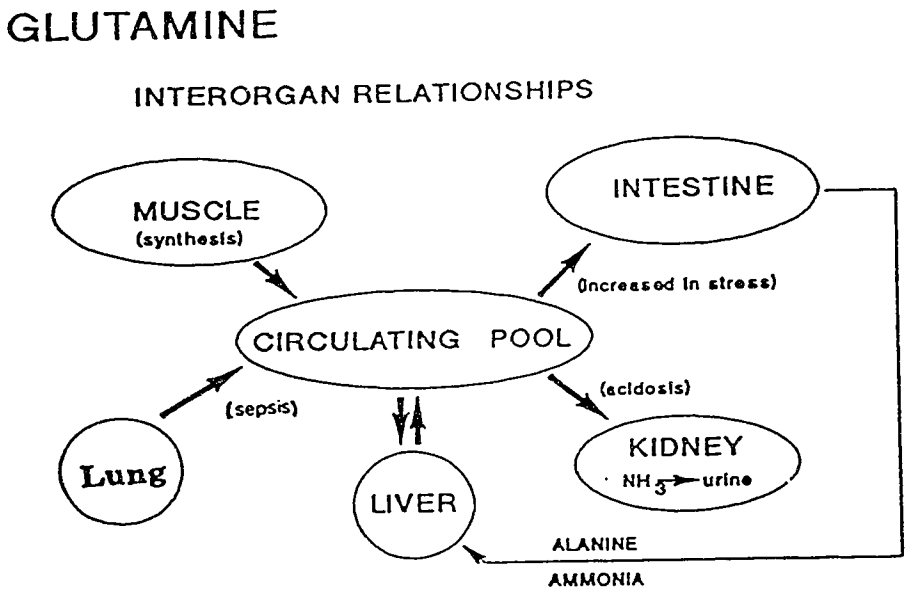


Figure 4: Interorgan relationships of glutamine. Bold arrows indicate predominant direction of glutamine transfer. (Adapted from Klimberg and Souba [18]).

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CHAPTER 3

SURGICAL ANASTOMOSIS INJURY AND GLUTAMINE

BACKGROUND

Glutamine has been shown to benefit the intestine in several injury models [1-8], most of which have demonstrated positive effects to morphological parameters. From the outset, our project sought to examine the effects of glutamine on intestinal function. The choice for initial assessment of bowel function was that of fluid absorption. *In vivo* fluid absorption loops were used to measure absorption of 154 mM sodium chloride solution [9]. The result is expressed in microlitres absorbed per centimetre of intestine per hour. If significant alterations in fluid absorption were noted, this could be followed up with more specific *in vitro* measurements of intestinal function such a sodium and glucose transport or chloride secretion.

INTESTINAL ABSORPTIVE FUNCTION

Intestinal absorptive epithelial cells all share in common Na,K-ATPase protein on the basolateral membrane [10-12]. This sodium pump, driven by

energy from ATP, maintains low intracellular sodium concentration and high intracellular potassium. An electrochemical gradient is preserved which is used for absorption of a variety of ions. An assortment of transport proteins specific for particular substrates exist in the apical membrane of the enterocyte. These proteins use the gradient created by Na,K-ATPase to drive solute transport. Both cotransport with sodium and exchange for sodium occur. Amino acids and glucose are absorbed with sodium down the electrochemical gradient. Hydrogen ion secretion is coupled with sodium absorption through a brush border exchange protein. Linkage between sodium and chloride absorption is less clear.

Chloride secretion accompanies loss of water into the intestinal lumen which occurs in diarrheal conditions [12]. Chloride from the blood is coupled with sodium movement across the basolateral membrane down the electrochemical gradient. It then passes freely through the chloride permeable membrane into the intestinal lumen. Chloride secretion is therefore ultimately dependent on Na,K-ATPase as it is this protein which is responsible for the electrochemical gradient. Chloride secretion occurs in the intestinal crypts while sodium and water absorption predominate in the villi.

The preponderance of net water movement across intestinal cells occurs as a passive process in response to osmotic forces. Thus, it is primarily dependent on active transport of solutes which themselves depend on Na,K-ATPase.

In vivo fluid absorption, the technique used to measure intestinal absorption in this project, expresses gut function as volume of saline absorbed per unit time per cm of intestine. It is therefore a reflection of many diverse processes which ultimately result in passive net fluid absorption.

CHOOSING A MODEL

The initial injury model selected was the acetic acid colitis model in rats. The aim was to examine the effect of glutamine on colon absorptive function in colitic rats. A secondary question was to determine whether there is a requirement for luminal contact of glutamine. This was to be addressed by creation of colitis in a Thiry-Vella fistula [13], allowing direct luminal infusion of glutamine into the diseased segment. Technical difficulties rendered this model (Appendix II) less practical. Also, glutamine has been shown to be beneficial whether supplied parenterally [1,14-20] or enterally [2-8, 21-23]. For these reasons, closer study of the surgical anastomosis model was undertaken.

Adaptive changes that occur over weeks to months following massive small bowel resection have been partially explained [24], however little is known about the injurious effects of transection and anastomosis alone. Our pilot studies demonstrated impaired fluid absorption around a segment of limited ileal resection. Clinical relevance of this absorptive dysfunction is not known, however if it is prolonged, it may be important. If recovery of impaired intestinal absorption could be enhanced, then perhaps patients could benefit by earlier resumption of a normal diet. We therefore decided to look more closely at the functional and morphological changes that occur near a surgical anastomosis.

Intestinal fuels have been studied with respect to healing of a colonic anastomosis [25]. Intraluminal infusion of short chain fatty acids resulted in a significantly stronger colonic anastomosis in rats. Along similar lines, it seemed reasonable to study the effects of the small intestinal fuel, glutamine, on intestinal absorption around a limited ileal resection and anastomosis.

EXPERIMENTAL DESIGN

The experiment consisted of two parts. The initial stage plotted a time-course of intestinal absorptive function against time. From this, a suitable time in the postoperative period was selected for closer examination. Part 2 carefully studies the effects of a surgical anastomosis on intestinal fluid absorption, morphometry and glutaminase levels with and without supplemental dietary glutamine (See Figs III-1,III-2).

MATERIALS AND METHODS

GENERAL

ANIMALS

Male Sprague-Dawley rats (200-300 g, Biotron, University of Alberta, Edmonton, Alberta, Canada) were used for experiments 1 & 2. Animals were allowed to acclimatize at least 48 hours in the animal facility prior to entry into the study. They were housed in individual cages, were maintained on a twelve hour light-dark cycle and were allowed water intake *ad libitum.*

CHEMICALS

Glutamine, glycine, alanine, Beta NAD (from yeast, FW=663.4), 2',5'&3',5' ADP (FW=427.2), glutamic dehydrogenase (type II, from bovine liver in 50% glycerol) were all purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. Other chemicals were reagent grade, purchased from either Sigma or Fisher Scientific, Nepean, Ontario, Canada

NUTRITION/FEEDING

Animals were fed standard chow (Purina Mills Inc., St. Louis, Mo.) until randomization to either a glutamine or isonitrogenous control enteral diet. Vital HN (Ross Laboratories, Montreal, Quebec, Canada) was available in powder form for oral intake, *ad libitum*. Each 79 g (1260 kJ) package was mixed with 5.1 g of glutamine powder to approximate glutamine intake in other studies [2-8]. Controls were supplemented with isonitrogenous amounts of glycine. Food containers were weighed and augmented daily in order to accurately record and control intake.

OPERATIVE PROCEDURES

Rats were anaesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Oxygen was given by mask and a warming lamp was placed over the operative field. The abdomen was shaved and after a midline laparotomy incision, a 10 mg dose of cefazolin (40 mg/kg) was administered in the peritoneal cavity.

The injury model anastomosis was performed by first isolating a 10-15 cm segment of terminal ileum. Under 2.5 times magnification, mesenteric vessels were ligated with 6-0 silk ties on both sides of a one centimetre segment of intestine. This portion of bowel was resected with scissors. The anastomosis was initiated over a macaroni stent [26] using interrupted sutures of 6-0 silk. On completion of half the anastomosis, the stent was removed and discarded. The remainder of the anastomosis was finished without the stent, making a total of about eight sutures. Saline was injected to mildly distend the intestinal lumen, checking for the possible need for additional sutures to control leakage. The

peritoneal cavity was washed with warm saline and the midline incision was closed with a running suture of 3-0 silk.

Sham-operated controls underwent an operative procedure to simulate surgery described above, apart from the anastomosis. Controls received identical anaesthetic and antibiotics. The terminal ileum was similarly isolated and the mesenteric vessels were surrounded by 6-0 silk without actually ligating them. The animals were kept under anaesthetic for 30 minutes with periodic manipulation and irrigation of the segment of bowel to mimic the handling which occurred with anastomosis rats. The abdomen was closed and the animals were allowed similar recovery conditions in a warmed chamber with supplemental oxygen.

MEASUREMENTS

IN VIVO ABSORPTION LOOPS

In vivo fluid absorption loops were performed to assess intestinal function as previously described [9]. Briefly, a 10-15 cm segment of intestine was isolated at laparotomy. Stool was gently manually expressed from the segment. The empty portion of bowel was tied at each end with a 3-0 silk tie, taking care to not interrupt vascular supply. This empty closed loop was filled by injecting 2 ml of warm saline through a 26 gauge needle. The abdomen was closed and the animal allowed to recover for a period of one hour. At the end of this time period, the animal was sacrificed with a lethal injection of pentobarbital. The closed loop of intestine was removed intact, and the length measured. The segment was weighed with and without the remaining luminal saline. Absorption of saline,

expressed in microlitres per cm intestine per hour, was calculated as an indication of bowel function.

HISTOLOGY

After *in vivo* absorption loops were completed, a 3 mm length of intestine (1 cm and 5 cm away from the anastomosis) was carefully cut and stored in formalin for later hemotoxylin-eosin staining and blinded evaluation of inflammation by a single pathologist. Inflammation was graded on a 3-point scale of 0 through 2.

MORPHOMETRY

Using a computerized videoplan and blinded to experimental group, morphometric parameters were measured and averaged from three separate fields of each section. Villus height, number of villi per centimetre and lamina propria area : perimeter index [27] were evaluated.

GLUTAMINASE ASSAY

Intestinal glutaminase activity reflects enterocyte support from dietary glutamine and was therefore measured. Soon after sacrifice, intestinal samples were rinsed with cold saline and then frozen by plunging a tube containing the intestinal segment in liquid nitrogen. These were stored at -70° C. until thawed for the assay. The glutaminase assay was performed after adapting methods by others [28,29] (see Appendix I). Homogenate protein was measured using the Bradford method [30]. Glutaminase activity was thus expressed as micromoles per mg protein per hour.

DNA CONTENT

DNA content of intestinal homogenates was measured as described by Hindgardner [31]. DNA was expressed as mg DNA/mg protein.

STATISTICAL METHODS

Descriptive statistics were applied to all measured parameters and results were expressed as means +/- standard error of the mean (S.E.M.). Weight gain and food intake were expressed as means +/- standard deviation (S.D.). Analysis of variance was applied to compare groups, and a p value of <0.05 was considered significant.

RECOVERY AFTER SURGICAL ANASTOMOSIS - TIME COURSE

This initial experiment determined intestinal absorptive function over a 10-15 cm segment around a surgical anastomosis during different times in the postoperative period (Figure III-1). Rats were assigned to either a glutamine or glycine-supplemented diet. Each rat was pre-fed the particular diet *ad libitum* for five days preoperatively and in the postoperative period until *in vivo* absorption loops were performed. Animals were not pair-fed between dietary groups. Either a surgical anastomosis or sham laparotomy was performed as previously described. *In vivo* absorption loops were performed on days zero through five in anastomosis animals and on days zero and one in the sham-operated rats. The operator was blinded as to the diet of each animal but not to the postoperative time that loops were performed. Animals were not strictly randomized, and replacements were arbitrarily entered into the study when necessary. Intestinal fluid absorption was calculated for all groups and was plotted against time.

Statistical comparison among groups of was not implemented. Results of this time course were used in planning the next experiment.

INTESTINAL FUNCTION ONE DAY AFTER SURGICAL ANASTOMOSIS

As intestinal absorptive function appeared to be impaired until day one, this time was chosen to examine more closely the effects of a surgical anastomosis. A more stringently controlled experiment, studying fluid absorption, intestinal morphometry and the effects of glutamine was designed.

Forty rats were randomly assigned to one of four groups: 1. glutamine sham, 2. glutamine anastomosis, 3. glycine sham, and 4. glycine anastomosis. No replacement animals were entered into the study. Rats were fasted (except water) for four days in order to induce villus atrophy and to deplete intestinal glutaminase [22]. They were then fed Vital HN powder diet with glutamine, and glycine supplemented animals were pair-fed to ensure isonitrogenous intake between dietary groups.

On day zero, animals underwent either sham or 1 cm ileal resection and anastomosis, with the operator blinded as to the dietary group. Rats were allowed to recover and were fed their respective diets for one more day. Postoperative intake was restricted to ten grams per 24 hours to ensure that sham and anastomosis groups had similar intake.

On day one, rats had assessment of bowel function by *in vivo* absorption loops (blinding to dietary group). Samples for histology and morphometry were taken from the middle of the 10-15 cm ileal loop in sham animals. Anastomosis

animals had similar samples taken both near (1 cm) and far (5 cm) from the resection margin. Whole intestine was stored at -70^{o} C for assay of glutaminase enzyme, protein and DNA content. These data were tabulated and compared among the four groups by two-way analysis of variance (ANOVA). Food consumption, glutamine intake and weight change were also compiled.

RESULTS

TIME COURSE

Performing *in vivo* fluid absorption loops around the surgical anastomosis was initially difficult due to adhesions to the suture line region. The loop had a tendency to leak at the anastomosis, especially if evaluated on day two. Greater care to perform absorption loops *in situ* had to be implemented after onset of experiment 1. Leakage during assessment of intestinal absorption necessitated replacement with another animal at the study entry-point.

Forty-four rats satisfactorily completed this pilot experiment which yielded a time course with respect to intestinal absorptive function around a surgical anastomosis. In Figure III-3, each bar represents fluid absorption averaged in four to six rats. Absorption is decreased around the anastomosis on days zero and one. Each of the groups in Figure III-3 is half represented by glutamine-supplemented rats, the remainder having received glycine. There was no readily apparent difference between dietary groups from the time course experiment (data not shown).

