GLUCOSAMINE SYNTHETASE
IN THE HUMAN GASTROINTESTINAL MUCOSA
IN HEALTH AND DISEASE

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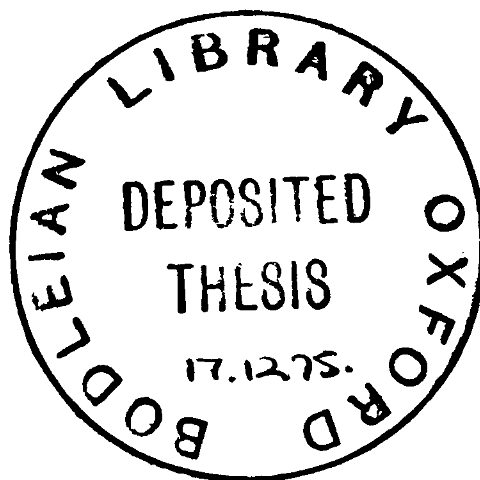

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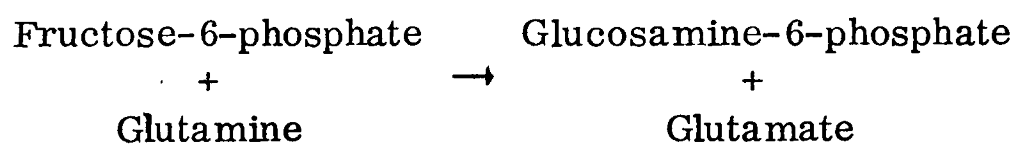
SECTION I

INTRODUCTION

CHAPTER 1

GASTROINTESTINAL MUCUS IN RELATION TO DISEASE

This thesis is concerned with the enzyme glucosamine synthetase, L-glutamine-D-fructose-6-phosphate aminotransferase, EC.2.6.1.16, which is a cytoplasmic enzyme catalysing the synthesis of glucosamine-6-phosphate from glutamine and fructose-6-phosphate:



The thesis describes the miniaturization of an assay of the enzyme for use with small specimens of human gastrointestinal mucosa and it reports the results obtained from the application of this assay to biopsy specimens and surgical operation specimens of the gastrointestinal tract, taken from patients with various gastrointestinal disorders.

Glucosamine synthesis is an essential step in the biosynthesis of glycoproteins and hence of gastrointestinal mucus, which is glycoprotein in composition. Glycoproteins consist of a protein chain and carbohydrate side-chains (Fig. 3). The carbohydrates of the side-chains are galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose and the sialic acids (Fig. 2). Glucosamine synthetase, by catalysing the formation glucosamine-6-phosphate, is the first step in the biosynthesis of N-acetylglucosamine, N-acetylgalactosamine and the sialic acids.

In this thesis, tissue levels of glucosamine synthetase have been studied in relation to gastrointestinal mucus. In particular, they have been studied in

those diseases in which it has been postulated that there may be abnormalities of mucus production.

Studies have also been made on liver tissue, described in an appendix to this thesis (Appendix C), to show the possibility of the application of the assay to liver biopsies. Glucosamine synthesis is considered in relation to hepatic glycoprotein synthesis, as one aspect of the biosynthetic function of the liver, and in relation to the formation in the liver of acid mucopolysaccharides, which, like the glycoproteins, contain N-acetylglucosamine.

The function of gastrointestinal mucus

The epithelium of the gastrointestinal tract from the cardiac sphincter to the anus is only one cell thick. This single cell layer has to withstand irritant chemicals and extremes of temperature from ingested food and drink; it has to resist its own secreted acid and digestive enzymes; and it is subject to abrasion by rough food in the stomach and by hard solid faeces in the rectum. It also has to provide a barrier against penetration by a potentially lethal flora in the colon.

Some protection is provided by the glycocalyx, a thin layer of glycoprotein along the luminal border of the mucosal cell (Fig. 1). However, much of the protection and lubrication of the mucosa is provided by the gastrointestinal mucus, a viscous secretion which tends to adhere to the mucosa.

Although mucus has been regarded as having only the physical functions of protection and lubrication, a recent paper by Shora et al (1975) has suggested a chemical role. Intestinal goblet cell mucus from the rat appeared to stimulate the proteolytic digestion of casein by trypsin and chymotrypsin *in vitro*.

The mucus-secreting cells

The mucus-secreting cell in the intestine is the goblet cell, first described by Henle in 1837. The luminal end of the goblet cell is seen histologically and with the electron microscope to be distended with droplets of mucus (Florey, 1962).

In the duodenum, mucus is also secreted by Brunner's glands.

In the normal human stomach, the entire surface epithelium consists of surface mucous cells. From the epithelium, there descend numerous pits, the foveolae, lined also by surface mucous cells. One or more simple or branched tubular glands, containing specialized secretory cells, open into the bottom of these pits.

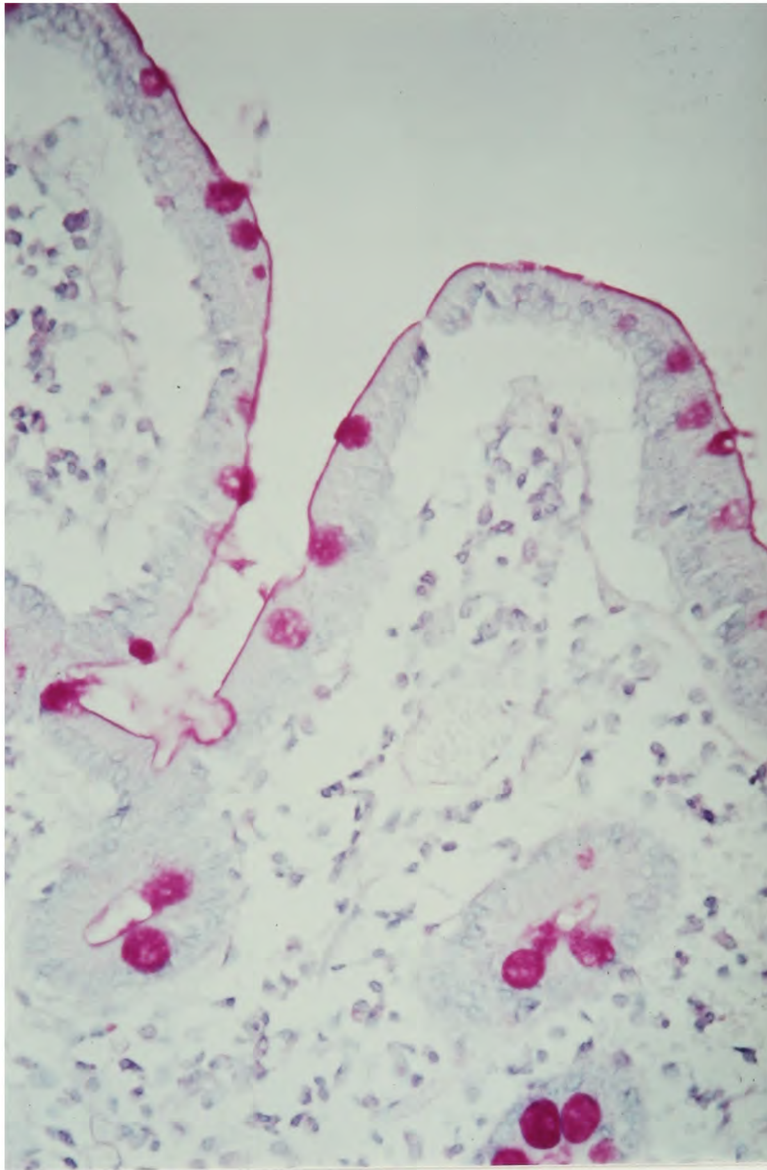


FIG. 1. The human duodenal mucosa stained with PAS to demonstrate the glycocalyx and neutral-staining mucus in the goblet cells.

The three histological types of gastric mucosa, the cardiac mucosa, the fundic or body mucosa, and the pyloric mucosa, have particular types of glands. The cardiac and the pyloric glands secrete mucus: the cardiac mucous glands are found in a narrow rim around the cardia, and the pyloric glands are found in the antral region. The fundic glands are the type found in the remainder of the stomach, including the body and the fundus. Within the fundic glands, the peptic and oxyntic cells secrete pepsinogen and hydrochloric acid respectively, but some of the cells around the necks of the fundic glands are mucus-secreting cells, known as the mucous neck cells. These cells and the mucus-secreting cells of the cardiac and pyloric glands are morphologically similar: they are small cells with relatively large nuclei and they tend to be deformed by adjacent parietal cells. The surface mucous cells are larger cells, with mucus granules often occupying much of their supranuclear cytoplasm. There are also ultra-structural differences between the two types of gastric mucous cell, in the appearances of their ribosomes and their endoplasmic reticulum (Ito, 1967).

The glycocalyx

This term was coined by H. S. Bennett in 1963 to describe a layer on the surface of intestinal cells which stains with PAS (Fig. 1). This layer had been observed by Leblond in 1950 in the microvilli of the small intestinal brush border, which at the time was known only as the "striated border" of the intestinal epithelium. Autoradiography in experimental animals has shown that the glycocalyx is composed of glycoprotein (Ito, 1969). Like the gastrointestinal mucus, it is synthesized in the Golgi apparatus (Ito and Revel, 1968).

The glycocalyx is prominent on the absorbing cells of the intestine and it is also present, although less marked, on the goblet cells and the undifferentiated columnar cells (Ito, 1965). In the stomach, it appears to be continuous along the surface epithelium and along the luminal surface of the cells in the gastric pits (Lambert et al, 1968).

In the fundus of the rat stomach, where there are no microvilli, the thickness of the glycocalyx has been estimated from electron micrographs to be 20-30 nm (Lambert et al, 1968), which probably represents just a single molecule of glycoprotein in thickness.

In the small intestine, the glycocalyx appears to be the site of brush-border sucrase and alkaline phosphatase activity (Weiser, 1973a) and of other

disaccharidases (Kelly and Alpers, 1973).

Although the glycocalyx and the secreted gastrointestinal mucus are both composed of glycoprotein, they are antigenically distinct. Forstner et al (1973c) found that a fluorescent antibody to the major portion of the cell sap glycoprotein of rat small intestine attached itself to the intestinal goblet cell mucus of the rat, to the adherent mucus layer, to the mucus-secreting cells of the stomach, and to the sublingual gland, but it did not stain the glycocalyx. Also, the glycocalyx and the mucus appear to turn over as separate intra-cellular pools of glycoprotein (Forstner, 1970).

MUCUS AND DISEASE

Peptic ulcer and the mucus protection theory

It has often been suggested that peptic ulceration is a consequence of an impairment in the quality or quantity of gastric mucus, which normally protects the epithelium from digestion by the secreted acid and pepsin. As Claude Bernard said in 1856, "La muqueuse stomacale enferme donc le suc gastrique ... comme dans un vase aussi imperméable que s'il était de porcelaine." ("The mucosa of the stomach encloses the gastric juice ... as in a vase as impermeable as if it were made of porcelain.") In 1898, Schiff inserted pieces of meat and egg through a gastrostomy into a dog's stomach and reported that no digestion occurred.

Kowalewski et al (1969) washed the stomachs of rats, via gastric fistulae, with 2M sodium chloride, a procedure which had been shown by Webster (1967) to deplete the surface cells of their mucus. Following infusions of histamine, the rats developed gastric ulcers, which did not happen to control rats which had had their stomachs washed with M sodium chloride.

Peptic ulcer and blood groups

Blood group O individuals have a 35% greater risk of developing a peptic ulcer than people of other blood groups (Aird et al, 1954), and non-secretors of ABO blood groups have a 40% greater incidence of gastric ulcer and an 80% greater incidence of duodenal ulcer than blood group secretors (Doll et al, 1961). Roughly 77% of the population in England are blood group secretors (Doll et al, 1961), whose ABO blood group activity can be detected in the saliva

and in the gastric juice, and also in the small intestinal secretions, in the bile, and in the breast milk, although not in the secretions from the distal colon (Glynn et al, 1957; Szulman, 1960).

The blood-group antigenicity of the gastric, intestinal and biliary secretions is carried by the mucus molecules and is determined by their terminal sugars (Fig. 4). All secretors produce glycoproteins which possess a fucose radicle attached to galactose at or close to the end of the side-chain. The Se gene directs the synthesis of the fucosyltransferase which is responsible for adding this fucose onto the mucus molecule. This completes the molecule in blood group O secretors. Group A secretors have an additional N-acetylgalactosamine, group B secretors have an additional galactose, and AB secretors synthesize molecules with both types of terminal group. These terminal sugars correspond to those of the blood group glycolipids of the erythrocyte surface membrane, which occur in secretors and non-secretors alike (Watkins, 1972). The A and B determinants can only be added to secreted blood group substances after the fucose radicle of the secretor status has been attached.