INTESTINAL FUNCTION ON DAY ONE

Of the forty rats randomized to diet (glutamine, glycine) and operative procedure (sham, anastomosis), five died during the four day fasting period. This was prior to initiation of the amino acid supplemented diet. These animals included three who were to receive glycine and sham laparotomies in addition to two planned for anastomosis (one glutamine and one glycine). Five additional rats were excluded from the study due to death or technical difficulties at the time of *in vivo* absorption loops. This group comprised of three anastomosis animals, all receiving glycine, and two sham animals (one from each dietary group). Results are presented for the thirty remaining rats except for weight gain and food intake, which includes those rats surviving to the final operative procedure.

FOOD INTAKE

Daily food intake during the five day preoperative feeding period demonstrated successful pair-feeding as intake was similar between dietary groups. Glutamine supplemented animals consumed 14.8 +/- 3.4 g/day while those in the glycine group ingested 15.1 +/- 3.0 g/day (mean +/- S.D.). This corresponded to a mean daily glutamine intake of 0.96 grams in the preoperative period.

WEIGHT CHANGE

All rats lost weight during the four day fasting interval and all subsequently gained during the five day feeding period. Mean +/- S.D. values are reported in table III-1 and are similar in each dietary group.

IN VIVO FLUID ABSORPTION LOOPS

Fluid absorption in the 10-15 cm ileal segment is detailed in table III-2 and graphed in Figure III-4. *In vivo* fluid absorption around the anastomosis was decreased compared to absorption of the ileal segment in sham-operated animals by two way ANOVA ($p<0.05$). There was no significant difference noted between glutamine and glycine in either operative group.

HISTOLOGY

Blinded evaluation of microscopic sections revealed varying amounts of serosal inflammatory infiltrate in both sham and anastomosis animals. No mucosal lesions were noted. There was no appreciable difference between glutamine and glycine groups with respect to inflammation. Dietary groups have been pooled to depict inflammation in anastomosis animals (Figure III-5). Inflammation of higher grades occurred more frequently in sections near to compared to far from the anastomosis.

MORPHOMETRY

No significant differences were noted between dietary groups with respect to villus height, number of villi per centimetre and lamina propria area : perimeter index. Similarly, these morphometric parameters did not differ between sham, near anastomosis or far anastomosis sections (Figures III-6 - III-8).

GLUTAMINASE

Intestinal glutaminase was also not significantly altered by diet or operative procedure in the ileal segments (Figure III-9, III-10). Because

glutamine showed no benefit to ileal segments, jejunal glutaminase was measured to determine whether dietary group was having any influence on proximal intestine. Jejunal glutaminase was somewhat higher in the glutamine-supplemented animals (p=0.08). Mean jejunal glutaminase was 4.70 +/- 0.43 micromoles/mg protein/h in glutamine-fed rats compared to 3.58 +/- 0.27 micromoles/mg protein/h in the glycine groups (Fig. III-11).

DNA CONTENT

Jejunal segments were analyzed to determine if glutamine-supplemented animals were deriving benefit to DNA content in the proximal gut. Jejunal homogenates showed a trend toward higher DNA in glutamine-fed rats (p=0.09). Expressed in mg DNA per mg protein, DNA was 0.294 +/- 0.037 in glutamine supplemented rats compared to 0.206 +/- 0.021 in those receiving glycine (Fig. III-12).

DISCUSSION

Intestinal absorptive function around a surgical anastomosis was impaired significantly on day 1 as assessed by *in vivo* fluid absorption loops. The time course revealed this expected intestinal dysfunction (Fig III-3), however the apparent return to normal absorption by the second postoperative day was somewhat earlier than expected. The decreased absorption around the anastomosis represents a gross measurement of intestinal function. Fluid is absorbed in accordance with osmotic forces which are ultimately driven by active processes based on Na,K-ATPase in the basolateral membrane [10]. A more specific evaluation of intestinal absorption around a surgical anastomosis may

reveal specific nutrient fluxes that are also depressed. However, in our study, we pursued the effects of glutamine on the gut, rather than specific mechanisms of intestinal function.

In vivo fluid absorption loops represent mean absorption over a given segment of intestine. Our measurements included 10-15 cm around a surgical anastomosis. It is likely that absorption was markedly impaired (possibly resulting in net secretion) immediately adjacent to the anastomosis, while function may have been near normal at the end of the loop.

The mechanism of impaired intestinal fluid absorption around the anastomosis may in part be due to local ischemia. A temporary decrease in local blood flow may reduce effectiveness of the sodium-potassium pump which maintains the electrochemical gradient. Local release of prostaglandins by inflammatory cells may also have a deleterious effect on intestinal function around the anastomosis.

The significance of impaired fluid absorption around the anastomosis in our model is questionable. It was of fairly short duration (until and including the first postoperative day) and was measured over a limited segment of intestine (10-20% of the total intestinal length). The effect of an anastomosis may also be quite different in the human, as sutures are placed much further apart.

Despite these uncertainties, the impaired fluid absorption around the anastomosis was statistically significant, and by day 2, returned to normal. This process of recovery of intestinal function was felt to be suitable to test for beneficial effects of glutamine. Along with impaired fluid absorption was a higher inflammation grade near compared to far from the anastomosis. This is

not surprising, however, the changes were noted to be relatively minor and only related to the serosal aspect of intestine.

Glutamine was of no benefit to fluid absorption around the ileal surgical anastomosis. This result initially indicated that either glutamine could not improve intestinal function in our injury model, or perhaps that glutamine was not present in sufficient quantities to support the ileum. Results for DNA and three morphometric parameters also failed to demonstrate benefit in the glutamine-supplemented animals. DNA and intestinal morphometry are architectural characteristics that have been improved by glutamine in other studies [1-8,22]. Our animals also showed no positive effect on intestinal glutaminase activity in the glutamine group.

Clearly, the negative results in such a variety of measurements, suggests that glutamine was having no effect on the ileum whatsoever. It is therefore difficult to conclude that unimproved fluid absorption is a feature attributable to glutamine. It is more likely that for some reason, the ileum was unaffected by glutamine in our model.

The quantity of glutamine (0.96 g/day) consumed was probably adequate. Other studies have demonstrated beneficial effects with similar amounts, in the range of 0.8-1.0 g/day [2-8].

The oral route of administration was not expected to be a problem, as glutamine has supported both morphometrics and glutaminase activity with feeding by mouth [22]. However, these benefits were noted in the jejunum, not the ileum. In fact, our results for jejunal glutaminase are in close agreement with those of Souba's group [22], (see Table III-3). As our animals appeared to have

increased glutaminase and DNA in the jejunum, we concluded that the rats consumed significant amounts of glutamine, however, the ileum was not able to respond.

Why the ileum was not responding to glutamine became the focus of attention. It may be that the ileum is incapable of deriving benefit from glutamine. However, this explanation seems less likely. Probably, the ileum in our experiments was not exposed to sufficient quantities of glutamine.

Although glutamine has been beneficial to jejunum [8,22], and colon [2,4,5] when supplemented through the gastrointestinal tract, it has only supported beyond jejunum when an element of malabsorption was present. In these models of enterocolitis, glutamine has probably been retained in the intestinal lumen, where it can act locally on the distal intestine. Glutamine has also improved gut morphology in residual ileum after a 60% small bowel resection in rats [32]. In this situation, presumably the shortened intestine allowed glutamine to reach the residual ileum through luminal transit.

Glutamine can be utilized by enterocytes, whether supplied arterially or luminally [33]. If, however, glutamine is absorbed and transformed by the jejunum, then it may not be able to act systemically on the ileum. Glutamine is largely transformed by the intestine, as indicated by portal blood analysis [34].

In our model, glutamine was likely utilized in the proximal gut, thereby eliminating enteral glutamine. This, coupled with an insufficient rise in blood glutamine, may explain why the ileum did not respond. The importance of proximity to glutamine entry into the GI tract may have important implications with respect to enteral feeding. This issue is addressed in the next chapter.

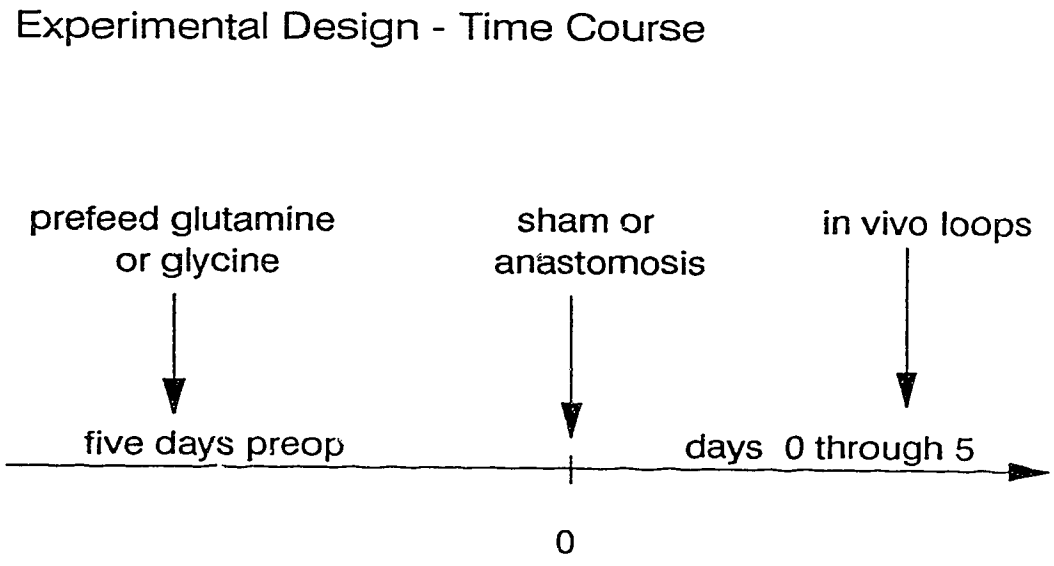

Figure III-1: Experimental design, mapping a time course of intestinal fluid absorption around a surgical anastomosis.

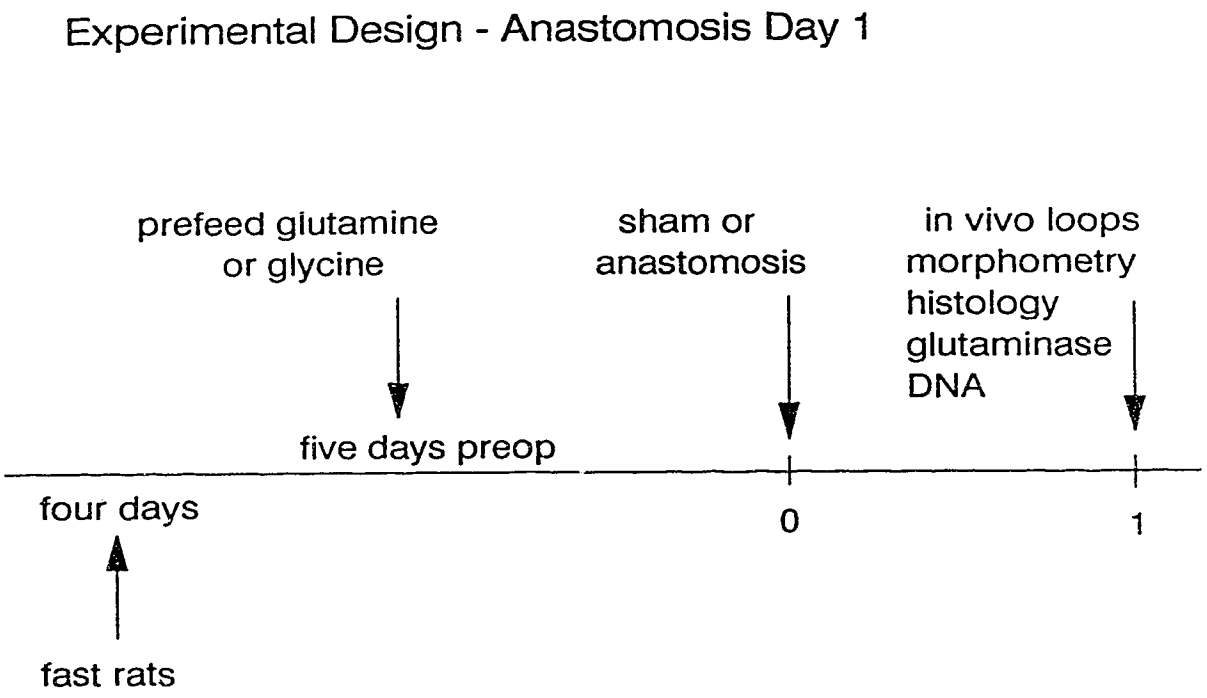

Figure III-2: Experimental design, comparing intestinal function around a surgical anastomosis between glutamine and glycine.

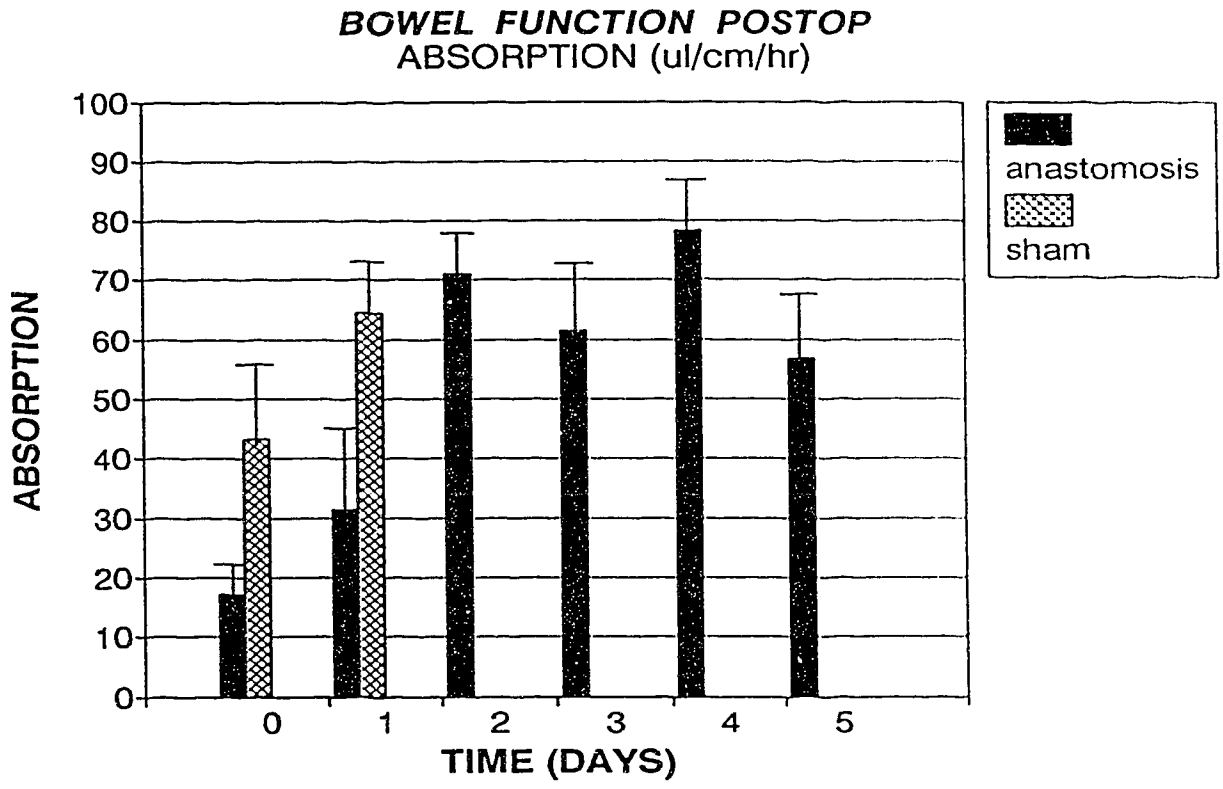

Figure III-3: Intestinal fluid absorption around a surgical anastomosis until the fifth postoperative day.

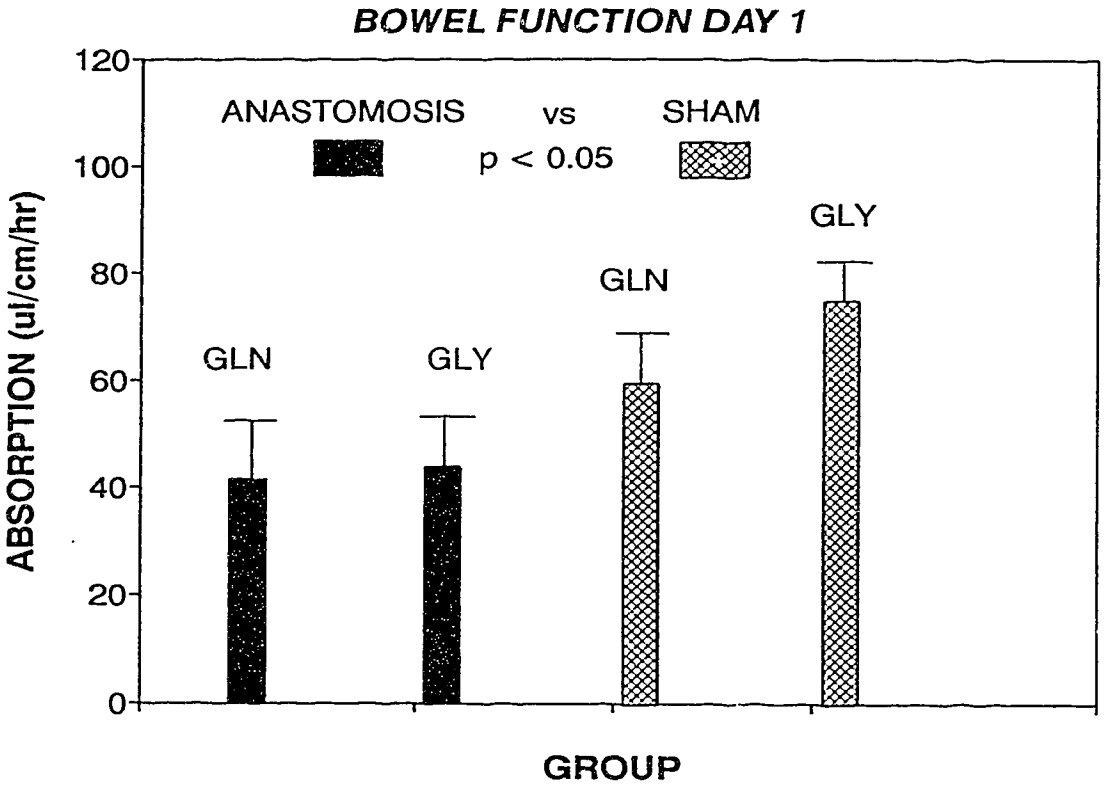

Figure III-4: Intestinal fluid absorption around a surgical anastomosis comparing anastomosis vs. sham and glutamine vs. glycine.

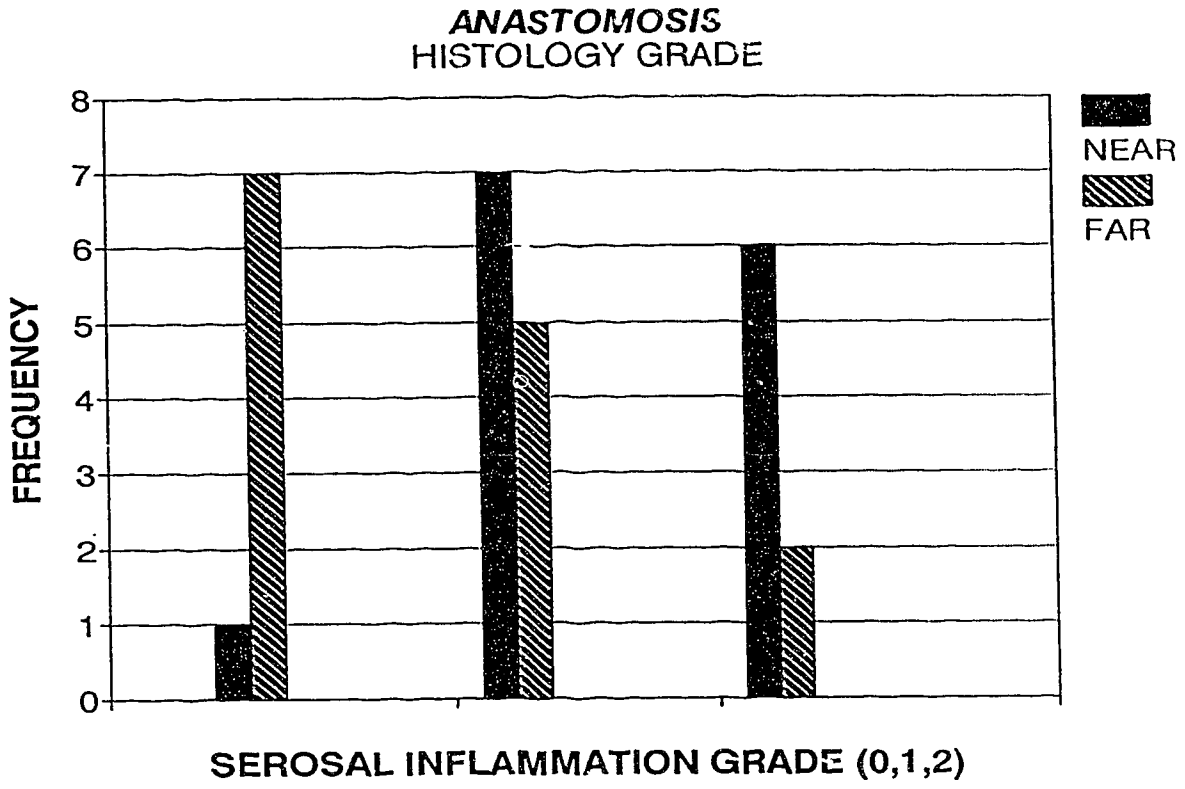

Figure III-5: Inflammation near (1 cm) and far (5 cm) from the surgical anastomosis (expressed in absolute numbers).

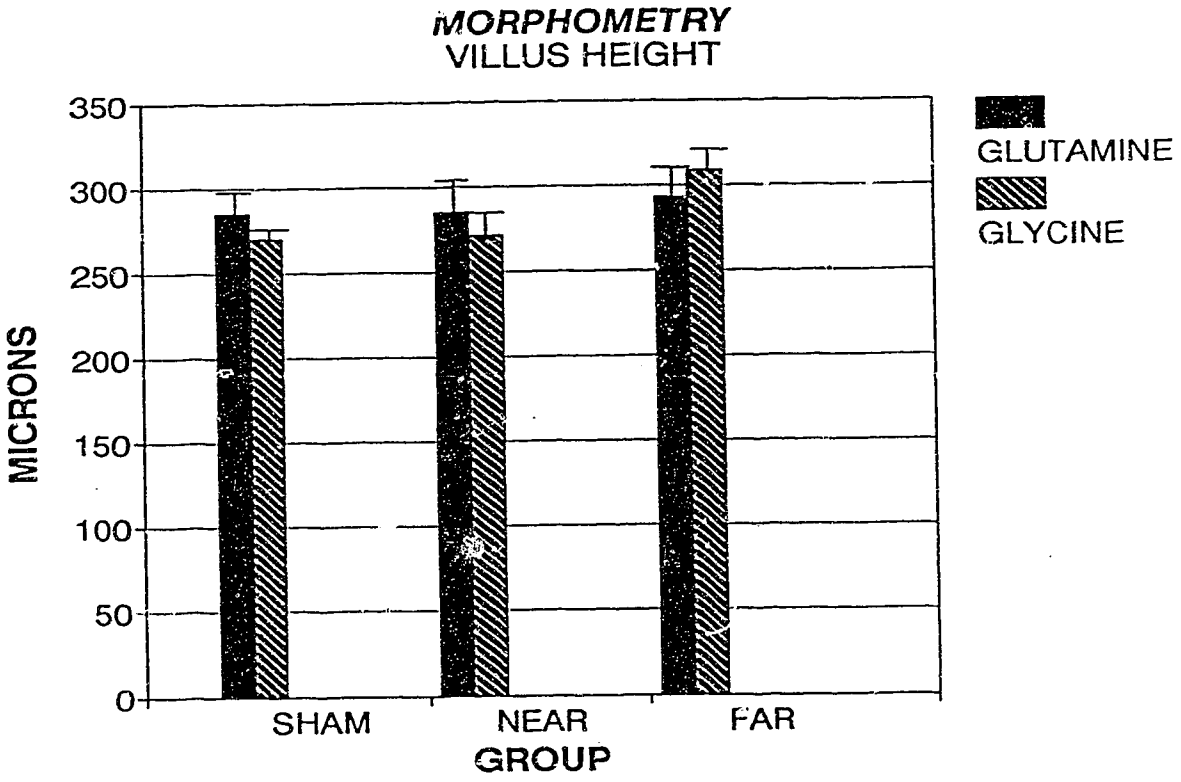

Figure III-6: Villus height in sham-operated controls and anastomosis (near and far) animals.

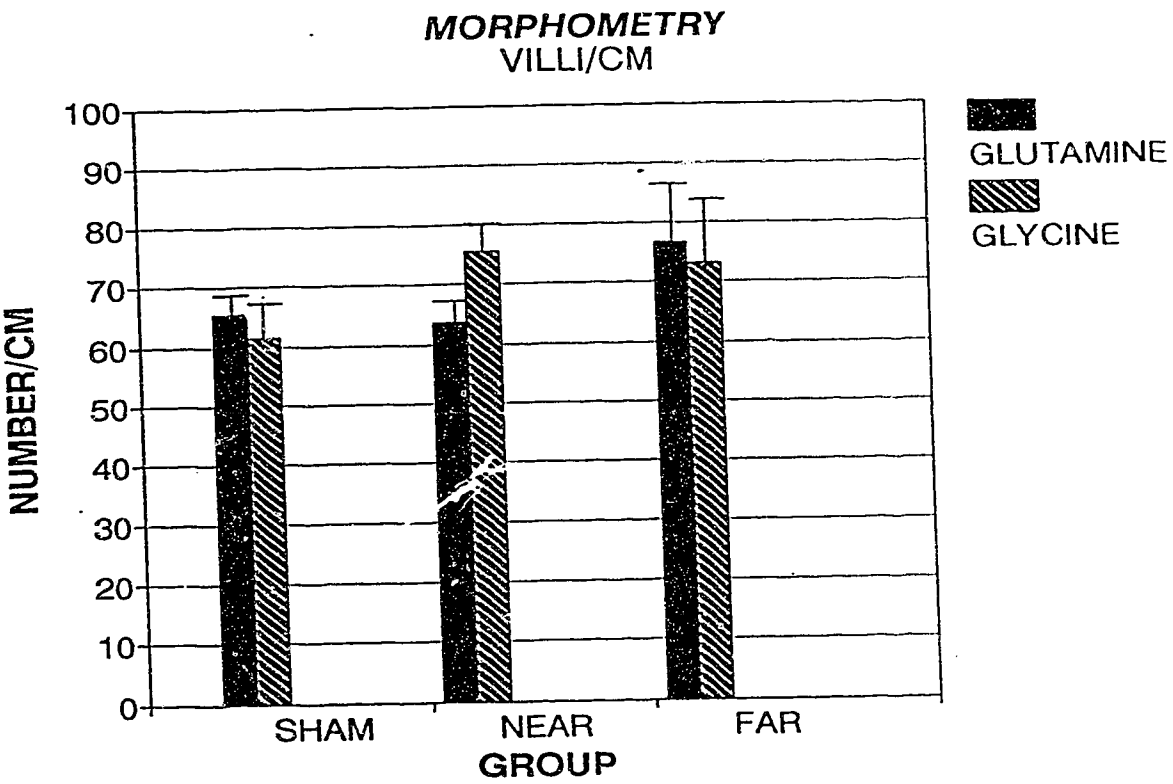

Figure III-7: Number of villi/cm in sham-operated controls and anastomosis (near and far) animals.