It is conceivable that the extra terminal side-chain of fucose that occurs in the mucus of blood group secretors imparts an additional resistance to peptic mucosal digestion. However, the additional protection conferred by A and B blood group status cannot be explained on a similar hypothesis because this protection occurs in non-secretors as well as in secretors, and non-secretors produce glycoprotein without the additional sugars which determine A and B activity.

Ulcerogenic drugs

Salicylates, corticosteroids, phenylbutazone, oxyphenbutazone and indomethacin are drugs that are liable to induce peptic ulceration. This ulcerogenic effect has been ascribed to damage by these drugs of the "gastric mucosal barrier", which has been taken to mean either the continuity of the surface of the mucosal cell layer (Davenport, 1967), which would probably include the glycocalyx, or the secreted gastric mucus, or both (Hollander, 1954).

The hexosamine content of gastric juice and the hexosamine in gastric mucosal scrapings have been used as estimates of the amount of gastric mucus secreted by experimental animals. By these methods, mucus secretion has been shown to be apparently diminished after the administration of aspirin

(Menguy and Masters, 1965), cortisone (Menguy and Masters, 1963), ACTH (Desbaillets and Menguy, 1967), phenylbutazone (Menguy and Desbaillets, 1967b) and indomethacin (Menguy and Desbaillets, 1967a). Salicylate has been shown by Kent and Allen (1968) to inhibit the incorporation of radioactive glucose, threonine and sulphate into sheep colonic glycoprotein and by Lukie and Forstner (1972b) to inhibit the incorporation of ^{14}C-glucosamine into the glycoprotein of rat small intestinal slices. Parke et al (1975) have shown inhibition of the incorporation of N-acetyl[^{3}H]glucosamine and other precursors of mucus into the gastric mucosal glycoprotein of ferrets treated with indomethacin and phenylbutazone. Depletion of PAS-staining mucous cells in the rat stomach was demonstrated following prednisone therapy by Robert and Nezamis (1964) and following calcium aspirin, oxyphenbutazone and indomethacin by Johansson and Lindquist (1971). Salicylates and phenylbutazone have also been shown to inhibit glucosamine synthetase (Perrey, 1968) but in Chapter 15 of this thesis doubt is cast on the relevance of this observation.

Carbenoxolone sodium

This drug has been shown to accelerate healing in human gastric ulcers (Doll et al, 1962) and some benefit has also been shown in duodenal ulcer (Brown et al, 1972). It has frequently been observed that patients taking carbenoxolone have an abnormally thick layer of mucus on their gastric mucosa (Goodier et al, 1967) and it has been proposed that the mechanism of action of the drug is through an effect on mucus synthesis resulting in improved mucosal protection. There is some evidence of increased mucus synthesis as a consequence of carbenoxolone therapy (Gheorghiu et al, 1971; Johnston et al, 1975).

Mucus in the stools

Excessive amounts of mucus are often reported by patients with inflammatory diseases of the colon such as ulcerative colitis and Crohn's disease. This is also a frequent feature in the irritable colon syndrome, which was in the past sometimes referred to as "mucous colitis", although it is not an inflammatory disease. Possible mechanisms of the production of increased quantities of faecal mucus are discussed in Chapter 5.

Membranous colitis

In this condition, which is also known as pseudomembranous colitis (Goulston and McGovern, 1965), a common feature is a particularly florid secretion of mucus into the stools. Membranous colitis sometimes follows the use of broad-spectrum antibiotics and it has recently been seen following the use of lincomycin and its derivative, clindamycin (Scott et al, 1973; British Medical Journal, 1974).

The histology of the colonic mucosa in this condition shows that the membrane, which is seen on sigmoidoscopy as plaques adherent to the inflamed mucosa, consists of fibrin, mucin and inflammatory cells, overlying a mucosa which often contains dilated glands filled with mucus (Fig. 82). The aetiology of the condition is obscure because antibiotics have not been used in all cases. A constant feature seems to be fibrin plugging of the submucosal capillaries (Whitehead, 1971).

Mucus depletion in ulcerative colitis

In ulcerative colitis, there is often a diminution in the number of crypts in the colonic mucosa and in the proportion of goblet cells in those crypts (Fig. 55). In Crohn's disease of the colon, on the other hand, the goblet cell pattern is usually normal (Fig. 57) and this fact is used by some pathologists in the histological differentiation of Crohn's disease from ulcerative colitis. This is an important distinction to make because the two forms of colitis are managed differently (Truelove and Lee, 1973a,b). The glandular mucus was severely depleted in 8 out of 23 colectomy specimens from patients with ulcerative colitis studied by Cook and Dixon (1973) compared with only 1 out of 19 patients with Crohn's disease of the colon. Mucus depletion in the rectal biopsy was considered by Hywel Jones et al (1973) to be one of the five best discriminant features in distinguishing between ulcerative colitis and Crohn's disease out of 107 clinical, radiological and pathological parameters in a series of 109 patients studied by numerical taxonomy.

Colitis cystica profunda and colitis cystica superficialis

These are rare and distinct conditions characterized by mucous cysts in the colon. In colitis cystica profunda, the cysts are submucosal mucous lakes (Goodall and Sinclair, 1957; Epstein et al, 1966). In colitis cystica superficialis,

the tubules containing goblet cells are dilated with cells filled with mucus (Morris, 1973). The aetiology of both these conditions is unknown. Colitis cystica superficialis has been described in pellagra (Denton, 1928) but Morris's case did not have vitamin deficiency.

Mucus-secreting tumours

Disordered epithelial cells produce disordered mucus, as can be shown when the mucus produced by gastric and colonic carcinomas is examined biochemically or histochemically (Schrager, 1972; Gad, 1969). Remarkably, the extracts of some gastric tumours have been shown to exhibit blood group activity different from that of the host. Häkkinen and Virtanen (1967) showed group A activity in the gastric carcinomas of 4 out of 7 group O patients and in 5 out of 5 group B patients. Schrager and Oates (1973) found by agglutination inhibition and by carbohydrate analysis that there was group A activity in the gastric tumours of 8 out of 14 group O secretors. All these patients have anti-A serum agglutinins, and the continued growth of the tumour in the presence of a circulating serum antibody is strong support for theories of a disordered immune response as a factor in the development of malignancy.

The mucosa adjacent to colonic tumours

Filipe and Dawson have found that in the morhpologically normal colonic mucosa adjacent to carcinomas there is an abnormal histochemical pattern of the goblet cell mucus (Filipe, 1969). They have termed this the "transitional" mucosa. The histochemistry of mucus is discussed in Chapter 4 and studies on the "transitional" mucosa are described in Chapter 12.

Fibrocystic disease

This congenital disease is characterized by the abnormal viscosity of the mucus of the gastrointestinal and bronchial tracts, and the pathological effects are the result of this. Children with this condition can be born with sticky mucus obstructing the intestine and causing a meconium ileus. The pancreatic ducts become blocked with mucus, leading to pancreatic exocrine insufficiency and malabsorption, and mucus plugs obstruct the bronchioles, giving rise to emphysema and repeated pulmonary infections. Cirrhosis can develop because of obstruction of the intrahepatic bile ducts by viscous mucus.

Although Parkins et al (1963) demonstrated excessive amounts of mucus and increased numbers of goblet cells in patients with fibrocystic disease, molecular analysis (Johansen, 1963), histochemistry (Johansen, 1970) and electron microscopy (Lillibridge et al, 1974) have failed to show any differences between the mucus in fibrocystic disease and normal gastrointestinal mucus, and Gugler et al (1968) found that submaxillary saliva from fibrocystic patients was identical to normal saliva on immunochemistry. It is well established that there is an abnormally high sodium content in the exocrine secretions in fibrocystic disease and it has also been shown that there is an abnormally high calcium content in fibrocystic mucus and saliva (Chernik and Barbero, 1961). If this excessive calcium is removed by dialysis, there remains a mucus which is nearly normal in viscosity (Gugler et al, 1967).

Heatley (1959) showed that when mucus from the stomach and duodenum of the pig was suspended in an aqueous medium containing no electrolytes, the viscosity was very low, but the addition of even small concentrations of sodium and calcium imparted an irreversible increase in viscosity.

Fibrocystic saliva and mucus are hyperpermeable to electrolytes when set up against a dialysing membrane (Gibson et al, 1970), and the addition of calcium ions makes normal saliva hyperpermeable (Gibson et al, 1971). In fibrocystic disease, there is a serum macroglobulin factor, known as the CF factor, which inhibits ciliary motility in cultured explants of respiratory epithelium (Spock et al, 1967; Lancet, 1973). When serum from fibrocystic patients is injected into normal rats, the mucus of these rats becomes hyperpermeable (Gibson et al, 1970). Thus the serum factor seems to be responsible for the hyperpermeability of the mucus in fibrocystic disease, probably by inducing the high calcium content of the mucus, which seems to be the cause of its high viscosity. At the cellular level, electron microscopy has demonstrated calcium-containing zymogen granules in the submandibular gland and in the saliva of patients with fibrocystic disease (Blomfield et al, 1973).

It is interesting to note that the pancreatitis of hyperparathyroidism may develop in a similar manner to that of fibrocystic disease. In hyperparathyroidism, there is blockage of the pancreatic ducts by PAS-positive protein, which is probably mucus which has become excessively viscid, and it is likely that this is the result of the high serum calcium level (Waller, 1975).

Vitamin A deficiency

The goblet cell pattern of the rat small intestine is much depleted in vitamin A deficiency (Manville, 1937; De Luca et al, 1969). Impaired glycoprotein synthesis has been demonstrated in the intestines of such rats (De Luca et al, 1970). However, intestinal glycoprotein synthesis has not been studied in human vitamin A deficiency.

GLUCOSAMINE SYNTHETASE IN RELATION TO THESE DISEASES

Glucosamine synthetase has been studied in this thesis because of its role in mucus synthesis. Its concentration in a tissue may be an index of the capacity of that tissue to synthesize mucus (see Chapter 6). If it can be shown to be depleted in clinical situations in which there is hypersecretion of mucus, then its estimation may prove to be of value in clinical diagnosis and in the elucidation of the aetiology of diseases in which disorders of mucus production may play a part.

Glucosamine synthetase was studied in the following gastrointestinal disorders:—

The irritable colon syndrome (Chapter 9).
Ulcerative colitis (Chapter 10).
Crohn's disease (Chapter 10).
Membranous colitis (Chapter 11).
Carcinoma of the colon (Chapter 12).
Gastric ulcer (Chapter 13).
Duodenal ulcer (Chapter 13).
Coeliac disease (Chapter 14).

Studies were also made of the effects of the following drugs which may affect mucus synthesis:—

Aspirin (Chapter 15).
Sodium salicylate (Chapter 15).
Hydrocortisone (Chapter 15).
Carbenoxolone sodium (Appendix C).

CHAPTER 2

THE CHEMICAL NATURE OF GASTROINTESTINAL MUCUS

The molecules of gastrointestinal mucus are glycoproteins. Glycoproteins consist of polypeptide chains to which carbohydrate side-chains are covalently linked (Fig. 3). The carbohydrate side-chains consist of oligosaccharides or polysaccharides of molecular weight of between 500 and 3500 daltons (Gottschalk, 1969).

Nomenclature

Simple chemical tests such as precipitation with salicylsulphonic acid showed the protein nature of submaxillary and tracheal secretions and of the mucin of ovarian mucinous cysts and these were known as "mucoproteins" in the early years of the century (Levene, 1925). Webster and Komarov (1932) compared the elemental analysis of the insoluble component of the gastric juice of several species with known "mucoproteins" and concluded that the gastric secretions also contained mucoprotein. The polysaccharide component of gastric mucus became known in the 1930's and it was considered to be a "mucopolysaccharide", although that term was used even at that time to designate particularly certain glycoproteins which contained uronic acid, such as chondroitin sulphate found in cartilage (Meyer, 1938).

In the 1960's, the mucopolysaccharides came to be defined as molecules in which the carbohydrate was predominant with relatively little protein (Kent, 1962). They include hyaluronic acid, the chondroitin sulphates (chondroitin-4-sulphate and chondroitin-6-sulphate), heparin, and keratin sulphate. These compounds are sulphated and their carbohydrates are acetylhexosamines

alternating with hexuronic acids such as glucuronic acid and iduronic acid (Gottschalk, 1972). Gastrointestinal mucus is not composed of mucopolysaccharide by this definition.

With regard to this thesis and to the enzyme glucosamine synthetase, it is important to note that the mucopolysaccharides do contain acetylhexosamines and that tissues which are composed of mucopolysaccharide, such as the connective tissues, contain glucosamine synthetase in concentrations of the same order as those in the gastrointestinal mucosa.