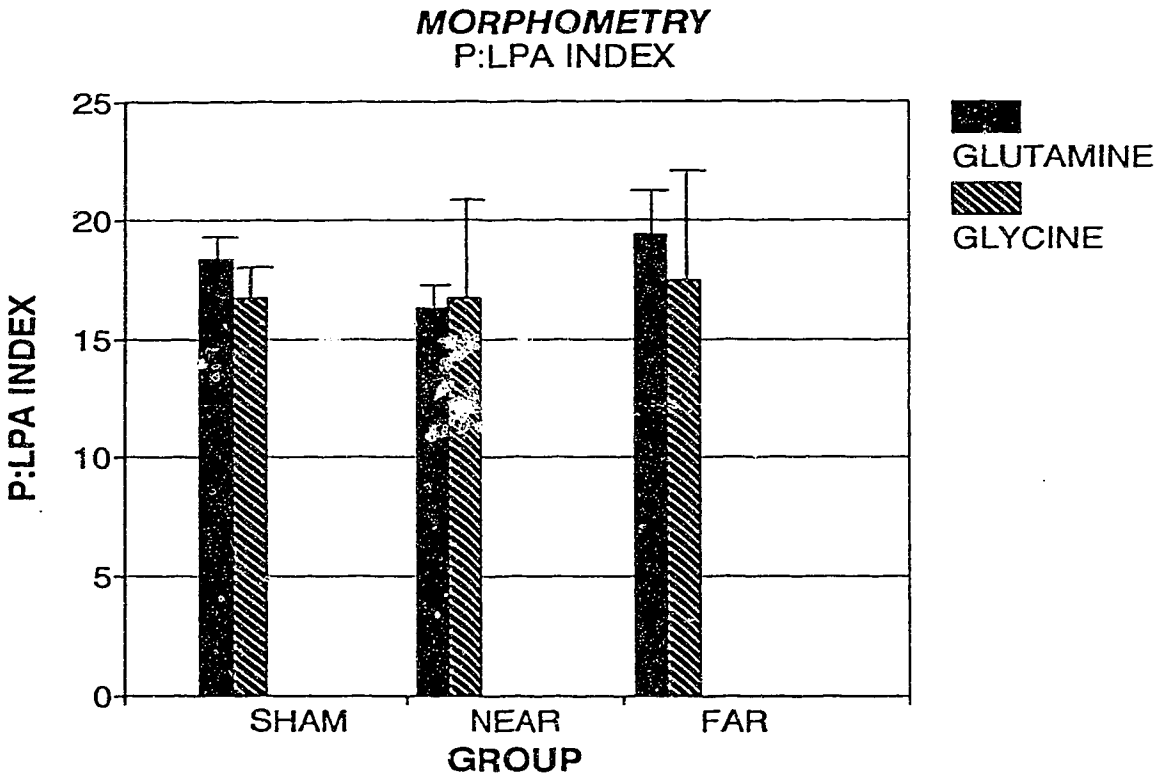

Figure III-8: Perimeter:lamina propria area index in sham and anastomosis (near and far) animals.

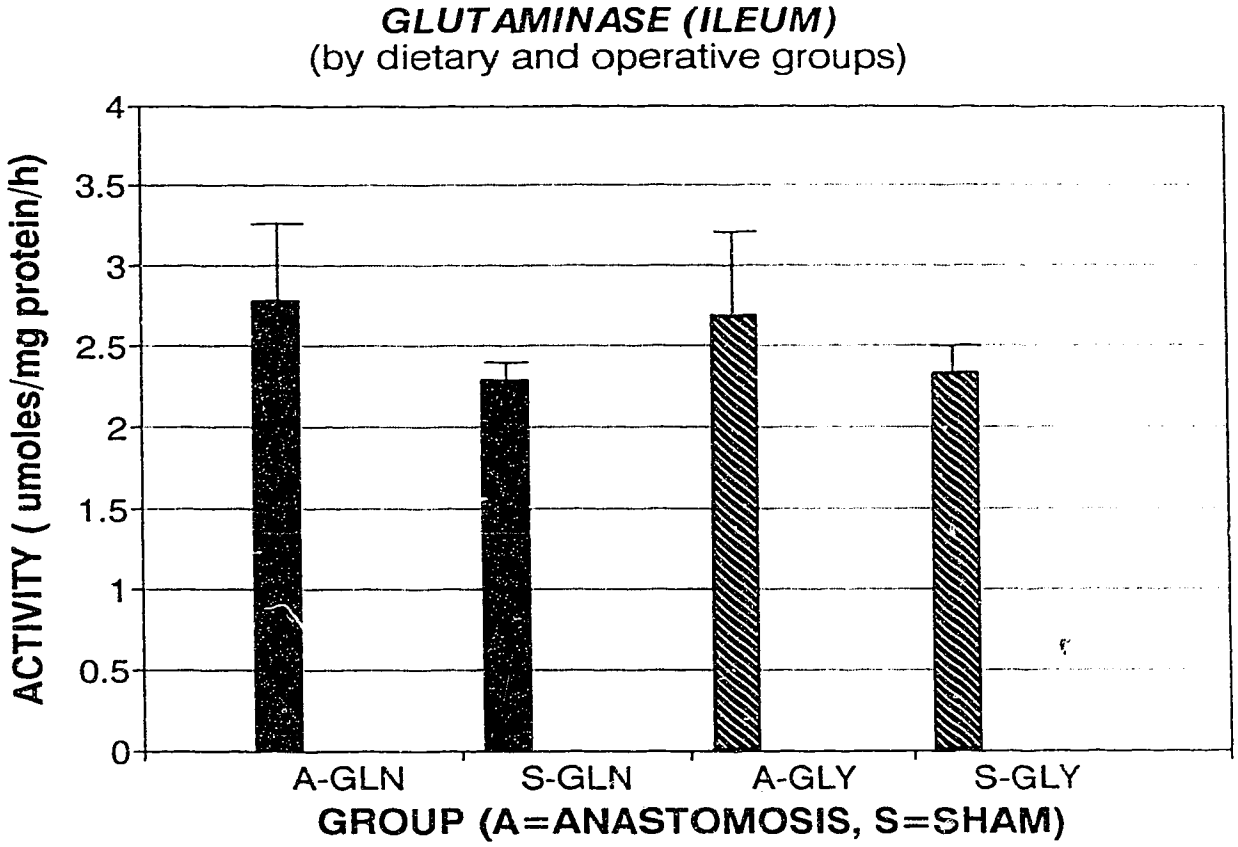

Figure III-9: Glutaminase activity in sham and anastomosis animals, comparing glutamine and glycine supplementation.

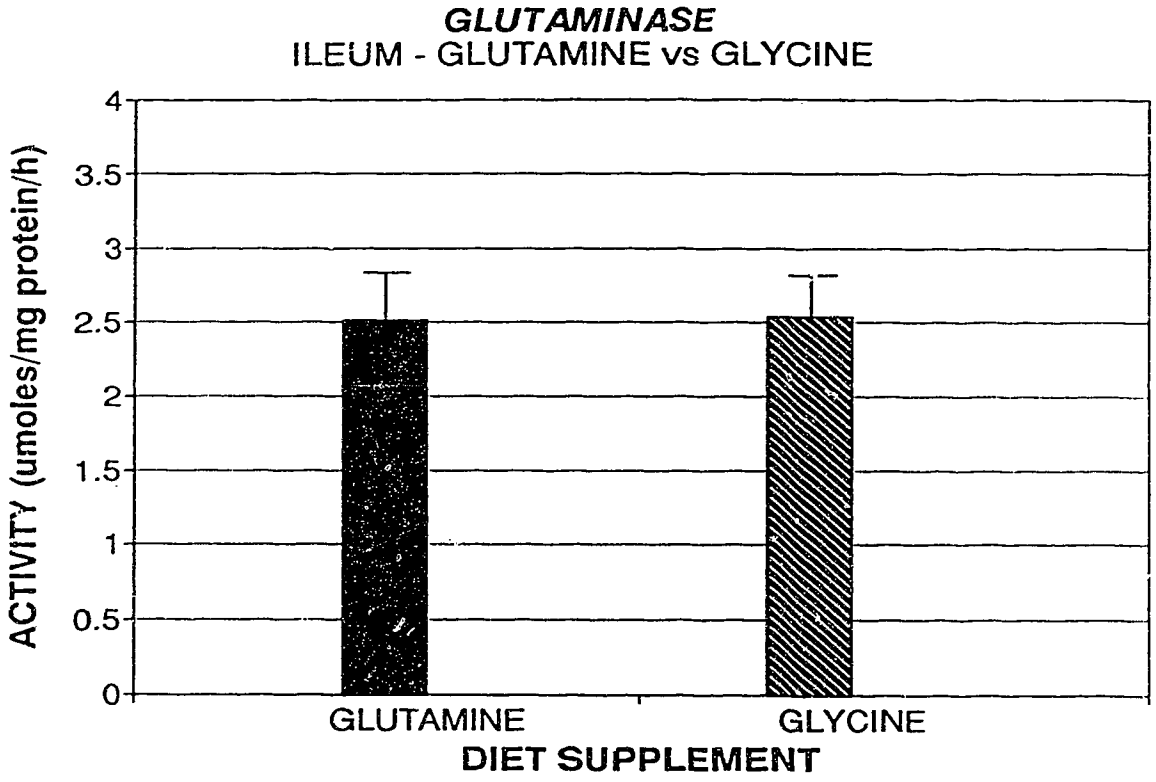

Figure III-10: Glutaminase activity in ileum. Results of sham and anastomosis groups are pooled.

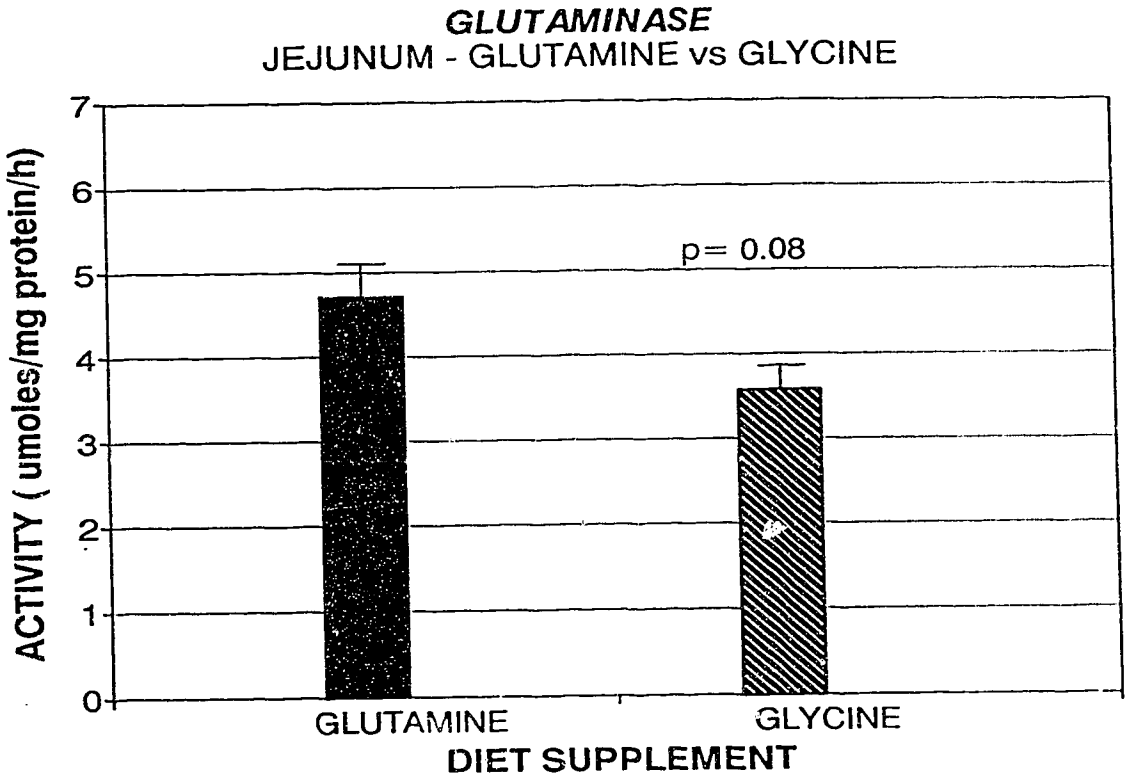

Figure III-11: Glutaminase activity in jejunum. Results for ileal anastomosis and sham animals are pooled.

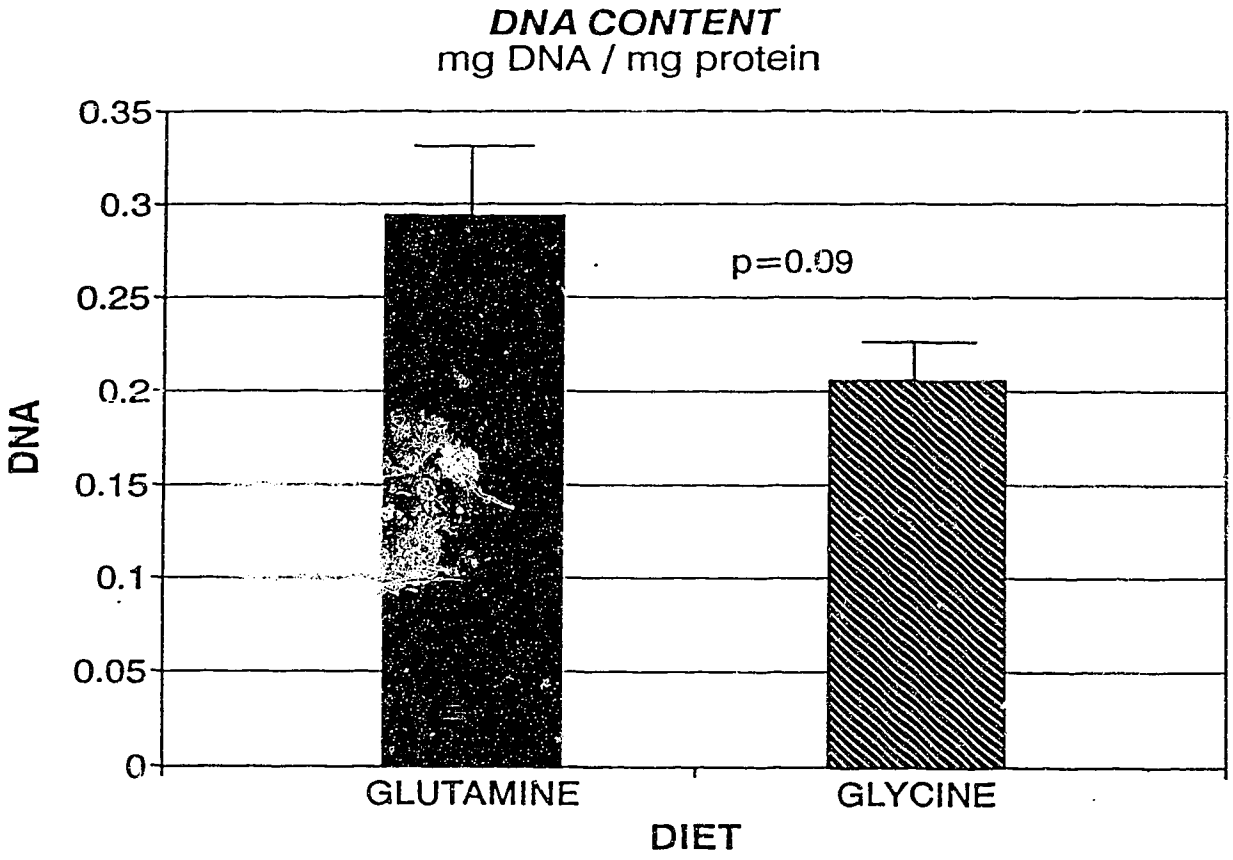

Figure III-12: DNA content in jejunum. Results of both sham and ileal anastomosis animals are pooled.

Table III-1

Preoperative Intake and Weight Change

	GLUTAMINE	GLYCINE
Intake (g/day)	14.8 +/- 3.4	15.1 +/- 3.0
weight loss (g) (fasting)	69.4 +/- 6.2	68.4 +/- 5.9
weight gain (g) (preop feeding)	37.4 +/- 12.5	48.1 +/- 7.7

mean +/ - S.D.

Table III-2

FLUID ABSORPTION - 1 Day Post-op

	ANASTOMOSIS *		SHAM	
	GLUTAMINE	GLYCINE	GLUTAMINE	GLYCINE
n	9	7	8	6
Mean (ul/cm/h)	41.1	43.9	59.1	74.7
S.E.M.	11.5	9.6	9.8	7.9

* $p < 0.05$ by ANOVA vs. SHAM

Table III-3

GLUTAMINASE RESULTS

micromoles / mg protein / h

	ILEUM	JEJUNUM	JEJUNUM (SOUBA)
GLN	2.52 +/- 0.33	4.70 +/- 0.43	4.31 +/- 0.23
GLY	2.54 +/- 0.29	3.58 +/- 0.27	3.56 +/-0.29

(means +/- SEM)

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CHAPTER 4

ILEAL INTERPOSITION AND EFFECTS OF GLUTAMINE

BACKGROUND

Glutamine has been demonstrated to support intestinal growth whether given parenterally or enterally, however, preferred routes of administration in various circumstances have not been fully delineated.

Although the experiments in Chapter 3 demonstrated no beneficial effect from glutamine on the anastomosis injury, this is likely because no glutamine was reaching the ileum. Our results of jejunal glutaminase assay are similar to those of Souba [1], however similar effects were not noted in the ileum, nor were their any of the expected morphometric changes.

Our negative results in the ileum are either explained by a lack of dietary glutamine exposure to the ileum, or an inability of the ileum to respond to glutamine in a similar manner as does the jejunum. The following experiment attempted to answer this question, as important clinical implications may be inferred, depending on the results.

By surgically moving a portion of ileum in continuity to the proximal small intestine, the interposed segment receives luminal contents early in transit

through the GI tract. This scenario allowed comparison of response to glutamine among jejunum, proximal (interposed) ileum and distal ileum and for the two dietary groups.

MATERIALS AND METHODS

Unless otherwise stated, materials and methods were employed in a similar fashion as explained in Chapter 3.

Twenty-five chow-fed rats (300g, Charles River, Quebec) underwent an interposition of ileum to the proximal jejunum (see Figure IV-1). The ileal segment was approximately 8 cm in length and was placed in an isoperistaltic direction. The interposed ileal portion was sutured to jejunum at both ends using a running 6-0 silk. Mid-ileum was joined to terminal ileum in a similar manner to restore intestinal continuity. Macaroni stents were used to complete all three anastomoses, leaving them to dissolve in the intestinal lumen in the postoperative period. Anaesthetic, antibiotics and perioperative care were similar to conditions in chapter 3.

The animals were allowed to recover for five days and survivors were randomized to either a glutamine or an isonitrogenous glycine/alanine - supplemented diet. Addition of alanine to the control dietary group was employed as it more closely approximates an isocaloric, isonitrogenous mixture than does glycine supplementation alone. Prior to feeding, rats were fasted (except water) for three days to induce villus atrophy and to deplete intestinal glutaminase. Animals were then pair-fed to ensure isonitrogenous, isocaloric intake between dietary groups. After five days of elemental feeding, rats were

sacrificed and intestinal samples were taken for morphometric analysis, DNA content and glutaminase activity assay. Morphometry (villus height only), DNA content and glutaminase activity were compared between dietary groups for jejunum, interposed ileum and surgically unaltered ileum.

RESULTS

SURVIVAL

Of the twenty-five rats undergoing ileal interposition, two died in the immediate perioperative period. Three more did not survive the three day fasting period. One additional rat (glycine/alanine group) died within twenty four hours of initiating feeding. The remaining nineteen rats (ten glutamine, nine glycine/alanine) survived for the duration of the study and data from these animals are presented in the results section.

INTAKE

Rats from both dietary groups ate similar amounts, although some rats had noticeably depressed intake. Glutamine-fed rats ate 12.2 +/- 2.43 grams while controls consumed 12.4 +/- 2.46 grams per day (mean +/- S.D.).

WEIGHT CHANGE

All rats lost weight during the fasting period, and all gained during the five days of elemental diet feeding. Weight gain was 18.6 +/- 9.2 (glutamine animals) and 26.1 +/- 10.0 (glycine/alanine).

INTESTINAL CHANGES - GROSS APPEARANCE

At sacrifice, rats were noted to have varying degrees of small bowel distension and mildly reddened intestinal serosa. The interposed ileal segments in particular were somewhat dilated. The changes appeared to be consistent with partial functional obstruction. There was no evidence of mechanical obstruction.

GLUTAMINASE

Glutaminase was increased in jejunal segments of the glutamine group of rats ($p<0.05$, Fig. IV-2). Although there was a trend toward decreased glutaminase levels in interposed ileal segment of glycine/alanine compared to glutamine-fed animals, there were no significant differences noted in glutaminase levels between groups (Figure IV-3).

VILLUS HEIGHT

Villus height was no different in jejunum between dietary groups. Interposed ileum had a significantly higher villus height when paired with native ileum ($p<0.05$). Dietary group did not alter villus height in interposed or native ileal segments. (See Figure IV-4).

DNA CONTENT

Glutamine resulted in no increase in DNA of jejunal, interposed ileal or ileal segments over glycine/alanine controls. Data are shown in Figure IV-5.

DISCUSSION

The ileal interposition model provided a mechanism to examine the effects of glutamine ingestion through the gastrointestinal tract. The literature and our previous results (chapter 3) provide little evidence that glutamine supports ileal mucosal integrity to the same extent as to jejunum. Why jejunum realizes significant improvements in gut morphometry, DNA and glutaminase activity as a result of enteral glutamine, and ileum does not, was the subject of our speculation. By interposing an ileal segment into the proximal intestine, the effect of distal location of ileum was eliminated. The interposed segment was presumably bathed in glutamine concentrations similar to those received by jejunum, and effects could be compared to the animal's own *in situ* ileum.

Our results supply some answers, however questions remain. Jejunal segments showed a response to oral glutamine over glycine/alanine supplementation with respect to glutaminase activity. This is in agreement with Souba's findings [1]. We, did not, however, observe accompanying jejunal villus height or DNA content enhancement. This would suggest that glutaminase activity is a more sensitive or an earlier responder to enteral glutamine than are villus height and DNA. In our experiment, probably the quantity of glutamine was insufficient to elicit a response in villus height. Animals who underwent ileal interposition consumed approximately 60% of the powder diet compared to healthy rats in previous experiments. This would also correspond to 60% glutamine intake compared to Souba's animals.

To expect similar changes to occur in interposed ileum, it would be desirable to see maximal effect in jejunum, which we unfortunately did not

obtain. Interposed ileal segments, did, however, demonstrate adaptive changes irrespective of dietary group. These segments, after approximately ten days in the enteral stream showed significant increases in villus height when paired with their native unaltered ileum. This adaptive change of ileum suggests that gut morphometry may be more attributable to environmental factors such as luminal contents compared to inherently programmed factors.

There were no significant differences between dietary groups in interposed or native ileum. Whether interposed ileum would have shown positive effects from glutamine if the exposure time or dose had been greater, remains speculation. In our model, oral glutamine does not evoke an equivalent response on interposed ileum and unaltered jejunum.

In addition to possible insufficient quantities of glutamine, other factors related to the model may have prevented glutamine effects on interposed ileum. There were varying degrees of dilation of interposed ileal segment. Accompanying edema and grossly visible inflammatory changes may indicate that the model itself not only altered enteral continuity, but it may have also been a variable injury to the intestine. Intestinal morphologic well-being in the interposed segment may have been more due to a chance erratic injury than as a result of dietary treatment. Glutaminase levels in ileal segments were more variable than jejunum which was likely related to an inconsistent injury to interposed and native ileum.

The ileal interposition model includes three small bowel anastomoses (two encompassing the interposed segment and one re-establishing continuity at the terminal ileum). Any one of these could cause partial impairment of luminal

flow. A longer period of adaptation may have reduced the high number of animals with dilated small bowel segments. Other factors may have caused the appearance of reduced small bowel transit. The ileal brake mechanism [2] responds to high concentrations of nutrients resulting in negative feedback on motility of the proximal small intestine. Interposed ileal segments retain venous drainage of the distal ileum, so this poorly understood feedback mechanism may have also contributed to increased intestinal transit time.

Enterally-fed glutamine has imparted morphologic benefit to jejunum in healthy [1] and sick [3-7] animals however, results in ileum have not been as compelling. Jejunal morphology has also been improved with parenteral glutamine [8,9]. A dose response was noted [8], suggesting that a critical amount of glutamine may be required by the enterocyte for it to derive positive effects.

Evidence suggests that ileum is capable of responding to glutamine, if present in adequate supply. Glutamine's rapid disappearance in the presence of intestinal cells was first demonstrated in rabbit ileum [10]. Enteral glutamine improved residual intestine in rats with a 60% mid small bowel resection [11,12]. In these animals, villus height and DNA content were similarly increased in remaining jejunal and ileal segments. Presumably, the ileum was receiving sufficient quantities of glutamine in the luminal stream. The effect was maximal when glutamine represented 25% of the total amino acids. When glutamine was 4-10% of the total amino acid pool, the effect was not noted.

Our ileal interposition model attempted to demonstrate a similar positive response in interposed ileum. This, combined with no effect in native distal ileum would demonstrate a diminishing benefit from enteral glutamine as one moves

distally. However, the quantity of glutamine consumed was not sufficient to induce beneficial changes in the interposed ileal segment.

In humans, glutamine is quantitatively absorbed in jejunum with a dose-dependent increase in plasma glutamine level [13]. It is likely that no glutamine reaches the ileum through the gastrointestinal tract. Whether *in situ* ileum can be positively affected by oral glutamine has not been conclusively demonstrated. In humans [14], approximately 85% of orally administered glutamine did not enter the systemic circulation. Presumably, a large proportion was metabolized by the splanchnic bed and liver. Therefore, high doses of oral glutamine may be required to ensure systemic or luminal delivery to the ileum. Whether such doses can or need to be achieved in practice is yet to be determined.

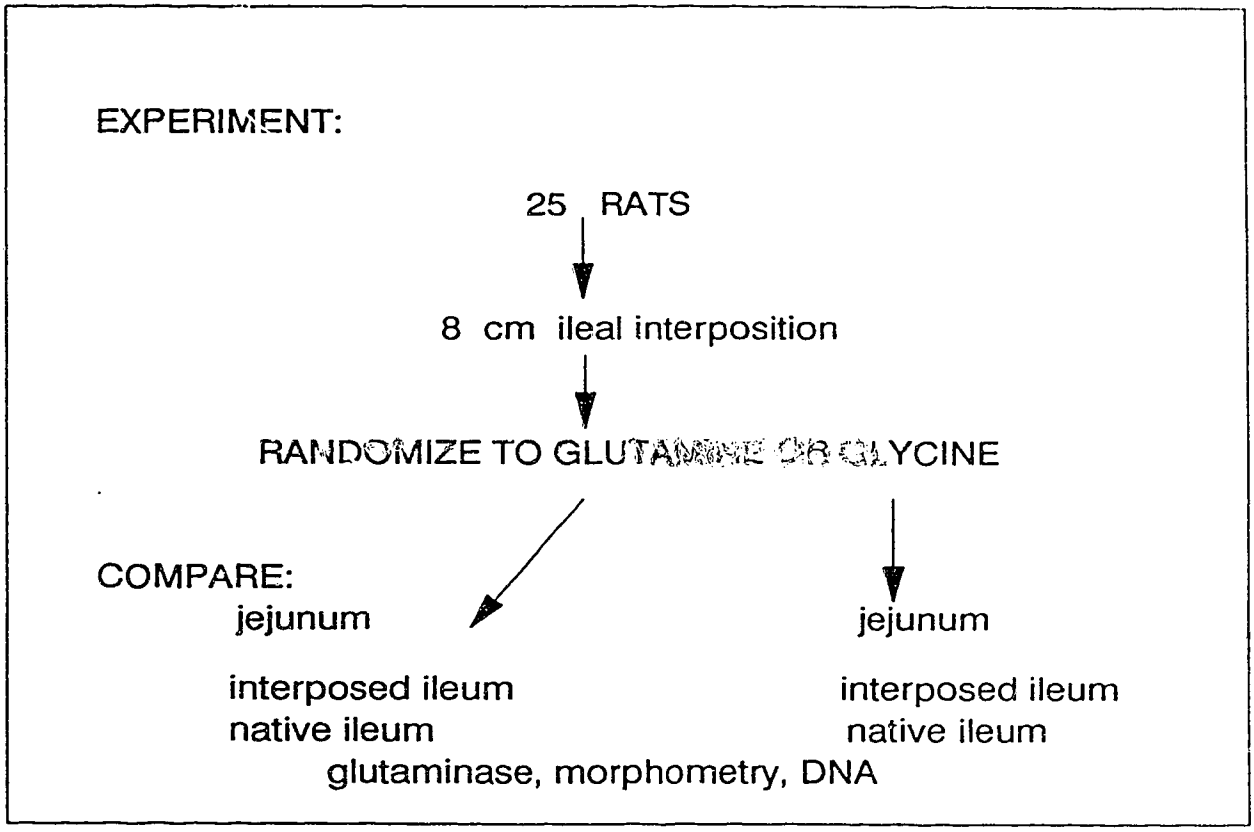

Figure IV-1: Experimental design, the effect of glutamine on intestinal segments in the ileal interposition model.