The mucopolysaccharides of connective tissue are now often referred to as the glycosaminoglycans (Jeanloz, 1960).

The identification of mucus: visible and soluble mucus

Difficulties have arisen in the collection of gastrointestinal mucus for analysis. The mucus is just one of the many secretions of the gastrointestinal tract, which include water, electrolytes and hydrogen ions, digestive enzymes, exuded serum proteins and secreted immunoglobulins. The gastrointestinal contents also include desquamated epithelial cells, saliva, food in the process of digestion, and bacteria. The secreted immunoglobulins and some of the serum proteins are themselves glycoproteins, as are some of the membranes of the desquamated cells, but they are not part of the mucus. Nevertheless, the glycoproteins of mucus are present in much greater amounts than other glycoproteins in the gastrointestinal tract and it is possible to analyse mucus in secretions and in homogenized mucosal scrapings.

The gastric and intestinal juices are not physically homogeneous. Gastric juice can be separated by filtration (Heatley, 1959) or by centrifugation (Glass and Boyd, 1949) into "visible mucus" (Webster and Komarov, 1932) and a soluble fraction. The visible mucus has the form of a gel. The soluble mucus is also a viscous fluid although not as viscous as the visible mucus.

In the past, some authors have ignored one or other of these two components of gastric mucus. Heatley (1959) only studied the visible mucus, as did Skoryna and Waldron-Edward (1967). Wise and Ballinger (1970) precipitated the cellular debris and the visible mucus and "all further estimations were made on the soluble mucus"; yet they were investigating the parasympathetic control of gastric mucus secretion and they ought to have collected all of the secreted mucus.

The identification of gastric mucus as "mucoprotein" by Webster and Komarov (1932) was based on the elemental proportions of the visible mucus. The soluble component of gastric juice appeared to be quite different on such an analysis. Glass propounded that there were three components of gastric mucus, namely, the visible mucus and two components of soluble mucus, the "glandular mucoprotein" and the "dissolved mucoproteose" (Glass and Boyd, 1949; Glass, 1967).

Schrager analysed both the visible and the soluble mucus by gas chromatography to elucidate their protein and carbohydrate content and he showed that they were of similar glycoprotein composition. Soluble mucus contained pepsin whereas visible mucus did not (Schrager, 1969, 1970; Schrager and Oates, 1971). It is interesting to note that Webster and Komarov had noticed a similarity between the soluble fraction and pepsin in their elemental analyses.

It thus appears that mucus in its gel form, the visible mucus, is digested by pepsin to become soluble in gastric juice.

THE CHEMICAL COMPONENTS OF MUCUS

The water content

As with most biological fluids, by far the largest component of gastrointestinal mucus is water. Boldyreff (1936) found that 30 ml of canine gastric mucus had a dry weight of 100 mg, giving a proportion for the dry weight of 0.3%, although it appears that Boldyreff was actually referring to the whole gastric juice. Heatley (1959) obtained visible mucus by filtration of the secretions of pyloric and duodenal pouches of experimentally prepared pigs and found the dry weight to be 0.1% for pyloric mucus and 0.15% for duodenal mucus. On these figures, the water content of mucus is about 99.8% but the meaning of such a statement is limited by the fact that visible mucus is a substance which is permeable to water and it is collected from an aqueous environment.

Methods of analysis

Modern analysis of the chemical composition of mucus is based on electrophoretic and chromatographic methods. Visible mucus presents difficulties in view of its gel nature. Schrager (1970) has found that analysis

is satisfactory if it is dissolved in saturated calcium chloride solution or in 8M urea, and Skoryna and Waldron-Edward (1967) found that 8M solutions of carbamide, formamide and acetamide were suitable solvents. Schrager (1974) now uses N-acetylcysteine to dissolve gastrointestinal mucus. Martin and his colleagues (1968) allowed the mucus to undergo autoproteolysis for 48 hours at 37°, a method which has been criticized but which may have some validity in view of the relationship between the visible and the soluble mucus.

Samples of mucus, from the aspirated soluble mucus, from visible mucus dissolved as described, and from mucosal homogenates dissolved in appropriate solvents, are fractionated on polyacrilamide gel columns such as Sephadex and Bio-Gel (Glass, 1967; Menguy et al, 1969). Schrager (1970) uses Bio-Gel P150.

The fractions of glycoprotein so obtained are analysed for their peptide and carbohydrate content. The sugars and amino sugars are identified by gas-liquid chromatography (Clamp et al, 1967; Clamp, 1974).

The amino acid composition

The peptide component of gastric and small intestinal mucus is approximately 15-20% of the molecule and the amino acids have a characteristic composition. Threonine and serine make up 45-50% of the amino acids and serine, threonine, proline, alanine and glycine constitute 75-80% of the amino acid content. Threonine and serine occur in a ratio of roughly 2:1 (Schrager, 1974).

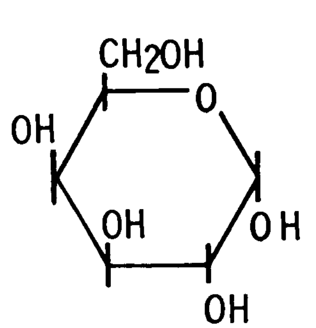
The carbohydrate composition

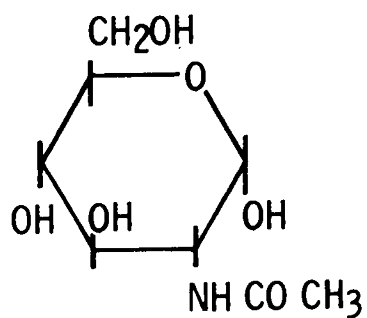
The carbohydrates of gastric mucus are D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and L-fucose (Fig. 2). Sialic acids are also present in small amounts (Häkkinen et al, 1965; Allen and Snary, 1972) although Schrager (1974) does not consider that sialic acid forms part of gastric mucus.

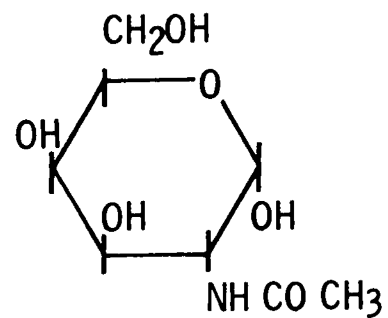
Small intestinal mucus contains the same carbohydrates, including significant amounts of sialic acid (Bella and Kim, 1972; Forstner et al, 1973b; Schrager, 1974).

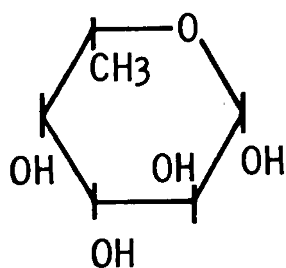
Colonic mucus from the sheep also has these same carbohydrates, with more sialic acid but less fucose than in gastric mucus (Marsden, 1964).

The sialic acids of intestinal mucus are N-acetylneuraminic acid (Fig. 2),









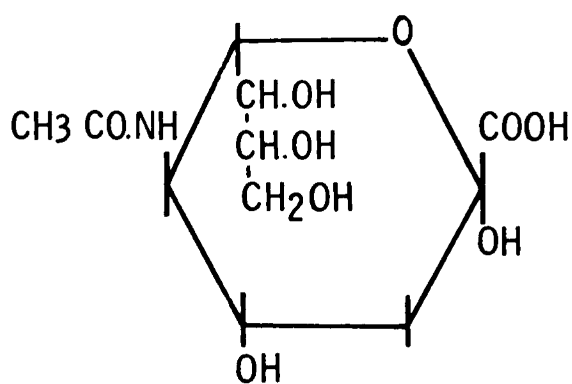


FIG. 2. The chemical structure of the carbohydrates of gastrointestinal mucus.

which is the principal sialic acid in intestinal mucus (Schrager, 1974), N-glycollylneuraminic acid, and O,N-diacetylneuraminic acid (Kent and Draper, 1968).

Mannose

Kent (1974) only found small amounts of mannose in sheep colonic mucus. However, Teague et al (1973) have reported finding much larger amounts of mannose in the carbohydrates of the mucous glycoproteins of human colonic and rectal mucus and of human rectal and colonic biopsies. Soergel and Ingelfinger (1964), by immunoelectrophoresis, found considerable quantities of serum glycoproteins in human rectal mucus. Serum glycoproteins, like the glycoproteins of parotid gland mucus and like immunoglobulin glycoprotein, contain mannose as an important carbohydrate constituent. The mannose found by Teague et al might have come from serum glycoproteins or from locally produced immunoglobulin. Their finding that the mannose content of the mucus was increased in ulcerative colitis would suggest this hypothesis for the source of the mannose (see Chapter 10).

Sulphate

There has been confusion and dispute over the presence of sulphate in gastrointestinal mucus. The confusion arose because of species differences. There is sulphate in the gastric mucus of the dog (Meyer et al, 1937; De Graef and Glass, 1968b and the hog (Slomiany and Meyer, 1972) but autoradiography of ^{35}S-sulphate uptake by the gastric mucosa has shown no significant sulphation of the gastric mucus of the pig (Snary and Allen, 1969), the sheep, the calf, the frog, and the guinea-pig (Pasternak et al, 1958). Belanger (1954) found that in the rat and the hamster there was ^{35}S-sulphate uptake by the mucous neck cells but not by the surface mucous cells. A single experiment in a human patient with melanomatosis showed that there was no uptake of radioactive sulphate by the gastric mucosa of man (Lambert et al, 1971).

Another cause of confusion has been that gastric aspirates are contaminated by the glycoproteins of the saliva and of the secretions of the oesophageal glands. Human salivary gland mucus does not appear to contain sulphate (Nisizawa and Pigman, 1960) but the oesophageal mucus does seem to be sulphated (Lambert et al, 1971). Human gastric aspirates have been found

to contain sulphated mucus (Schrager, 1970; Martin et al, 1968) but there was no sulphated mucus in two oesophagectomized patients studied by Lambert et al (1971).

Histochemical studies (see Chapter 4) have demonstrated sulphated mucus in human large intestinal mucus but not in small intestinal mucus (Lev and Spicer, 1965; Goldman and Ming, 1968). Chemical analyses have not been reported on human large intestinal mucus. Schrager (1974) found no sulphate in human small intestinal mucus. Chemical analyses of the small intestinal mucus of the rabbit (Nemoto and Yosizawa, 1969) and the rat (Bella and Kim, 1972) have shown that there is sulphate in the small bowel mucus in these species and Marsden (1964) showed that ester sulphate constituted 4% of a purified fraction of sheep colonic mucus.

THE MOLECULAR STRUCTURE OF MUCUS

The molecular weight of gastrointestinal mucus has been calculated by several workers to be approximately 200 000 daltons (Marsden, 1964; Kent, 1967; Schrager, 1970; Allen and Snary, 1972). Hough and Jones (1972) give a molecular weight of over 300 000 daltons for human gastric mucus.

From the results of light-scattering measurements, Schrager (1970) considers that the molecules of gastric mucus are rigid rod-shaped molecules, the larger ones being about 1000 nm in length.

Allen and Snary (1972) separated the visible pig gastric mucus into two components of identical chemical composition, with molecular weights of 200 000 and 110 000, the larger component being much more viscous than the smaller component. There was a 75% reduction in the viscosity of both components when treated with N-acetylcysteine and with mercaptoethanol and they concluded that the smaller component is formed of four units, each of molecular weight 27 700 daltons, linked by S-S bridges, and that it polymerizes to form the larger component.