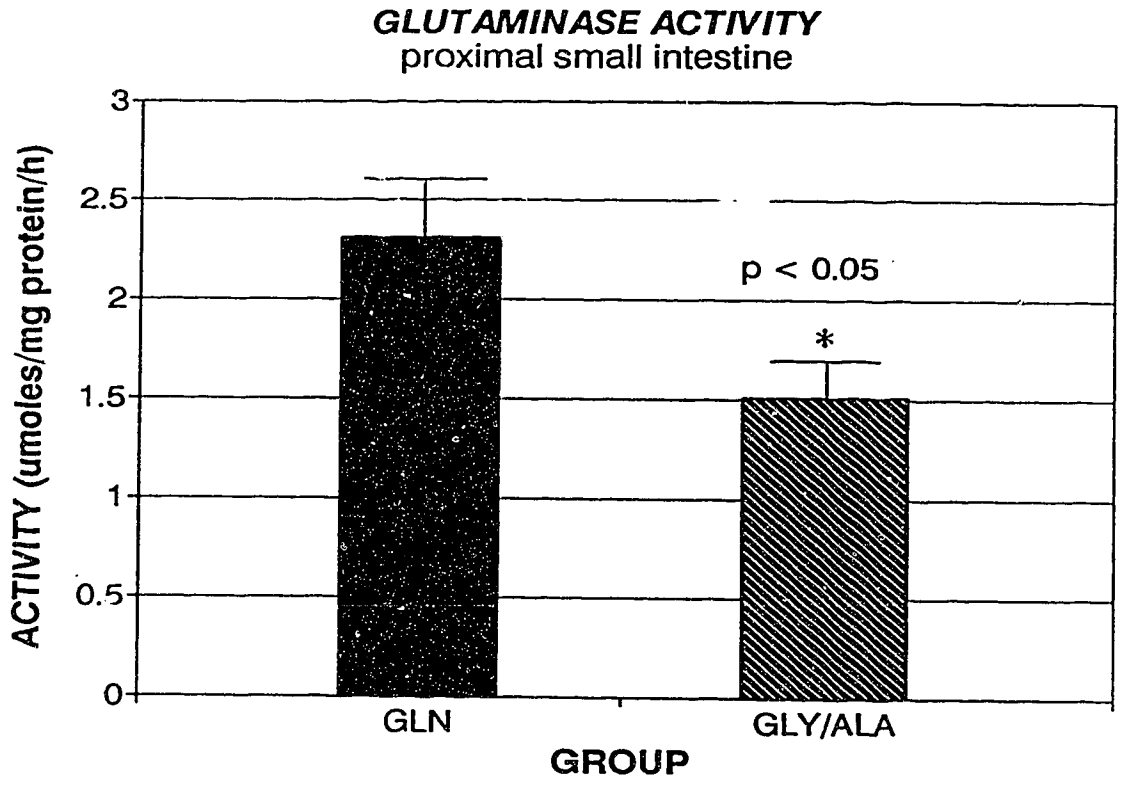

Figure IV-2: Glutaminase activity in jejunum of ileal interposition animals.

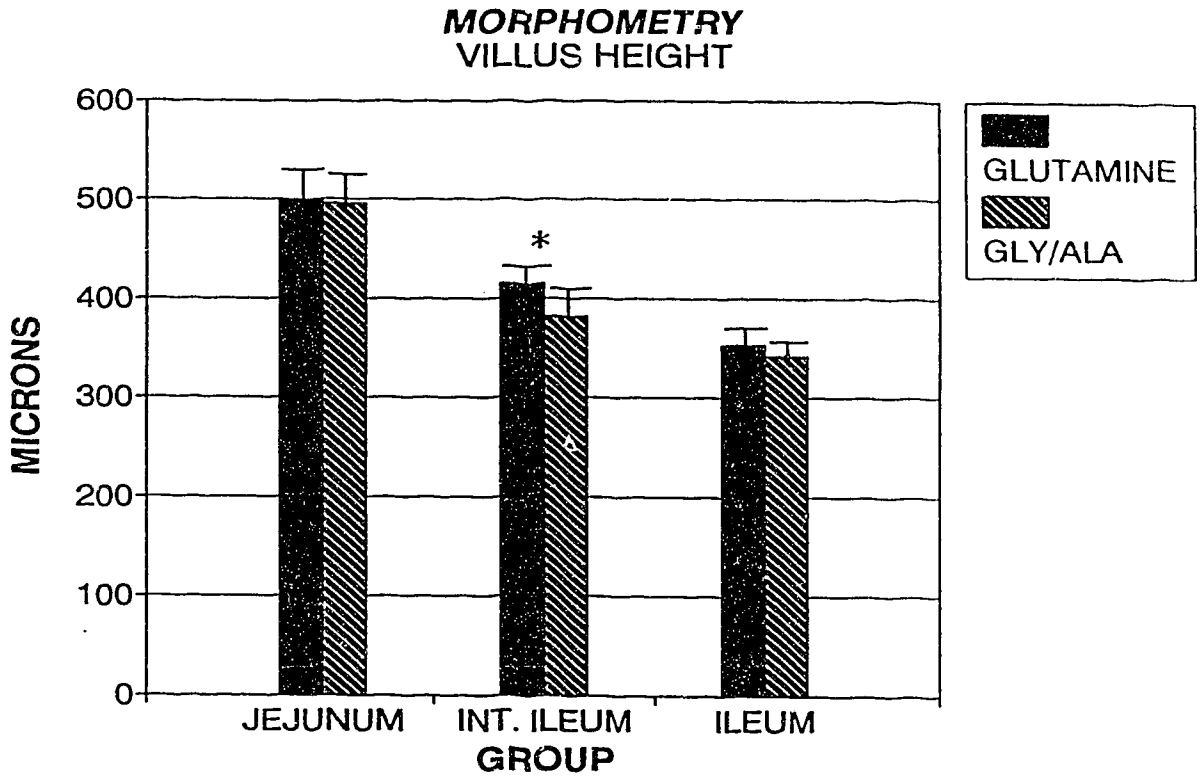

Figure IV-3: Villus heights in ileal interposition animals. * Interposed ileum has significantly higher villus height than native ileum by paired t-test. (Int. ileum = interposed ileal segment).

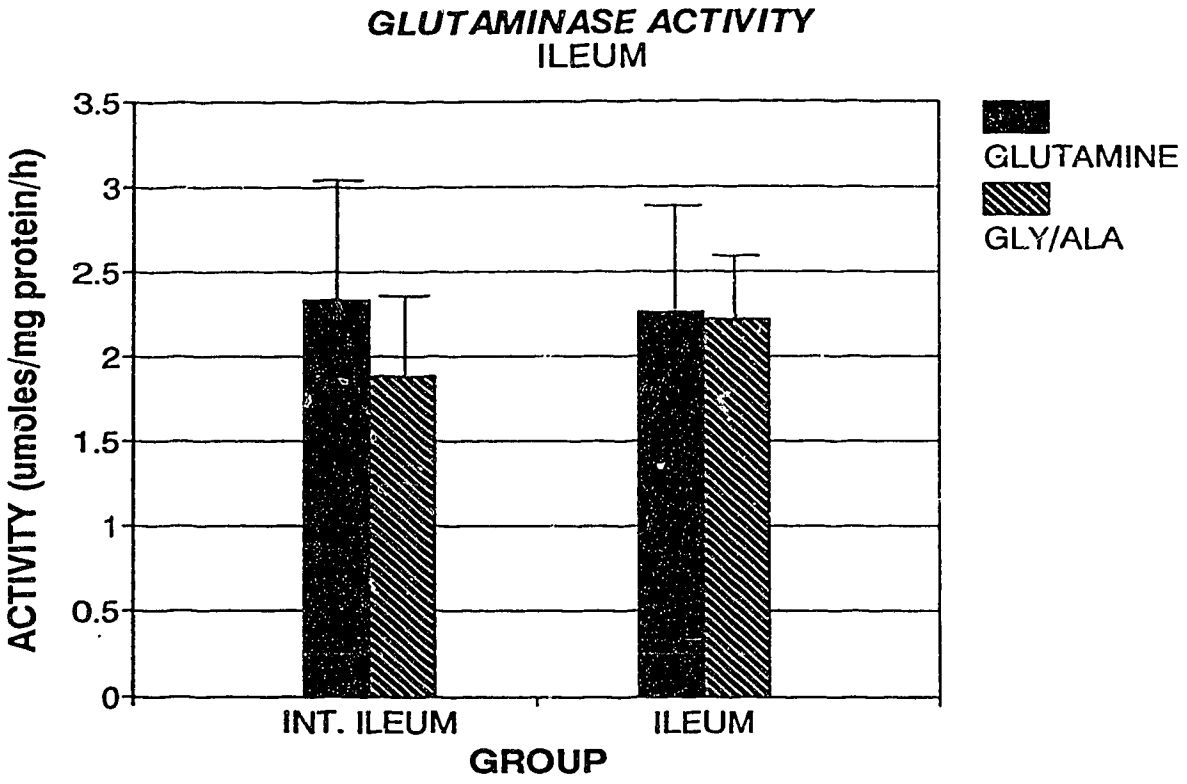

Figure IV-4: Glutaminase activity comparing interposed and native ileal segments.

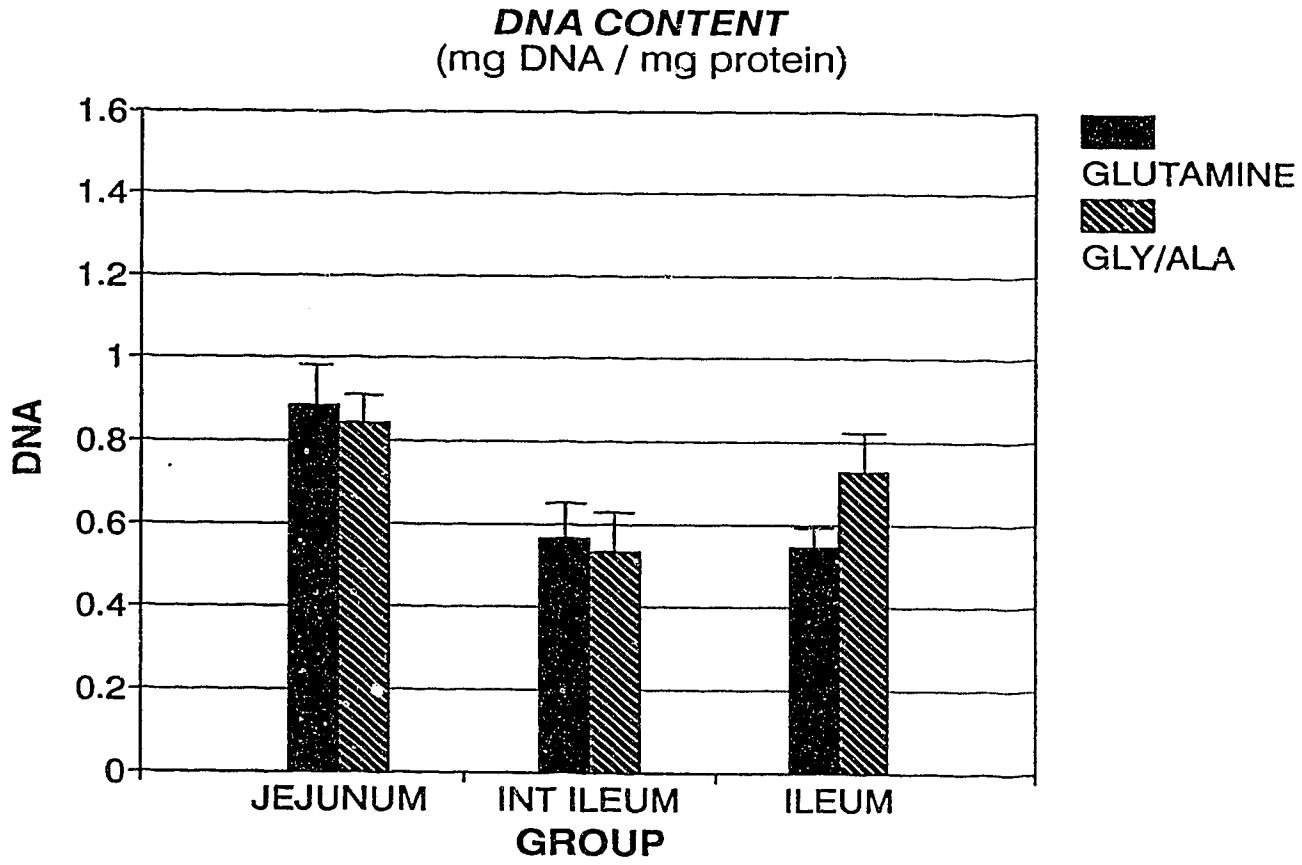

Figure IV-5: DNA content in ileal interposition animals.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

The subject of glutamine and its effects on healing of intestinal injury touches on several areas of nutrition and intestinal metabolism. The project set out to focus on intestinal function and how it was affected by glutamine. Also, effects on the oral route were studied, in order to derive conclusions with respect to glutamine and its role as an enteral nutrient.

Our experiments demonstrated that fluid absorption is transiently impaired around a surgical anastomosis in rats. This ileal segment returns to normal by the second postoperative day. We failed to demonstrate any benefit to fluid absorption by glutamine, however this is probably because glutamine was not reaching the ileum in sufficient quantities. Our anastomosis animals showed increased jejunal but not ileal glutaminase activity and DNA content in glutamine-supplemented rats. The jejunal glutaminase results were very close to those of Souba et al.

Our next step was to investigate this discrepancy where oral glutamine afforded an effect on jejunum without improving ileum. The ileal transposition

model eliminated the factor of distal location. Now proximal, the interposed segment was bathed in enteral stream similar to that of jejunum. The interposed ileal segments showed significant increases in villus height, but there was no differential improvement between dietary groups. Given more time and higher doses of glutamine, improvement in the interposed ileum of glutamine-fed animals may have occurred.

Glutamine improves intestinal morphometry, DNA content and glutaminase activity in animal models [1,2]. Its effects on functional aspects of the intestine are less clear, although it has demonstrated benefit in reducing positive bacterial cultures and increasing IgA levels in bile [3,4]. Glutamine has also been shown to reduce pancreatic atrophy and hepatic steatosis normally associated with TPN [5].

The relative merits of intravenous and enteral glutamine supplementation have not been fully clarified. Intragastric glutamine resulted in more weight gain in rats than similar doses by the intravenous route [6]. This apparent advantage must be tempered by the fact that oral glutamine is 85% transformed before entry into the systemic circulation [7]. Oral glutamine, therefore, may not be as potent in exerting systemic effects as parenteral glutamine. The effect of oral glutamine may also be limited to the proximal intestine, as glutamine is absorbed quantitatively in the proximal jejunum [8] in humans. This concept is in agreement with our results, where a positive effect was noted in jejunal but not ileal glutaminase.

Glutamine's central role in metabolism has largely been worked out and has recently been reviewed [9]. Current questions are concentrating on specific

indications, routes of administration, doses and potential side effects of glutamine in parenteral and enteral solutions.

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APPENDIX I - GLUTAMINASE ASSAY

BACKGROUND

The phosphate dependent glutaminase assay for this project was a compilation of three sources [1-3], although other methods were considered [4,5]. Measurement of glutaminase depends on two reactions:

1. glutamine --------> glutamate + ammonia

2. glutamate + NAD ---------> alpha keto glutarate + NADH

The first reaction is catalyzed by glutaminase. It proceeds for a set period of time, allowing the rate of product formation to be calculated. The second reaction is pushed towards completion. Formation of NADH is measured at 340 nm in a spectrophotometer. Endogenous glutamate in tissue samples must be measured as blanks to subtract from the total glutamate measured. The blanks are run by adding homogenate after reaction 1 is stopped in samples by addition of HCl.

Calculations [3]:

$C = A/E$ where C= concentration

A = change in absorbance

at 340 nm, $E = 6.22 \times 10^3$ (molar absorption coefficient)

C represents concentration of glutamate, therefore glutamate produced per hour is C x 3, because incubation of reaction 1 is for 20 minutes. Three dilution factors (7, 5, 1.2) also enter the equation:

Glutaminase activity = A x 3 x 7 x 5 x 1.2 / 6.22

= A x 126/6.22

in micromoles/ hr/ ml homogenate

Glutaminase activity is expressed per mg protein. Preparation of the tissue homogenate requires a range of protein concentration in which the assay is accurate. Figure VI-1 demonstrates that with increasing amount of homogenate in the assay, the yield is no longer linear. Since this homogenate contained approximately 10 mg/ml of protein, a safe range of protein is 0.25 - 2.5 mg/ml. The curve is nearly linear to a volume of 100 microlitres, which corresponds to a protein concentration of 5 mg/ml in a 200 microlitre sample. By using 10 cm of intestine with 30 ml of buffer, protein concentrations were in the acceptable range.

Reaction 1 must occur at a steady rate. Rate of product formation was constant up to 25 minutes (Figure VI-2). This determined our incubation time to be 20 minutes compared to 30 minutes as reported by others under slightly different conditions [2].

Reaction 2 tends towards glutamate utilization, rather than production, therefore conditions must be modified to push the reaction to the right. A relatively alkaline medium (pH = 8.8), high concentrations of NAD and hydrazine hydrate all favour glutamate production. Hydrazine acts by reacting with alpha keto glutarate, thereby continuously removing this product.

Glutamate standards are used to confirm linearity of product formation and to assess comparability of results between assays. Measurements of

glutamate from 0 to 2 g/l concentrations reveal linearity as demonstrated in Figure VI-3. Higher concentrations of glutamate resulted in diminishing yield. Calculation of glutamate yield is approximately 65% of the known standards added to the assay mixture. It was decided to calculate glutaminase activity rather than reading off the glutamate standard curves. Glutaminase levels for jejunum by this method were comparable to those reported by others [1,6].

GLUTAMINASE ASSAY - PROTOCOL

1. Solution 1: Tris Buffer with glutamine.

For Stock solution, add to one litre of water:

6.055 g Tris base

20.4 g Potassium phosphate (monobasic)

0.074 g EDTA

Adjust pH to 8.6. This solution is stable for at least four weeks, however pH should be checked before each use. This stock is used for intestinal homogenate and, after addition of glutamine, for reaction 1. Add 2.92 g of glutamine per litre of stock on the day of the assay to complete solution 1.

2. Solution 2:

For stock solution, add 6.055 g Tris acid to one litre of water. On the day of the assay, to 100 ml stock solution add 4 ml hydrazine hydrate and adjust pH to 8.8. Add 66.6 mg NAD, 6.4 mg ADP and 75 microlitres of glutamate dehydrogenase in glycerol. The absorbance of this solution changes within hours, so it is important to use fresh solution and to use the same batch for zeroing the spectrophotometer as for the samples tested.

3. Preparation of intestinal homogenate:

Add 10 cm of intestine to 30 ml of solution 1 without glutamine. Homogenize with Polytron at speed 8 for 30 seconds. Immediately prior to assay, sonicate ten bursts with W-375 sonicator at maximum output, pulsatile mode. Ideally, the homogenate should be frozen in aliquots to ensure extra frozen samples if problems arise. Tissue or homogenate can be thawed and frozen only once, when assaying for enzyme activity.

4. Preparation of glutamate standards:

Glutamate standards of 0, 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 g/l are prepared. These are assayed in duplicate to confirm linearity and to compare from one assay to the next. The zero standard (water) is run through the entire assay and used for zero calibration of the spectrophotometer.

5. Reaction 1:

Each sample is run in triplicate with a corresponding blank which accounts for endogenous glutamate. To 1 ml solution 1, add 200 microlitres of homogenate. Incubate for 20 minutes at 37^{o} C. Add 200 microlitres 2 N HCl to precipitate protein and stop the glutaminase reaction. Homogenate is now added for the blanks, as reaction 1 cannot proceed. Centrifuge at 3000 rpm for ten minutes.

6. Reaction 2:

Remove 200 microlitres of supernatant from blanks and samples and add to 1 ml of solution 2. Incubate for 30 minutes. Read absorbance at 340 nm.

7. Calculations:

Change in absorbance is calculated by subtracting blanks from samples.

Glutaminase activity in micromoles / h / ml homogenate

= change in absorbance x 42 x 3 / 6.22

Activity can be expressed in:

micromoles / h / mg protein by measuring protein / ml homogenate.

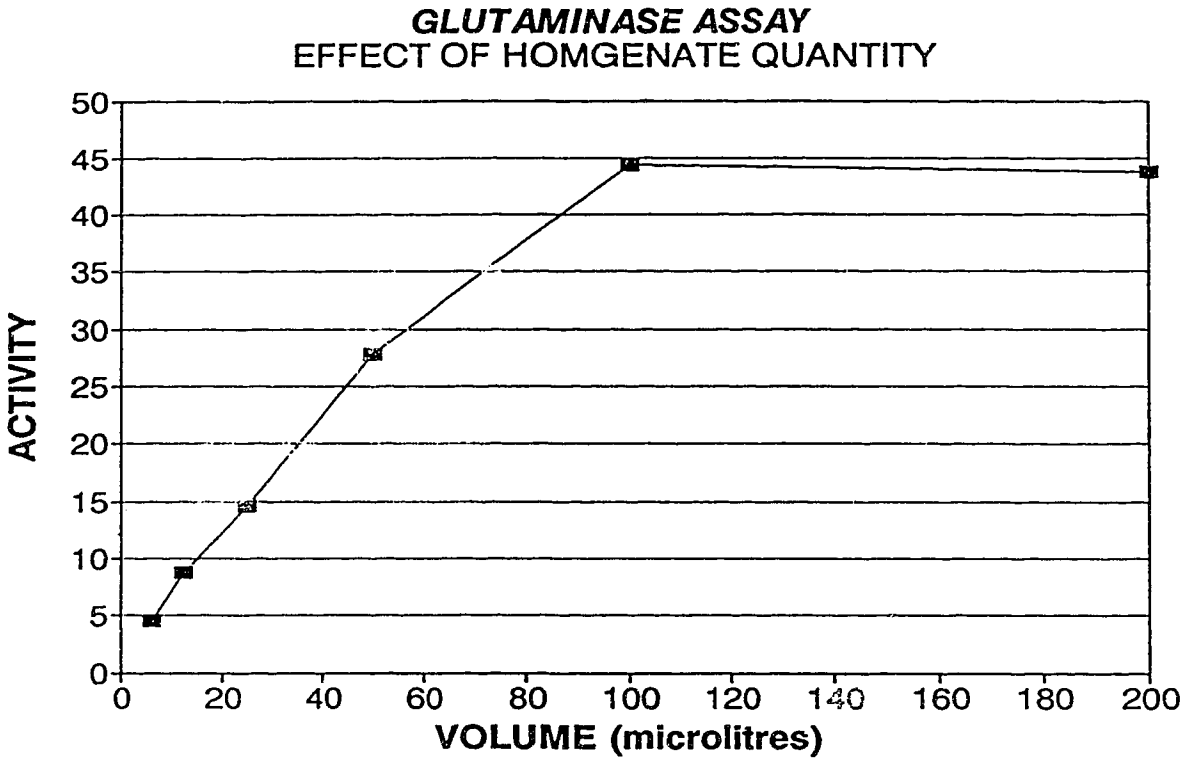

Figure V-1: The effect of homogenate quantity on measured activity. Glutaminase must be measured using quantities on the linear sloping portion of the curve.

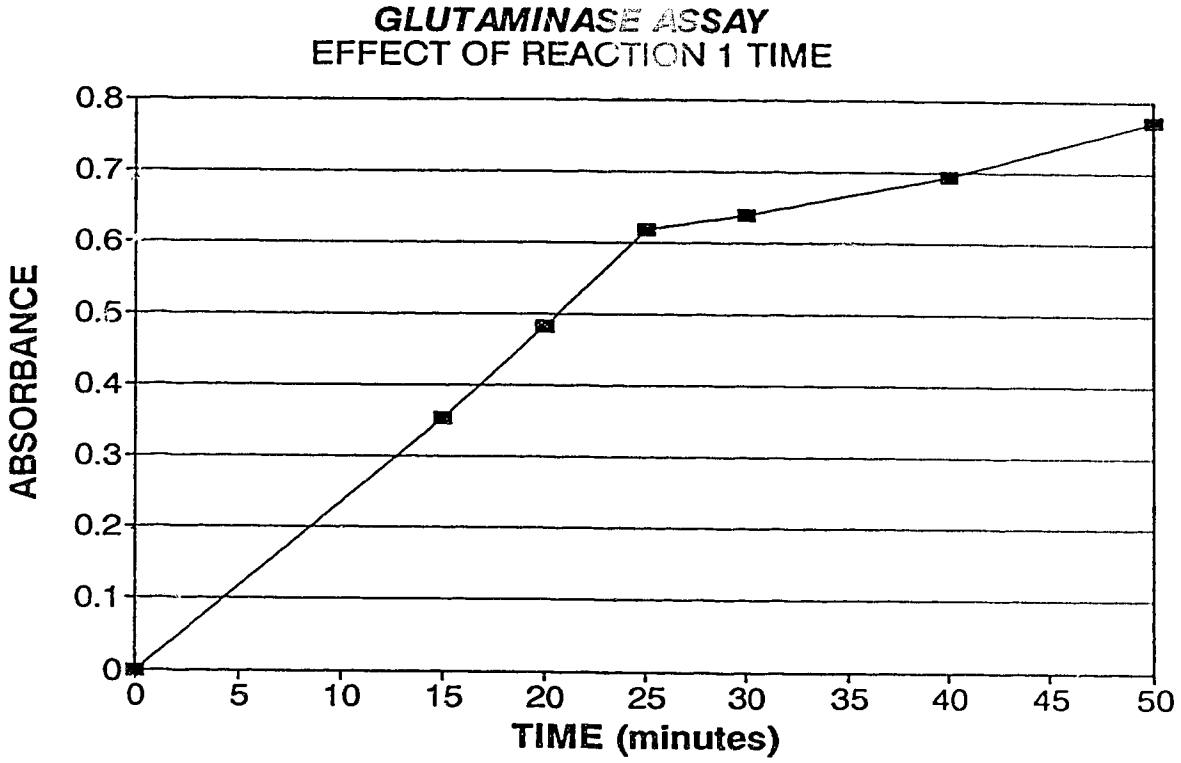

Figure V-2: Increasing the incubation time of reaction 1. This yields diminishing returns beyond 25 minutes.