The molecule of mucus consists of a peptide backbone with attached carbohydrate side-chains (Fig. 3). The elucidation of its molecular structure has involved the sequential removal of the sugars from the ends of the carbohydrate side-chains and the determination of the composition of the residual structure. Eventually only the peptide chain remains (Kent, 1967; Schrager,

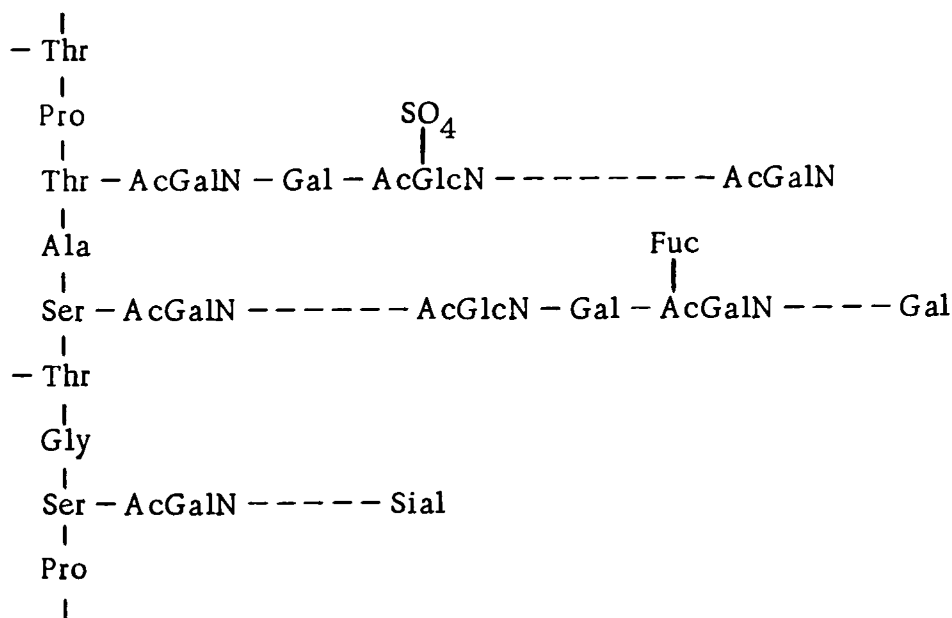


FIG. 3. Diagrammatic representation of a molecule of gastrointestinal mucus. (AcGalN = N-acetylgalactosamine; AcGlcN = N-acetylglucosamine; Ala = alanine; Fuc = fucose; Gal = galactose; Gly = glycine; Pro = proline; Ser = serine; Sial = sialic acid; Thr = threonine.)

1974). One standard method is the Smith degradation, in which alternate periodate oxidation and borohydride reduction remove the terminal sugars (Clamp and Hough, 1964; Kent, 1967). Fucose and sialic acid are easily removed from the molecule, even by mild acidic hydrolysis (Schrager and Oates, 1968). These sugars are only found as terminal sugars at the ends of the carbohydrate side-chains. They may occur as branches off these side-chains.

Careful analysis of the proportions of the various carbohydrates and peptide components of mucus has revealed important facts about their structure. When Schrager (1970) measured the molar ratios of the carbohydrates in the gastric mucus of his patients, he found variation from patient to patient, but the ratios of galactose, galactosamine, glucosamine and fucose remained fairly constant through several aspirations in an individual patients. The differences between patients were related to their ABO blood group and their secretor status. In non-secretors, the ratio of galactose:glucosamine:galactosamine appeared to be stoichiometric, at 4:3:1. The same ratio was found in group O secretors. The ratios observed for secretors of groups A, B, and AB were not entirely stoichiometric but could be explained if 4:3:1 remained the basic ratio for galactose:glucosamine:galactosamine, with a small additional amount of galactosamine for group A, of galactose for group B, and of both galactose and galactosamine for AB. All secretors had a similar amount of

fucose, whilst non-secretors had considerably less fucose.

The terminal sugars of gastric and small intestinal mucus carry ABO blood group specificity in blood group secretors. There is no blood group activity in the mucus from the distal colon (Szulman, 1960). The structures of the blood-group-active carbohydrates are shown in Fig. 4. These are the same as the

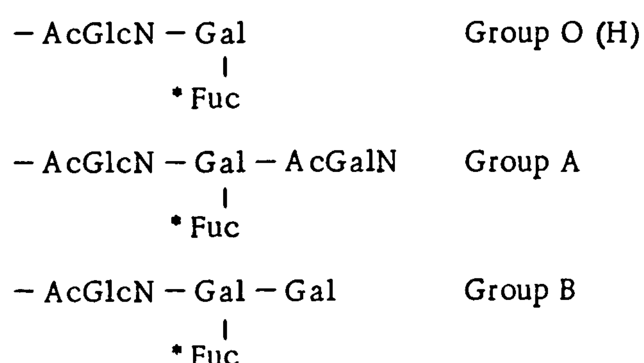


FIG. 4. The chemical structure of the terminal carbohydrates which determine the ABO blood group activity of glycoproteins and glycolipids. • Secretor status determined by the presence of this fucose. (Abbreviations as in Fig. 3.)

terminal carbohydrates of the glycolipids of the red cell surface membrane (Watkins, 1972, 1974; Koscielak et al, 1973).

For human small intestinal mucus, Schrager (1974) has found a ratio of 3:2:1 for galactose:glucosamine:galactosamine. In both gastric and small intestinal mucus, Schrager interprets his findings as indicating an alternation of galactose with either acetylgalactosamine or acetylglucosamine.

Repeated digestion of the glycoprotein molecule eventually leaves residues consisting of only a single species of sugar. In ovine submaxillary gland mucus, this sugar is N-acetylgalactosamine and it is found in glycopeptides in which the amino acids are only serine and threonine (Bhavanandan et al, 1964). The glycopeptide bond is susceptible to the lysosomal enzyme N-acetylhexosaminidase obtained from bovine spleen. The structure of the carbohydrate-peptide linkage in gastrointestinal mucus is presumed to be similar, with acetylgalactosamine always closest to the peptide chain and linked to serine or threonine. These two amino acids can be shown in vivo to be acceptors of acetylgalactosamine for the synthesis of mucous glycoproteins (Marshall, 1974). The bond between serine or threonine and acetylgalactosamine is an O-glycosidic bond (Kent, 1967), a serine-β-xyloside linkage of the type that was first demonstrated by Lindahl and Roden (1966) in chondroitin-4-sulphate.

Schrager (1974) showed that the sum of the serine and threonine content was equal to the content of acetylgalactosamine in gastric and small intestinal mucus, except in blood group A secretors, who have additional amounts of acetylgalactosamine as terminal groups. Thus the acetylgalactosamine is linked to serine and threonine in the peptide chain and, to fit in with the ratios Schrager has found for carbohydrates in the side-chains of gastric and small intestinal mucus, he proposes the chemical structure for these as shown in Fig. 5.

```
(a) Thr
    or  – AcGalN – Gal – AcGlcN – Gal – AcGlcN – Gal – •X
    Ser     |                          |       |
          AcGlcN                      Fuc    •Fuc
            |
           Gal

(b) Thr
    or  – AcGalN – Gal – AcGlcN – Gal – •X
    Ser     |       |       |
          AcGlcN   Fuc    •Fuc
            |
           Gal
            |
           Sial
```

FIG. 5. Proposal of Schrager (1974) for the structure of the carbohydrate side-chain (a) in human gastric mucus and (b) in human small intestinal mucus. (Abbreviations as in Fig. 3.) •Present only in blood-group secretors. X = AGalN in blood group A, Gal in blood group B, absent in blood group O.

Marsden (1964) found that the sulphate groups in sheep colonic mucus were attached to amino sugars, that is, to acetylglucosamine or acetylgalactosamine, close to the peptide chain (Kent, 1967). In the sulphated gastric mucus of the hog, Slomiany and Meyer (1972) have shown that the sulphate is attached to the acetylglucosamine closest to the peptide chain, as shown in Fig. 3.

Microheterogeneity

A characteristic feature of glycoproteins is their microheterogeneity (Winzler, 1973; Beeley, 1974). Molecules from the same source are not necessarily identical and the molecules themselves may have heterogeneous rather than regularly occurring structures. It is unlikely that the variations occur in the peptide backbone, which is synthesized on a messenger-RNA template. The microheterogeneity is apparent with the carbohydrate side-chains. The short

disaccharide side-chains of salivary gland mucus do not seem to display much heterogeneity, which is more marked with longer carbohydrate side-chains (Montgomery, 1972). Schrager (1974) has found no significant microheterogeneity in the non-terminal carbohydrates of gastric and small intestinal mucus but he has observed it with respect to the amount of terminal blood-group specific carbohydrate that is present.

Kent (1974) has suggested that, rather than being distributed uniformly along the peptide chain, the carbohydrates might occur mainly at one end of the chain, making the molecule polar, hydrophilic at the carbohydrate end and hydrophobic at the other end. Such a model would seem a reasonable suggestion for membrane glycoproteins. It is an interesting speculation for the structure of secreted mucus but there is as yet no evidence to support it.

CHAPTER 3

THE BIOSYNTHESIS OF MUCUS

Glycoprotein synthesis appears to conform to the same pattern in all mammalian cells. All cells synthesize glycoprotein, if only for their own membranes and membranous structures. Current theories are derived from studies of the synthesis of liver glycoprotein, of salivary gland and gastro-intestinal mucus, of immunoglobulin, and of the glycosaminoglycans of connective tissue.

The first stage is the assembly of the peptide chain in the ribosomes. This is in the usual manner of protein synthesis, with the amino acids being assembled in sequence along strands of messenger RNA.

The first acetylhexosamine sugar is attached to the peptide chain while the latter it is still on the ribosomes. Galactose and more acetylhexosamine are added in the rough and smooth endoplasmic reticulum, and the terminal carbo-hydrates, fucose and sialic acid, are added in the smooth endoplasmic reticulum of the Golgi apparatus. Sulphate is added at an early stage, probably in the rough endoplasmic reticulum.

This theory is based on experiments in which radioactive serine, threonine, glucosamine, galactose, fucose and sialic acid were incorporated into glyco-proteins _in vivo_, in the rat intestinal mucosa (Louisot et al, 1967), in the bovine submaxillary gland (Lawford and Schachter, 1967), and in the rat liver (Gottschalk, 1969), and the radioactivity was then detected in subcellular fractions (Kim et al, 1971). In gastrointestinal mucus, the first acetylhexos-amine sugar is acetylgalactosamine, which is added in the ribosomes, and the remainder of the carbohydrate side-chains are added in the endoplasmic

reticulum. Support for this theory of the cellular assembly of glycoproteins comes from electron microscopic autoradiography of the glycoprotein precursors, ^{3}H-glucose, ^{3}H-galactose (Neutra and Leblond, 1966a,b) and ^{3}H-fucose (Bennett and Leblond, 1970) as well as from subcellular fractionation experiments (Gottschalk, 1969). In the intestinal goblet cell, the droplets of synthesized mucus are first seen under the electron microscope at the Golgi apparatus and they then coalesce to form intracellular mucus globules (Freeman, 1962).

Neutra and Leblond (1966a), by autoradiography of the rat colon using ^{3}H-glucose, demonstrated the time course of these events. Five minutes after injection, the label is in the flattened saccules of the Golgi apparatus; at 20 minutes, the radioactivity is in mucus granules near the Golgi apparatus; and by 40 minutes, nearly all the mucus granules are labelled.

The carbohydrates are supplied to the carbohydrate chain from nucleotide sugars in the cytoplasm, namely, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-galactose, GDP-mannose, GDP-fucose, and CMP-sialic acids (O'Brien and Neufeld, 1972). Transfer of the sugars from the nucleotides to the glycoprotein molecule is catalysed by specific transferases, which seem to be firmly attached to the membrane of the endoplasmic reticulum. For each linkage in each glycoprotein, there appears to be a specific transferase and the order of attachment of the carbohydrates on the glycoprotein is probably dictated by the order of the transferases along the membranes (Hagopian and Eylar, 1968).

A number of transferases have been isolated from disrupted cells, including some directing the attachment of the terminal carbohydrates of blood group specificity, such as the N-acetylgalactosaminyl transferase found by Hearn et al (1968) in the submandibular gland of human blood group A secretors. Hagopian and Eylar (1969) have characterized a polypeptide-N-acetylgalactosaminyl transferase in bovine submaxillary gland, which is responsible for attaching N-acetylgalactosamine to the peptide core. Baker and Munro (1971) identified an enzyme in canine respiratory tissue which transfers galactose from UDP-galactose to acetylgalactosamine residues; this must represent the building up of the carbohydrate side-chains. Bella and Kim (1971) have demonstrated a fucosyltransferase which transfers fucose from GDP-fucose to a glycoprotein isolated from rat small intestinal mucosa.

The biosynthesis of the nucleotide sugars

These are synthesized in the cytoplasm from glucose-6-phosphate and the phosphate nucleotides. The synthesis of UDP-galactose is shown in Fig. 6 and that of GDP-mannose and GDP-fucose is shown in Fig. 7. UDP-N-acetylglucosamine is synthesized from fructose-6-phosphate and L-glutamine by the series of reactions shown in Fig. 8 (Warren, 1972).

Hardingham and Phelps (1968) measured in neonatal rat skin the labelling from ^{14}C-glucose of the intermediates in the synthetic pathway of UDP-N-acetylglucosamine. They could not detect any glucosamine-6-phosphate nor any N-acetylglucosamine-1-phosphate. N-Acetylglucosamine-6-phosphate was detected but it had a rapid turnover time (4.0 ± 0.3 min) compared to that of UDP-N-acetylhexosamine (76.0 ± 9.0 min). The turnover rates of acetylglucosamine-6-phosphate and UDP-acetylhexosamine were similar (2.0 and 1.5 μmole/min/g wet wt respectively). Hence it seems that glucosamine-6-phosphate is rapidly converted to N-acetylglucosamine-6-phosphate, which itself is soon converted first to N-acetylglucosamine-1-phosphate and then immediately to UDP-N-acetylglucosamine.

Lukie and Forstner (1972a) studied the incorporation of ^{14}C-glucosamine into the same intermediates. They found only small amounts of glucosamine-6-phosphate compared to the other intermediates, but they did not measure acetylglucosamine-1-phosphate and acetylglucosamine-6-phosphate separately.

UDP-N- acetylgalactosamine is formed by the epimerization of UDP-N-acetylglucosamine (Maley and Maley, 1959). This is the source of galactosamine for the glycoproteins (Fig. 9).

The sialic acids are synthesized from UDP-N-acetylglucosamine (Fig. 10) via N-acetylmannosamine (Comb and Roseman, 1958; Warren and Felsenfeld, 1961). N-Acetylneuraminic acid (Fig. 2) is formed. The formation of N-glycollylneuraminic acid is by the oxidation of N-acetylneuraminic acid (Schauer et al, 1968) and O,N-diacetylneuraminic acid is formed by the O-acetylation of N-acetylneuraminic acid. The three sialic acids are converted to the CMP-sialic acids (Fig. 10) for utilization in glycoprotein synthesis (Kean and Roseman, 1966).

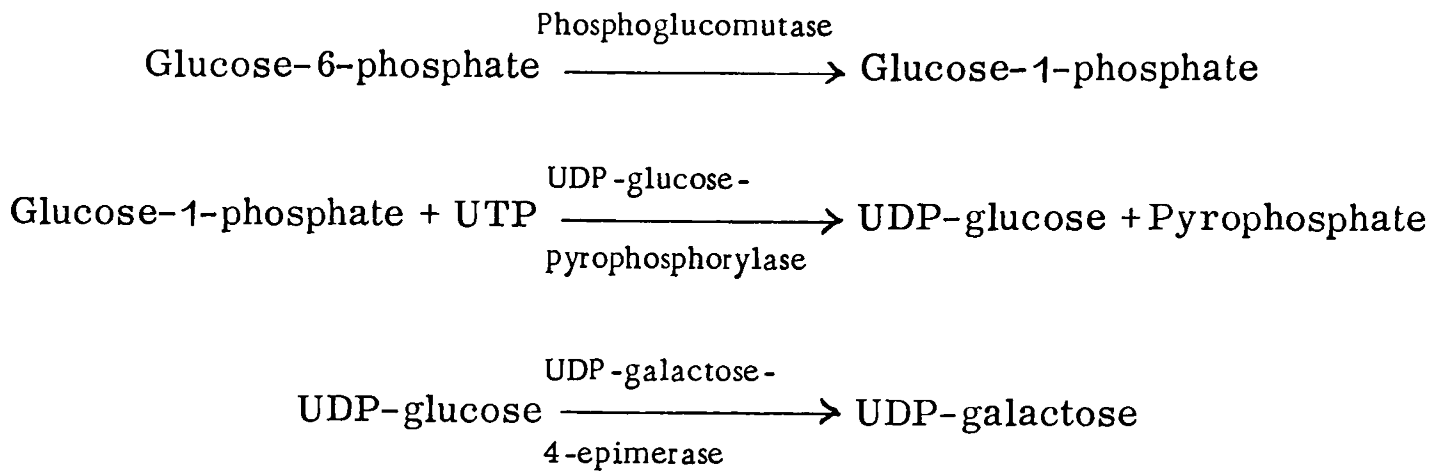


FIG. 6. The biosynthesis of UDP-galactose.

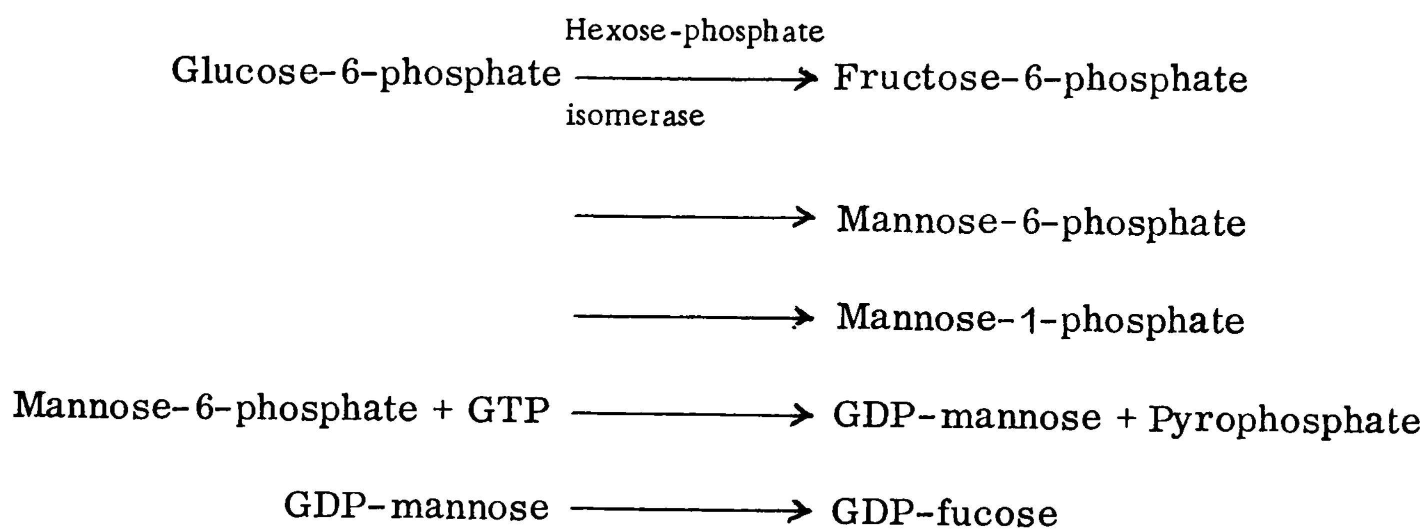


FIG. 7. The biosynthesis of GDP-fucose and GDP-mannose.

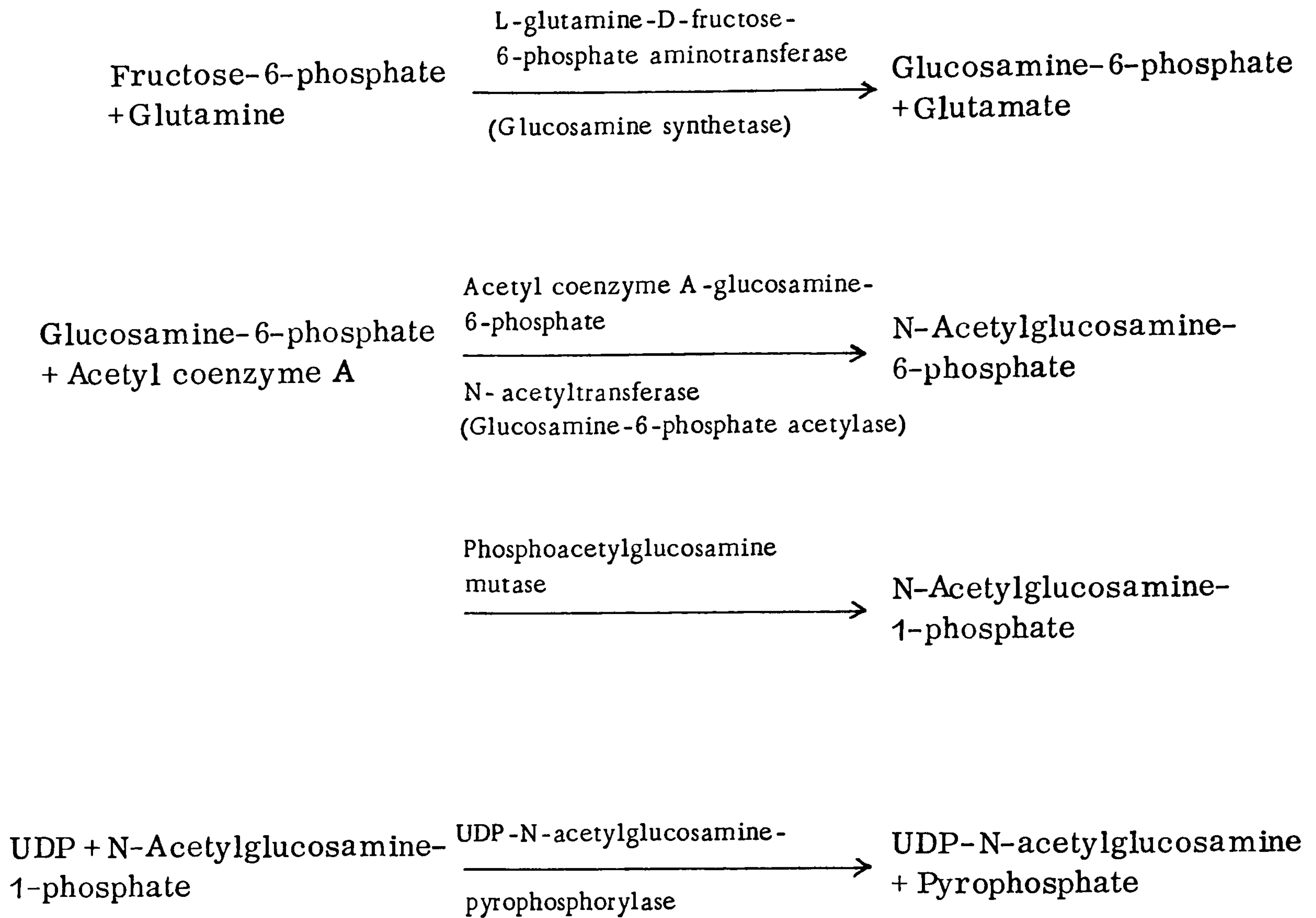


FIG. 8. The biosynthesis of UDP-N-acetylglucosamine from fructose-6-phosphate and glutamine.

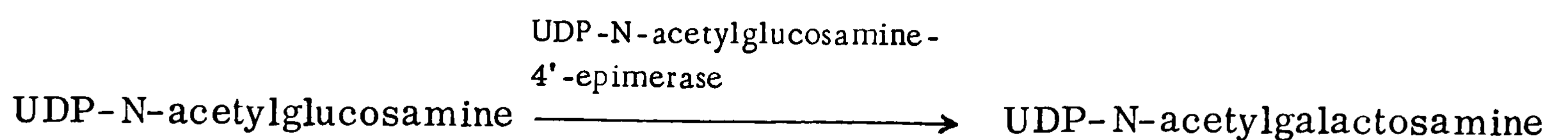
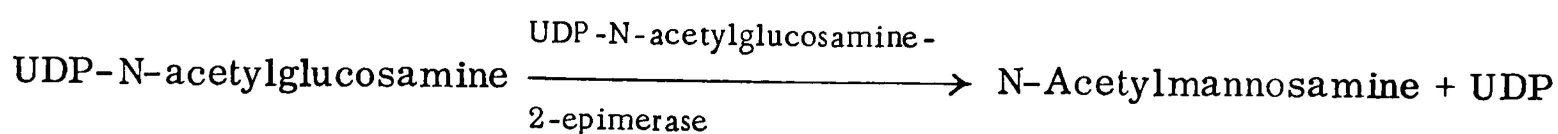
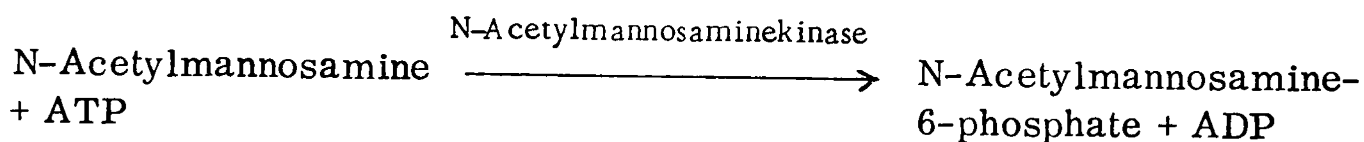
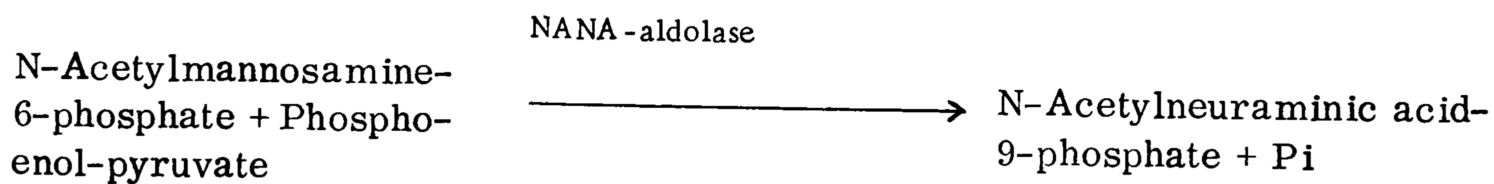
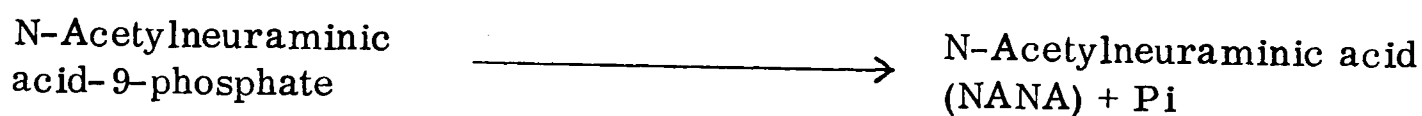


FIG. 9. The formation of UDP-N-acetylgalactosamine.