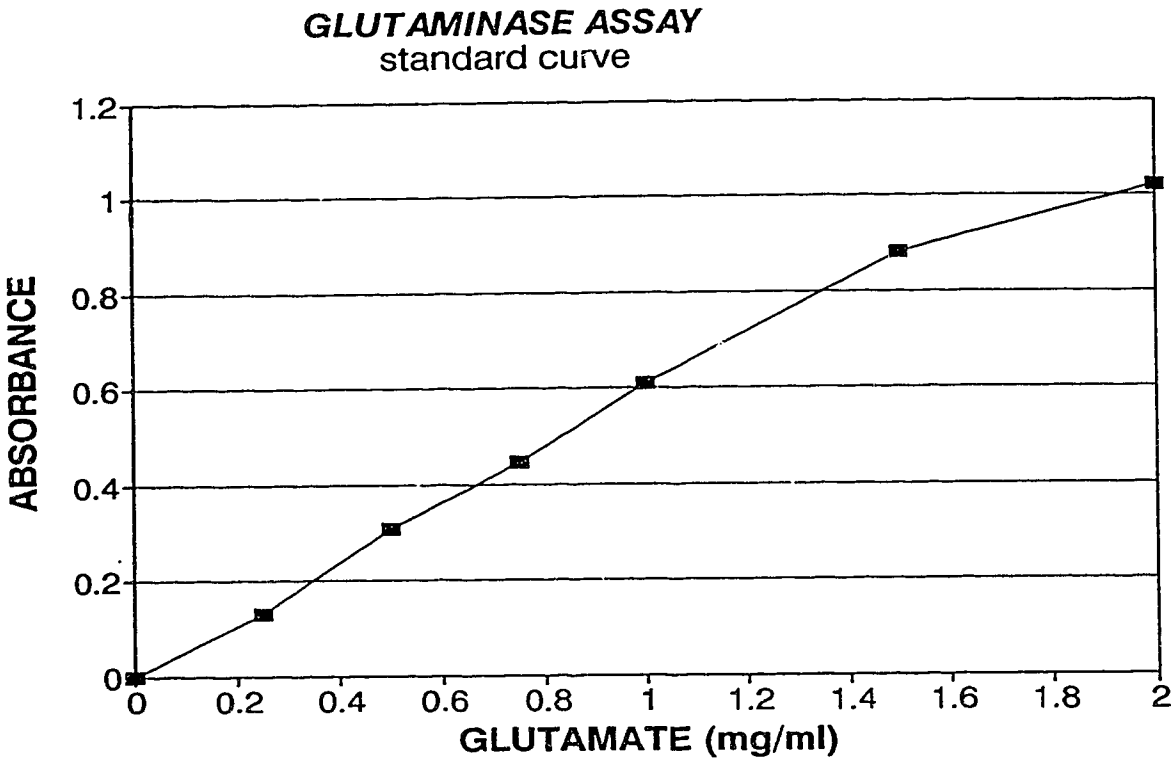

Figure V-3: Glutamate standards are used to confirm linearity of reaction 2 and to assess comparability between assays.

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APPENDIX II

ACETIC ACID-INDUCED COLITIS AND GLUTAMINE

Another model studying the effects of glutamine on healing of intestinal injury was the acetic acid colitis model in rats. The aim was to focus on colon function (*in vivo* fluid absorption) in rats with experimental colitis. The question posed was whether glutamine had any beneficial effect on colon function. This experiment differed from others [1-3] in that it specifically examined functional changes to the colon. Difficulties in achieving an adequate model were encountered. Therefore, the experiment is presented briefly with emphasis on these problems and how they related to ensuing experimental design of the surgical anastomosis injury model.

MATERIALS AND METHODS

Rats were fed an elemental diet (Vital HN) with glutamine or glycine supplementation. They were given experimental acetic acid colitis by injecting acid into the colon at laparotomy [4]. The animals were allowed to recover and after one week of dietary treatment, they were sacrificed and underwent *in vivo* fluid absorption loops. Several pilot experiments were performed in order to standardize the colitis injury and to ensure that glutamine and glycine-supplemented rats were receiving adequate and equal amounts of the amino acid:

1. colitis , chow ad lib
2. no colitis, liquid elemental diet
3. colitis, liquid elemental diet
4. no colitis, powder elemental diet

5. colitis, powder elemental diet

6. no colitis, gravity tube feeding liquid elemental diet

7. colitis, pump tube feeding liquid elemental diet

The above trials were monitored with respect to food and glutamine intake, animal survival and colon function.

RESULTS

FEEDING

The greatest difficulty was encountered in feeding rats such that glutamine and caloric intake was both known and controlled. This predicament was compounded in those rats with colitis, as they were systemically ill enough to make intake problematic.

When rats were allowed to drink liquid elemental diet, they gained weight. It was, however, very difficult to quantify their exact intake. Bottles were observed to leak to varying degrees, and for this reason, liquid diet was temporarily abandoned.

The same diet in powder form was much more easily quantified. Food containers were weighed and food was replenished on a daily basis. Rats gained weight on this diet and average intake was approximately 20.6 grams per day in healthy rats (range 18.1-24.1 grams per day). However, colitis induction decreased daily oral intake to 5.7 grams (range 1.4-11.3 grams).

Intermittent tube feeding liquid elemental diet by gravity carried unacceptably high rates of mortality (gastric distention) in colitic animals. Gastrostomy tube blockage was also frequent. Therefore a pump with swivel and harness apparatus

was utilized. With colitis, gastric distention was again responsible for a high mortality rate approaching 50%.

FLUID ABSORPTION

Two trials reached completion, comparing colon function between glutamine and glycine-supplemented rats with acetic acid colitis. The first trial involved amino acid supplementation only during the time period after induction of colitis. The second trial included a prefeeding period where animals received glutamine or glycine for five days prior to acetic acid colitis. Results of these trials are presented in Figures VII-1 and VII-2: Glutamine demonstrated no beneficial effect over glycine on colon function in rats with colitis.

DISCUSSION

The bulk of time spent on this experiment was devoted to feeding: both control and accurate monitoring of intake. For two groups to be comparable, they must be similar in all respects except for the factors (in this case two amino acids) being compared. If the diets are not isonitrogenous and isocaloric between the two groups, then comparison is flawed.

Powder elemental diet was the simplest and most accurate in terms of monitoring food consumption. It was also easy to control and therefore accurate pair-feeding was possible. However, with colitis, rats had a substantial but variable drop in food intake, rendering accurate pairing unfeasible.

The liquid elemental diet was difficult to monitor unless fed through gastrostomy tube. Enteral tube feeding was possible with healthy rats, however it proved to be difficult when the animals had colitis. The high mortality from acute gastric dilation was perhaps not insurmountable. It appeared to be a functional as opposed to mechanical obstruction and was often accompanied by luminal fluid accumulation and massive dilation of the small intestine. Such measures as gradual onset of feeding and reduction of colitis severity were helpful, however it was apparent that a great deal of fine tuning would be required.

Most other models of enterocolitis used to study enteral glutamine [1-3,5-7] employed a glutamine prefeeding period prior to injury. This probably decreased the need to advance feeding soon after surgery and may have contributed to better tolerance of tube feeding in their studies. Prefeeding by gastrostomy with our model of colitis, however, would have increased the number of laparotomies to three per rat which in itself may have been excessive stress on the animals.

The difficulties encountered in controlling and monitoring food intake in the sick colitic animals favoured a model in which the animals were not systemically ill. The surgical anastomosis injury model was preferential from this point of view as ad lib feeding could be employed while maintaining accurate pairing between dietary groups.

Our limited amount of data did not demonstrate benefit to colon function by glutamine in rats with colitis. This was likely due to several reasons: Firstly, the animals may not have received enough glutamine. This was most likely when a prefeeding period was not employed. These rats had no significant benefit from glutamine (Fig VII-1), however intake was markedly decreased and skewed

towards the late postoperative period. When glutamine prefeeding was undertaken (Fig VII-2), the lack of a difference may have been due to inadequate amounts of glutamine or inadequate numbers of animals.

Another possible reason for lack of glutamine benefit in our colitis model may have related to the route of administration. Other models of enterocolitis where glutamine was administered through the gastrointestinal tract have had an element of small bowel involvement. Malabsorption may have allowed luminal transit of glutamine to the colon where benefit occurred [1-3]. The acetic acid colitis model is a fairly local injury. Healthy small bowel could absorb and transform glutamine before it had opportunity to support the colon.

Glutamine may not have helped in this model because its role is less important in the colon. Short chain fatty acids are more valuable energy substrates in the colon compared to glutamine [8,9]. Intestinal glutaminase is highest in jejunum and ileum with much lower activity in the colon [10]. A lower intrinsic glutamine requirement by colon may have contributed to our lack of demonstrated effects by glutamine.

Most of the improvements noted by glutamine in enterocolitis models relate to architectural or morphological changes [1-3,5-7]. Perhaps the lack of positive results in our model is because the assessment of function was unlikely to yield success from glutamine.

Several of these factors probably influenced experimental results of the acetic acid colitis model and glutamine. It is difficult to draw definite conclusions as to whether glutamine would be if the model were modified. The ileal surgical anastomosis injury model circumvented several of the above

problems and therefore the preponderance of time was devoted to it. The experience with acetic acid colitis and glutamine, however, did provide questions and opportunity to take a closer look at glutamine and intestinal injury with the alternate model.

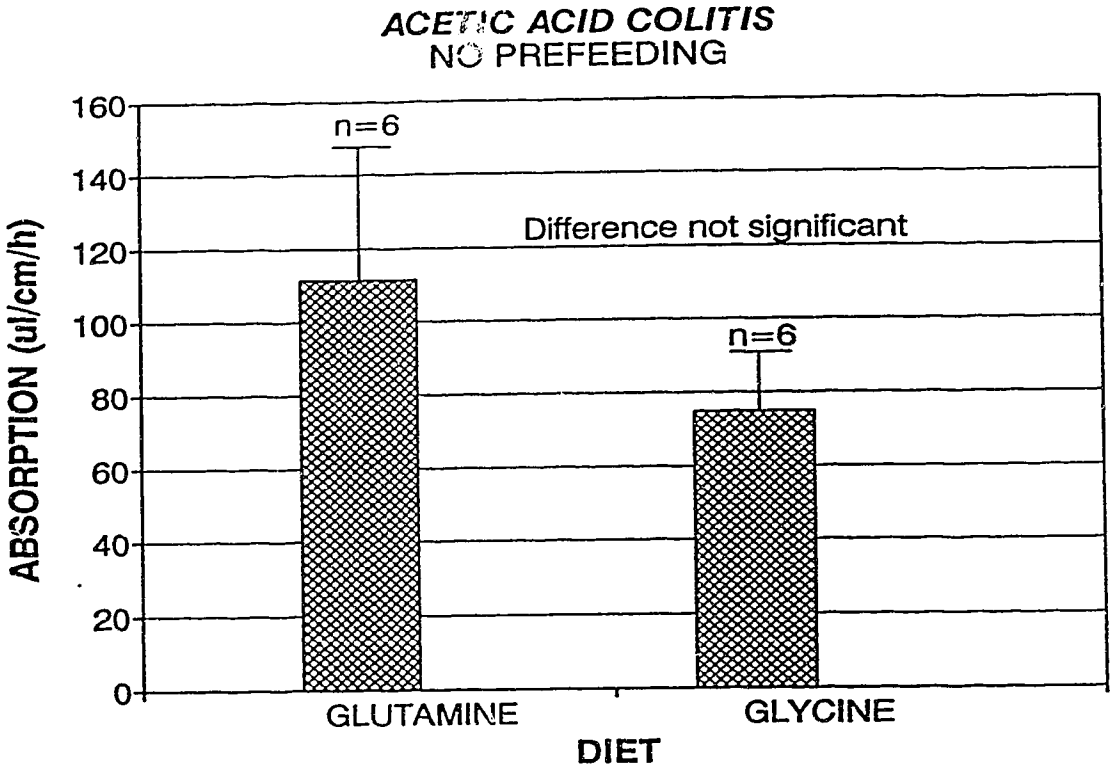

Figure VI-1: Intestinal absorptive function (colon) in animals with acetic acid colitis. No prefeeding period of glutamine and glycine was employed.

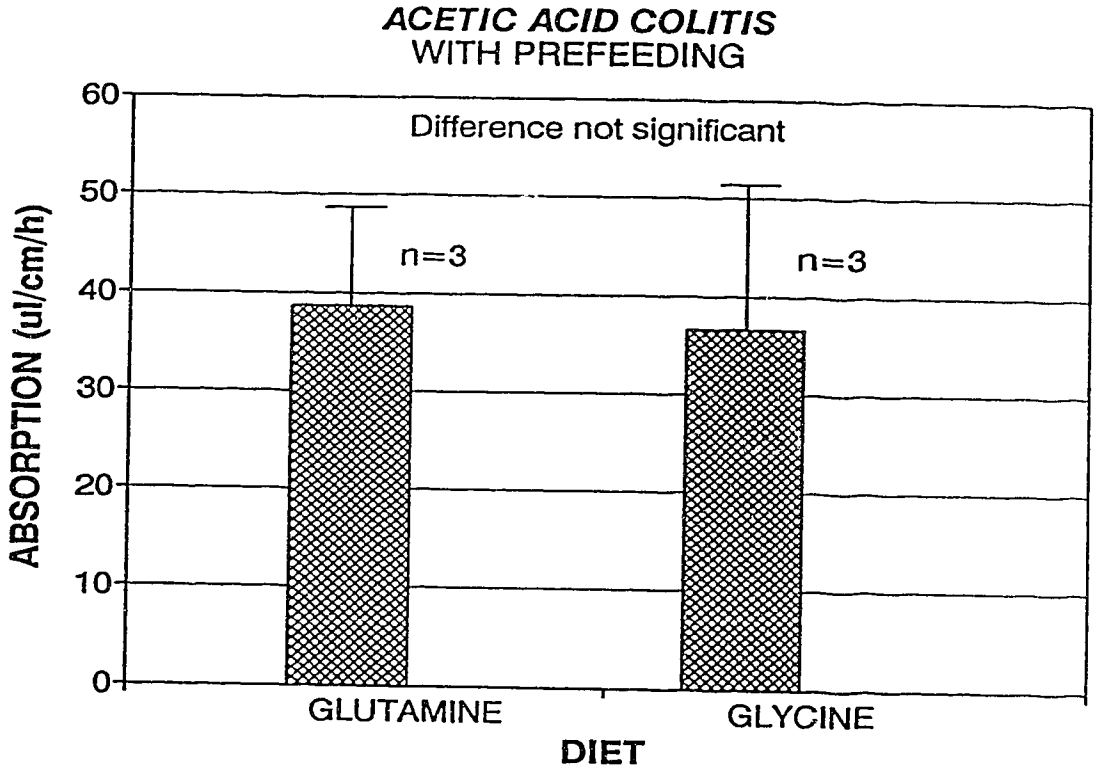

Figure VI-2: Intestinal absorptive function (colon) in animals with acetic acid colitis. A five day prefeeding period of glutamine or glycine was employed.

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