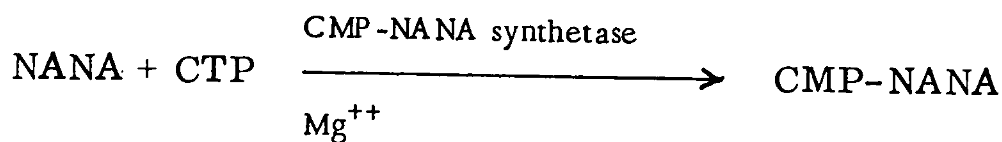


FIG. 10. The biosynthesis of CMP-N-acetylneuraminic acid (CMP-NANA), a CMP-sialic acid.

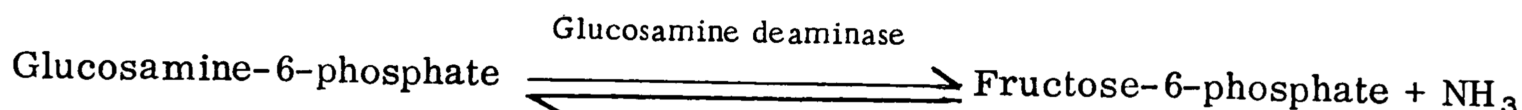


FIG. 11. The deamination of glucosamine.

Glucosamine deaminase

Leloir and Cardini in 1956 found glucosamine deaminase (Fig. 11) in the pig kidney. They showed that glucosamine-6-phosphate could be synthesized by this enzyme, although the reaction generally proceeds towards deamination of glucosamine-6-phosphate. Glucosamine synthetase (Fig. 8) rather than glucosamine deaminase appears to be the major pathway for glucosamine synthesis (Warren, 1972).

Intracellular pools of glycoprotein

All cells contain membrane glycoprotein and in the intestinal cell, particularly in the small intestinal cell, this is prominent in the glycocalyx of the brush border. The glycoprotein of the glycocalyx needs to be distinguished from the mucus glycoprotein. Forstner (1970) demonstrated two separate pools of glycoprotein in the rat small intestine. The glycoprotein was labelled with [1-^{14}C]glucosamine and mucosal scrapings were homogenized and the subcellular components were fractionated. There was a pool derived from the plasma membranes with a rapid turnover and with a peak of radioactivity in glycoprotein-bound glucosamine at 90 min. The major fraction was a pool derived from the cell sap, which turned over more slowly and in which the peak of radioactivity of the glycoprotein occurred at 150 min. This fraction corresponded to the luminal mucus collected by perfusion. During the incubation of tissue slices in which the glycoprotein had already been labelled, there was found to be a loss of mitochondrial and cell sap radioactivity, which was accounted for by radioactivity appearing in the bathing medium, whereas the brush border radioactivity did not change. The labelled glycoprotein in the bathing medium was identified as being similar to the glycoprotein of the luminal mucus.

Feedback inhibition

Kornfeld et al (1964) demonstrated feedback inhibition at two points in the biosynthesis of glycoprotein in the rat liver:

1) UDP–N–acetylglucosamine inhibits glucosamine synthetase (see Chapter 6).
2) CMP-N–acetylneuraminic acid inhibits UDP-N-acetylglucosamine 2-epimerase which forms N-acetylmannosamine from UDP-N-acetylglucosamine (see Fig. 10).

It is assumed that such inhibition also occurs in other mammalian cells which synthesize glycoprotein. Mazlen et al (1969) showed inhibition of glucosamine synthetase by UDP-N-acetylglucosamine in the bovine retina.

Lipid intermediates in glycoprotein biosynthesis

Interest in the role of lipid intermediates in glycoprotein synthesis has recently been stimulated by the work of Hemming and his colleagues on the dolichol phosphates (Hemming, 1973). These are lipids which act as acceptors of mannose from GDP-mannose (Richards et al, 1971). The mannose is then transferred from dolichol phosphate-mannose to glycoprotein. The dolichol phosphate accelerates uptake of mannose from GDP-mannose to the growing glycoprotein (Richards and Hemming, 1972).

This is the only example of such lipid-accelerated transfer so far described but other lipid accelerators of glycoprotein synthesis are being sought.

Retinol, derived from vitamin A, is a lipid and it is speculated that the impaired glycoprotein synthesis observed in vitamin A-deficient rats (De Luca et al, 1970) may be due to the lack of retinol as a lipid intermediate in glycoprotein biosynthesis (Kent, 1974).

The measurement of mucus synthesis

The rate of mucus synthesis by a tissue is difficult to ascertain separately from the rate of total glycoprotein synthesis. This can be done with sophisticated kinetic experiments with subcellular fractionation, as in the work of Forstner (1970). Forstner's results have shown that the major component of glycoprotein synthesis in the mammalian intestine is the synthesis of the mucus. Human studies generally assume that glycoprotein synthesis as measured represents the rate of mucus synthesis.

The rate of incorporation of labelled precursors of glycoprotein into non-dialysable or protein-bound forms is the usual method of measuring glycoprotein synthesis. Many workers prefer to identify the labelled macromolecule as glycoprotein. ^{14}C-Glucosamine incorporation is most commonly used (Forstner, 1970; MacDermott et al, 1974). Ellis and Stahl (1973) studied L-[G-^{3}H]fucose and L-[U-^{14}C]serine incorporation. Parke's group have studied the incorporation of N-acetyl[^{3}H]glucosamine, [1-^{14}C]galactosamine, ^{14}C-galactose, ^{3}H-fucose and N-acetyl[1-^{14}C]neuraminic acid into gastric glycoprotein (Lindup

et al, 1974; Johnston et al, 1975). These workers have attempted to assess separately the total synthesis of glycoprotein, by the rate of incorporation of the non-terminal sugars such as N-acetylglucosamine, and by the rate of completion of the carbohydrate side-chains, as reflected by N-acetylneuraminic acid incorporation.

In the classical studies on the mechanism of glycoprotein synthesis, ^{14}C-glucose incorporation was frequently used (Draper and Kent, 1963; Allen and Kent, 1968; Hardingham and Phelps, 1970) but it was necessary to identify the glycoprotein chemically.

In experimental animals, the radioactive label can be injected in vivo. For human studies, biopsy material needs to be studied in tissue culture in vitro, a compromise which hinders the assessment of glycoprotein synthesis in human pathological conditions. Nevertheless, meaningful results have been obtained by such means (MacDermott et al, 1974; Johnston et al, 1975).

Hormonal influences on mucus synthesis

Forstner et al (1973a) demonstrated hormonal effects on mucus biosynthesis by studying the rate of incorporation of ^{14}C-glucosamine into slices of rat small intestine in vitro. Beta-adrenergic agents increased the rate of glucosamine incorporation, as did cyclic AMP.

N-Acetylhexosaminidases

These are lysosomal enzymes which cleave the O-glycosidic linkages between the peptide core and the carbohydrate side-chains in glycoproteins (Gottschalk and Buddecke, 1972). N-Acetylgalactosaminidase is the principal acetylhexosaminidase (Bhavanandan et al, 1964). It is found in the lysosomes of mammalian organs such as the liver, the kidney and the spleen (Mahadevan and Tappel, 1968) and its assay has been used as a marker of lysosomal activity in human jejunal biopsies in tissue culture (Mitchell et al, 1974).

These enzymes are lysosomal, whilst glycoprotein synthesis occurs away from the lysosomes. The N-acetylhexosaminidases do not seem to bear any relationship to glycoprotein biosynthesis.

CHAPTER 4

THE HISTOCHEMISTRY OF MUCUS

Mucus can be identified histochemically and the stains can be used to ascertain the pH of the mucus and to detect the presence of accessible sulphate radicles (Table 1). Such procedures are frequently used by histopathologists in the diagnosis of mucosal lesions.

TABLE 1. Histochemical stains for mucus

Sulphated mucus (strongly acidic)	Acidic non-sulphated mucus (predominance of sialic acid)	Neutral mucus (predominance of fucose)
Aldehyde-fuchsin High iron-diamine Alcian blue at pH 0.5	Alcian blue } at pH 2.5 Alcian yellow } Maxwell stain (Alcian green at pH 1.5) (These also stain sulphated mucus)	PAS Maxwell stain (Alcian yellow at pH 7)

The Alcian stains – blue, yellow and green – can in fact be used at any pH.

The first stain to be used for mucus was the PAS (periodic acid-Schiff) reaction (McManus, 1946). Periodic acid acts on the 1,2-glycol linkage (-CHOH-CHOH-) in sugars, which are oxidized to aldehydes (-CHO, -CHO). Monosaccharides so treated are washed out of sections by aqueous reagents but the aldehyde groups from polysaccharides such as glycoproteins remain <u>in situ</u>. The Schiff reagent is basic fuchsin which has been bleached by sulphurous acid. The magenta colour of fuchsin is restored by the aldehydes produced by periodic acid (McManus, 1948). All polysaccharides are stained by PAS, including glycogen, but the only polysaccharides in mucosae are the glycoproteins. PAS is a neutral stain, so that the PAS reaction stains only

neutral mucus (Fig. 1).

The Alcian stains — Alcian blue, Alcian yellow and Alcian green — can be used at any desired pH. Alcian blue (Fig. 53) was first used by Mowry in 1956 to stain acidic mucus. In the Maxwell stain (Maxwell, 1963; Whitehead, 1973), Alcian yellow is used at pH 7 for neutral mucus and Alcian green at pH 1.5 for acidic mucus. Generally, however, the Alcian stains are used at a low pH to detect acidic mucus. At a pH of 0.5, only strongly acidic mucus is stained, whereas at pH 2.5, more weakly acidic mucus is also stained.

Mucus which stains with Alcian blue at pH 0.5 is in fact sulphated mucus, as demonstrated by specific stains for sulphate. Such stains include aldehyde fuchsin, which stains sulphated mucus an intense purple colour (Spicer and Meyer, 1960), and the high iron-diamine reaction (Fig. 86), in which ferric chloride is added to a mixed diamine reagent to stain the sulphated mucus black (Spicer, 1965).

Of the chemical components of mucus, the amino acids and the hexoses are neutral, the hexosamines are basic but being acetylated they too are neutral, and it is the sialic acids and the sulphate groups which give acidic mucus its low pH. Presumably, during the staining process, some of the ester sulphate bonds are hydrolysed, liberating sulphuric acid which makes sulphated mucus strongly acidic. Weakly acidic mucus contains sialic acid but is not sulphated, at least histochemically. Excluding sulphated mucus, the histochemical pH of the mucus is weakly acidic when the predominant terminal sugar is sialic acid, as it is in the intestine and particularly in the colon, and it is neutral when the predominant terminal sugar is fucose, as in the stomach.

The anatomical distribution of the histochemical types of mucus

Although mucus of any pH below 7 can be identified, it is customary for histopathologists to classify mucus into three histochemical types that have been referred to:—

1) Neutral mucus, stained by PAS or by an Alcian stain at pH 7.
2) Acidic non-sulphated mucus, stained by Alcian blue at pH 2.5 but not at 0.5.
3) Sulphated mucus, stained by the specific methods for sulphated mucus but more commonly identified by staining with Alcian blue at pH 0.5. (Alcian blue at pH 2.5 stains both sulphated and weakly acidic mucus.)

In the human stomach, the surface mucous cells contain neutral mucus. The mucous neck cells and some of the surface mucous cells deep in the foveolae contain acidic non-sulphated mucus but in the body of the stomach such cells are much less numerous than cells containing neutral-staining mucus (Lev, 1966; Goldman and Ming, 1968). In the pyloric glands, the mucus is acidic and mainly non-sulphated although Goldman and Ming (1968) have detected occasional cells containing sulphated mucus. The conclusion that sulphated mucus is absent from the human stomach is based on data from the body mucosa. Lambert et al (1971), for instance, did not study the pyloric mucosa.

In areas of intestinal metaplasia in the stomach, acidic non-sulphated mucus and sometimes sulphated mucus are found (Goldman and Ming, 1968).

In the duodenum, Brunner's glands contain only neutral mucus. The goblet cells of the small intestine contain only acidic non-sulphated mucus (Lev and Spicer, 1965; Gad, 1969).

In the caecum and the colon, acidic non-sulphated mucus predominates in all parts, but there is also sulphated mucus and neutral mucus, although in the ascending colon there is very little sulphated mucus (Gad, 1969). Beyond the ascending colon, sulphated mucus is secreted deep in the crypts, with acidic non-sulphated being found in the more superficial goblet cells (Greco et al, 1967; Gad, 1969). In the sigmoid colon and in the rectum, the lower two-thirds of the crypts contains predominantly sulphated mucus, whilst in the upper one-third and in the superficial epithelium there is a mixture of sulphated and acidic non-sulphated mucus (Fig. 86) (Filipe and Dawson, 1970).

It seems unlikely that the mucus molecules of the more mature goblet cells of the upper parts of the colonic crypts lack the sulphate that is present in the lower parts of the crypts. Weiser (1973a,b) has demonstrated in the small intestine that the mucus molecule is built up as the cell migrates along the crypt and along the villus. A likely possibility for the colonic goblet cell is that the sulphate moiety of the mucus molecule, which is close to the peptide chain, becomes less accessible to staining procedures specific for sulphate as the molecule acquires longer side-chains.

The glycocalyx

Membrane glycoproteins, including the glycocalyx, are neutral (Kent, 1974) and stain with PAS (Fig. 1).

The measurement of mucus production by histochemical means

Histological sections provide only a static picture of a tissue. The histochemical demonstration of an excess of mucus implies that there has been increased synthesis of mucus but no accurate assessment of the rate of mucus synthesis can be made, even using quantitative histology.

Mucus synthesis as measured biochemically may prove to correlate with the density of mucus in histological sections. Such a correlation has apparently been shown in regard to the effect of ulcerogenic drugs in the stomach (Robert et al, 1963; Robert and Nezamis, 1964). Rather than correlating with the visible volume of mucus, the rate of mucus synthesis may correlate with the number of mucus-secreting cells, which may only contain small amounts of mucus yet which may be secreting at a high rate. The difficulty would then be to identify mucus-secreting cells totally depleted of mucus.

Other applications of histochemistry

Sialic acid can be removed by the use of neuraminidase, so that staining before and after treatment with this enzyme can identify mucus containing sialic acid (Filipe, 1969). An increase in PAS reactivity induced by saponification with potassium hydroxide (Culling et al, 1971) has been shown to be due to an O-acyl terminal sialic acid residue (Culling et al, 1974). Such methods may prove useful in demonstrating the anatomical distribution of structural variants in the mucus molecule.

The prospect for histochemistry

Histochemistry is mainly limited to the study of terminal groups and of chemical structures which can be converted into terminal radicles, as in the PAS reaction. Great progress in the determination of the molecular structure of mucus is not to be expected by these methods.

CHAPTER 5

THE SECRETION OF MUCUS

The stomach

The physiological control of the secretion of mucus in the stomach has been most widely studied in the dog. Acetylcholine and vagomimetic drugs stimulate the secretion of mucus in this animal (Janowitz et al, 1951). Histologically, there is extrusion of mucus followed by a period of increased synthesis in the cell (Gerard et al, 1968).

Various irritative stimuli can induce the secretion of mucus, presumably via cholinergic neural stimuli. Pavlov in 1901 observed mucus secretion by the stomach pouch of a dog in response to the application of 10% silver nitrate solution. The mechanical effect of a cannula must not be ignored. Hollander and Stein (1943) found that pilocarpine appeared to stimulate mucus secretion from the dog stomach but that much of this increased secretion was caused by the irritation of the collecting tube.

Reports of the effect of histamine have been contradictory. In the dog, there is diminished secretion of mucus as measured by the typical chemical components (De Graef and Glass, 1968b; Ley et al, 1969). In the human stomach, Glass et al (1969) found that histamine diminished the concentration of acidic non-sulphated mucus in the gastric juice, in other words, that more acid than mucus was secreted in response to histamine. In histological studies in the dog, Gerard et al (1968) observed increased mucus production in the crypt cells of the body and in the pyloric glands in response to histamine and to gastrin. It seems that substances such as histamine which stimulate gastric acid secretion also induce mucus secretion, but to a lesser degree. The mucus secretion may be a secondary response to the acid output.

Experimentally, one must beware of ignoring the visible mucus, as did Piper et al (1965) and Wise and Ballinger (1969). The increased acid production induced by histamine will alter the partition of gastric mucus between the visible and soluble components so that there would appear to be more soluble and less visible mucus.

The intestine

Florey and Webb in 1931 showed histologically the rapid loss of mucus from the goblet cells of the rat intestine following the application to the mucosa of an irritant, mustard oil. Within a few days, the cells were seen to contain mucus again. Merely passing physiological saline through the rat colon led to a reduction in the goblet cell mucus (Florey, 1962).

Werner (1953) showed that the secretion of mucus in the human small intestine could be increased by acetylcholine and by prostigmine. This could be a primary effect or it could be a response of the mucosa to increased bowel movements induced by these drugs.

The relationship to mucus synthesis

The apparent rate of the production of mucus, as judged, for example, by the amount appearing in the stools, is the resultant of the rate of the intracellular synthesis of mucus, the rate of release from intracellular stores, and the rate of disposal of the mucus. Following a brief mechanical, chemical or neurological stimulus, there may be a release of stored mucus without any change in the rate of synthesis, but over a period of days or more, as would pertain in a subacute or chronic disease state such as peptic ulcer, the rate of production of mucus must be parallelled by its rate of synthesis.

Electron microscope autoradiography shows a close time-relationship between the synthesis and the release of mucus. Injected radioactive precursors, ^{3}H-glucose, ^{3}H-galactose and ^{35}S-sulphate, are taken up rapidly by the Golgi apparatus of the goblet cell. They then appear in the intracellular mucus and there is secretion of radioactive mucus from the cell within one hour. By 24 hours, nearly all the labelled mucus has been secreted (Jennings and Florey, 1956; Neutra and Leblond, 1966a, b).

The measurement of mucus secretion

Methods have now been devised to study separately the synthesis and the secretion of intestinal mucus. MacDermott, Donaldson and Trier (1974) studied the kinetics of ^{14}C-glucosamine in the culture medium during the tissue culture of mucosal biopsies of rabbit colon and human rectum. During the first 12 hours of culture, most of the labelled glucosamine was taken up by the tissue, but this reached a plateau at about 18 hours and at this time radioactive glycoprotein began to appear in the medium in increasing amounts. "Pulse-chase" experiments were performed, in which the tissue was taken out of the culture medium and placed in fresh medium, identical except that the glucosamine was now unlabelled and was present in excess, at 20 mM. After a further 3 hours, the tissue was placed in another fresh medium, again with unlabelled glucosamine at 20 mM, but in this medium there were also drugs which were believed to affect mucus secretion. By this means, MacDermott et al were able to show that acetylcholine stimulated mucus secretion and that this effect was blocked by atropine.

MacDermott et al have used their technique to study mucus synthesis and mucus secretion in rectal biopsies from patients with ulcerative colitis. They were able to show a small but significant increase in mucus synthesis and a much greater increase, a fourfold increase, in mucus secretion compared to biopsies from normal patients.

The degradation of mucus

Mucus is synthesized and secreted in the gut but some of it is also destroyed in the gut. Some is digested like any other protein in the lumen. In the colon, there is degradation of the mucus by the bacteria.

The germ-free rat excretes several times more hexosamine into its colon than does a normal rat, even allowing for the larger caecum in the germ-free animal (Lindstedt et al, 1965). Hoskins and Zamchek (1968) incubated the faecal mucus of germ-free rats with rat faecal organisms for 48 hours and observed that 75-90 % of the original non-dialysable carbohydrate appeared as dialysable carbohydrate in the incubation medium. Chemical analysis of the colonic mucus from germ-free rats shows that it is similar in quality to the mucus of normal rats (Wold et al, 1974).

It follows that diminished bacterial degradation of mucus could be a mechanism for the occurrence of excessive faecal mucus in some human pathological conditions. There have been no studies to test this hypothesis. In ulcerative colitis and in other forms of colitis in which there is excessive mucus in the stools, it may be that there are disturbances in the colonic flora. The antibiotics lincomycin and clindamycin kill 98% of the colonic bacteria and these drugs, like some other broad-spectrum antibiotics, can cause membranous colitis, in which there is florid faecal mucus excretion. Another possibility is that faecal hurry through the colon does not leave enough time for the normal degree of bacterial decomposition of the mucus. Such faecal hurry is observed in the inflammatory conditions of the bowel. In the irritable colon syndrome, some patients have faecal hurry, but some patients have slow colonic motility and Ritchie (personal communication) finds that it is the patients with slow motility who most frequently complain of passing large amounts of mucus from the bowel.

CHAPTER 6

GLUCOSAMINE SYNTHETASE IN RELATION TO GLYCOPROTEIN BIOSYNTHESIS

The synthesis of glucosamine from glucose was first shown in animals by Becker and Day in 1953. In the same year, Leloir and Cardini (1953) demonstrated an enzyme in the fungus Neurospora crassa that catalysed the synthesis of glucosamine-6-phosphate from hexose-6-phosphate and L-glutamine. Boström et al, in 1955, observed that glutamine accelerated the synthesis of chondroitin sulphate, a mucopolysaccharide which contains hexosamine. In 1956, Pogell discovered glucosamine synthetase in rat liver.

Pogell (1956) found that glucosamine-6-phosphate could be synthesized from either glucose-6-phosphate or fructose-6-phosphate. As there is always sufficient hexosephosphate isomerase in the cell to convert as much glucose-6-phosphate to fructose-6-phosphate as is required for glucosamine synthesis, it has been assumed since 1960 that fructose-6-phosphate is the substrate for glucosamine synthetase (Gryder and Pogell, 1960; Ghosh et al, 1960). Warren (1972) still doubts the finality of this view.

Ghosh et al (1960) could demonstrate no cofactor requirements for glucosamine synthetase but Clarke and Pasternak (1962) found that EDTA activated the enzyme.

Glucosamine synthetase is a cytoplasmic enzyme. Its full name is L-glutamine-D-fructose-6-phosphate aminotransferase and it has been numbered EC.2.6.1.16 by the Enzyme Commission.

Feedback inhibition and substrate interactions

Kornfeld et al (1964) demonstrated feedback inhibition of glucosamine synthetase by UDP-N-acetylglucosamine, to which glucosamine-6-phosphate is rapidly converted after its synthesis from fructose-6-phosphate and glutamine (see Chapter 3). Miyagi and Tsuiki (1971) found that there was less inhibition when the enzyme was purified, to remove glucose-6-phosphate which increases the inhibition by UDP-N-acetylglucosamine (Winterburn and Phelps, 1971b). It is postulated that this enables glucosamine synthesis to proceed at an even rate despite variations in fructose-6-phosphate concentration. As the concentration of hexose-6-phosphate increases, for instance after a meal, the fructose-6-phosphate will tend to accelerate the reaction but this will be inhibited by the glucose-6-phosphate (Kornfeld et al, 1964; Winterburn and Phelps, 1971b).

The glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON) inhibits glucosamine synthetase but this inhibition is less powerful if the enzyme is already inhibited UDP-N-acetylglucosamine (Bates and Handschumacher, 1969). Winterburn and Phelps (1971c) consider that this implies that UDP-N-acetylglucosamine affects the structure of the glutamine binding site.

The inhibition of glucosamine synthetase by UDP-N-acetylglucosamine is enhanced by glutamine (Bates and Handschumacher, 1969). Glutamine is more inhibitory at lower concentrations of fructose-6-phosphate (Winterburn and Phelps, 1971b).

Glutamine itself is directly inhibitory to glucosamine synthetase in bovine tracheal mucosa but not in rat liver (Ellis and Sommar, 1971).

UTP activates glucosamine synthetase and relieves inhibition induced by UDP-N-acetylglucosamine. ATP inhibits the enzyme. AMP enhances UDP-N-acetylglucosamine inhibition (Winterburn and Phelps, 1971b).

Winterburn and Phelps (1971c), from studies of ligand binding, consider that fructose-6-phosphate is attached to the enzyme before glutamine is attached.

The enzyme reaction in rat liver at 37° at pH 7.5 follows Ping Pong Bi Bi kinetics, according to Winterburn and Phelps (1971a). This gave a V_{max} of between 0.25 and 0.30 μmole/h/mg protein, with a K_m of 2.4×10^{-4} M for fructose-6-phosphate and a K_m of 6.9×10^{-4} M for glutamine. These parameters were determined in a purified preparation of the enzyme but similar results were obtained with a 105 000 g supernatant.

Winterburn and Phelps (1971a) could find no evidence for the reversibility of the glucosamine synthetase reaction.

Nearly all these kinetic studies have been performed on glucosamine synthetase from rat liver. Ellis and Sommar (1971), however, have investigated substrate inhibition and feedback control in glucosamine synthetase from the bovine trachea. On the whole, the kinetics were similar but they found that glutamine was inhibitory to glucosamine synthetase from this tissue at concentrations of greater than 5 mM whereas glutamine was not inhibitory to the enzyme from rat liver even at 20 mM.

On the basis of this last result, Ellis and Sommar suggested that glucosamine synthetase from the bovine tracheal mucosa is chemically different from the enzyme in rat liver. Mazlen et al (1969) found similar substrate and feedback interactions in glucosamine synthetase from the bovine retina to those in rat liver, but again the kinetics were different.

The relevance of the observations on the rat liver enzyme to glucosamine synthetase from the human intestine thus remains an open question. In Chapter 8 of this thesis, in the development of the microassay for glucosamine synthetase in the human colon, studies are described of the substrate interactions with the enzyme in this situation.

The molecular structure of glucosamine synthetase

In order to investigate the molecular structure of glucosamine synthetase, Winterburn and Phelps (1971a) purified the enzyme from rat liver. They used two procedures. In the first method, a 105 000 g supernatant was prepared at 4°. This was fractionated on DEAE-cellulose and then with hydroxyapatite. This increased the specific activity of the enzyme from 0.34 μmole/h/mg protein in a 1:3 homogenate to 4.33 μmole/h/mg protein in the purified preparation. In the second method, the enzyme was purified in the presence of glucose-6-phosphate, which yielded fructose-6-phosphate which is known to stabilize the enzyme during purification (Gryder and Pogell, 1960). The second method was similar to the first but with the addition of glucose-6-phosphate and with a final ammonium sulphate fractionation. Winterburn and Phelps were unable to dissociate fructose-6-phosphate from this more stable preparation, so they used for their studies the enzyme prepared by the first method.

Winterburn and Phelps (1971a) determined the molecular weight of glucosamine synthetase by gel filtration and by sedimentation and found it to be between 360 000 and 400 000 daltons. This compares with 340 000 given by Kornfeld (1967) for glucosamine synthetase from rat liver and from HeLa cells, determined by sucrose density gradient measurements, and with 350 000 suggested by Ellis and Sommar (1972) for glucosamine synthetase from bovine trachea. The molecular weight of bacterial glucosamine synthetase from *E. coli* and *B. subtilis* is approximately 100 000 daltons (Kornfeld, 1967).

The pH optimum for glucosamine synthetase from rat liver was found to be 7.5 by Winterburn and Phelps (1971a, Fig. 25) Ellis and Sommar (1972) found a broad pH optimum between 6.5 and 7.5 for glucosamine synthetase from bovine trachea. The pH relationship of glucosamine synthetase in the human colon is described in Appendix B (and see Fig. 26).

The purified forms of the enzyme prepared by Winterburn and Phelps were susceptible to feedback inhibition of UDP-N-acetylglucosamine. Winterburn and Phelps (1971c) found that the binding site for UDP-N-acetyl-glucosamine was different from the catalytic site of the enzyme. The binding sites for glucose-6-phosphate and for AMP, both of which enhance this feedback inhibition, are different from the catalytic and the UDP-N-acetylglucosamine inhibitory sites.

The 50% inactivation time of the enzyme at 4° was determined by Winterburn and Phelps (1971a) as 15-20 hours. Storage at -15° did not improve the stability. The addition of fructose-6-phosphate increased the stability of the enzyme and the 50% inactivation time under these conditions was 8 days. Studies on the decay of glucosamine synthetase in the human colon are described in Chapter 8.

GLUCOSAMINE SYNTHETASE AS A RATE-LIMITING STEP IN GLYCOPROTEIN BIOSYNTHESIS

Glucosamine synthetase catalyses the formation of glucosamine-6-phosphate, hence of UDP-N-acetylglucosamine. About half of the carbohydrate moiety of glucoprotein consists of acetylglucosamine, acetylgalactosamine and the sialic acids. Acetylglucosamine is supplied to the glycoprotein by UDP-N-acetyl-glucosamine, and acetylgalactosamine and the sialic acids are supplied by nucleotide sugars derived from UDP-N-acetylglucosamine.

In the discussion on the biosynthesis of the nucleotide sugars in Chapter 3, the work of Hardingham and Phelps (1968) and of Lukie and Forstner (1972a) was quoted to support the view that glucosamine synthetase is the rate-limiting step in the synthesis of UDP-N-acetylglucosamine. The glucosamine-6-phosphate formed is rapidly acetylated to N-acetylglucosamine-6-phosphate, which itself is soon converted to N-acetylglucosamine-1-phosphate and then to UDP-N-glucosamine. Izumi (1965) found that glucosamine synthetase had the lowest activity of all the enzymes involved in the synthesis of UDP-N-acetylglucosamine.

The feedback inhibition of glucosamine synthetase by UDP-N-acetylglucosamine implies that the rate of formation of UDP-N-acetylglucosamine depends upon its utilization. Glycoprotein and mucopolysaccharide synthesis uses up UDP-N-acetylglucosamine, feedback inhibition of glucosamine synthetase is relieved, and more UDP-N-acetylglucosamine is produced. Whatever induces or represses glycoprotein synthesis, the supply of UDP-N-acetylglucosamine will be critical, especially as N-acetylgalactosamine, formed from UDP-N-acetylglucosamine, is the first carbohydrate in the side-chain in the mucous glycoproteins and in other glucoproteins such as immunoglobulin (Marshall, 1974).

Whether glucosamine synthetase is a rate-limiting step in the biosynthesis of glycoproteins is a more difficult question. This must depend on whether it can be shown that the supply of UDP-N-acetylglucosamine is rate-limiting for the synthesis of glycoprotein. It does not seem that the supply of excessive amounts of UDP-N-acetylglucosamine can accelerate glycoprotein synthesis. Bates et al (1966) fed orotic acid to rats, which accumulated large amounts of UDP-N-acetylhexosamine in their livers, about five times as much as normal, apparently as a result of a defect in the feedback inhibition of glucosamine synthetase by UDP-N-acetylglucosamine, but there was no increase in glycoprotein synthesis in these rats. UTP is synthesized from orotic acid (Hurlbert and Potter, 1954) and Winterburn and Phelps (1971b) showed that UTP relieves the inhibition of glucosamine synthetase by UDP-N-acetylglucosamine.

On the other hand, inhibition of glucosamine synthetase does inhibit glycoprotein biosynthesis and this inhibition is relieved by the supply of exogenous glucosamine. 6-Diazo-5-oxonorleucine (DON), an analogue of glutamine, inhibits glucosamine synthetase in E. coli, in rat liver (Ghosh et al, 1960), in the ovine submaxillary gland (Harbon et al, 1966) and in the mammalian colonic mucosa (Pasternak, 1961), as does another analogue of glutamine, O-diazoacetyl-L-serine (Pasternak, 1961; Winterburn and Phelps, 1971c). Bates et al (1966) found that Duazomycin A, which is N-acetyl-6-diazo-5-oxonorleucine, inhibited glucosamine synthesis in vivo, although Winterburn and Phelps (1971c) could find no inhibition of their purified preparation of glucosamine synthetase and suggested that Duazomycin A is deacetylated in vivo to DON, the inhibitory compound.

Duazomycin A inhibits glycoprotein synthesis in the rat liver and this inhibition is relieved by the addition of glucosamine (Bates et al, 1966). Thus, the inhibition of glucosamine synthetase also inhibits glycoprotein biosynthesis. This experiment is the principal piece of evidence for the proposal that glucosamine synthetase is a rate-limiting step in glycoprotein synthesis.

Recently, tunicamycin, a compound which contains glutamine, has been shown to be an inhibitor of glycoprotein synthesis in yeast, possibly by competing for glucosamine in the synthetic pathways (Kuo and Lampen, 1974).

Glutamine itself is inhibitory to glucosamine synthetase in bovine tracheal mucosa (Ellis and Sommar, 1971). Ellis and Stahl (1973) found that in canine tracheal explants in tissue culture the incorporation of radioactive fucose and serine into the secreted glycoprotein was inhibited by 20 mM glutamine, which could be explained by the inhibition of glucosamine synthetase.

Glucosamine synthetase competes for its substrates with other metabolic pathways, particularly with the glycolytic pathway for fructose-6-phosphate. Hexosamine synthesis in vivo by the rat liver and by neonatal rat skin is approximately 0.1 μmole/h/g wet wt (Spiro, 1959; Hardingham and Phelps, 1968). With added substrates in vitro, the rat liver enzyme can synthesize glucosamine at 2 μmoles/h/g wet wt (Winterburn and Phelps, 1971b) and, by the suitable adjustment of substrate concentrations, an even higher rate of synthesis has been demonstrated in human colonic mucosa in this thesis (Chapter 8).

The object of this thesis has been to measure glucosamine synthetase levels in normal and pathological tissues, as a possible index of mucus biosynthesis. When glucosamine synthetase is induced to function at, say, 20 times its physiological rate, does it follow that variations in glucosamine synthetase activity under such conditions parallel the rate of function of the enzyme in vivo? This would require the measurement of glucosamine synthesis in vivo by a more sensitive method than by the estimation of glucosamine synthesis under non-physiological conditions. A radioisotope method might be suitable. Results by such a method could then be correlated with results obtained by the estimation of glucosamine synthesis at high substrate concentrations in order to elucidate the significance of glucosamine synthetase levels in relation to hexosamine and glycoprotein biosynthesis. Although this has not been done in the present work, the later chapters will indicate some pathological conditions in which glucosamine synthetase levels are abnormal and in which it will be important to study the true rate of hexosamine synthesis.

The relationship of glucosamine synthetase levels to glycoprotein synthesis is a further step removed. This depends on the relationship between UDP-N-acetylglucosamine formation and glycoprotein synthesis. Lukie and Forstner (1972a) found that the synthesis of glycoprotein by the rat small intestine in vitro was proportional to the glucosamine concentration of the tissue culture medium, up to 18 mM glucosamine. If glucosamine synthetase levels correlate with the synthesis of glucosamine in vivo, then they should correlate with the synthesis of glycoprotein.

Puromycin, an inhibitor of protein synthesis at the ribosomal level (Yarmolinsky and de la Haba, 1959), inhibits glycoprotein synthesis (Molnar et al, 1964; Allen and Kent, 1968; Ellis and Stahl, 1973). Cyclohexidine almost completely inhibits protein synthesis and MacDermott et al (1974) demonstrated this in terms of the incorporation of ^{14}C-leucine incorporation into rabbit and human colonic mucosa in tissue culture. On the other hand, ^{14}C-glucosamine continued to be incorporated during 24 hours of tissue culture in cyclohexidine, although at a diminished rate. The conclusion is that glycoprotein molecules in which the peptide backbone had already been formed continued to be assembled when protein synthesis was inhibited.

Thus the supply of glucosamine appears to be rate-limiting for the assembly of the carbohydrate component in glycoprotein synthesis. Other factors control the synthesis of the protein component, which is formed before the carbohydrates are added. MacDermott et al (1974) measured ^{14}C-leucine incorporation in their investigations into human rectal tissue in pathological conditions and they were able to take into account the factor of protein synthesis in their assessment of overall glycoprotein synthesis.

Inhibitory effects of glucosamine

One feature in the relationship between glucosamine synthesis and glycoprotein synthesis which must be mentioned in regard to the rate-limiting role of glucosamine synthetase is the inhibitory effect of glucosamine on glycoprotein synthesis (Ellis and Stahl, 1973).

Quastel and Cantero in 1953 reported that D-glucosamine inhibited tumour growth. Bekesi et al (1969a) found that glucosamine at 25 mM was inhibitory to protein synthesis in normal and neoplastic tissues. Galactosamine, mannosamine and mannose were also inhibitory but N-acetylglucosamine was not inhibitory.

Ellis and Stahl (1973) found that glucosamine in concentrations of more than 20 mM inhibited the incorporation of fucose and serine into glycoprotein by canine tracheal mucosa in tissue culture. They suggested, as Bekesi et al (1969b) had done, that this was due to inhibition of glucosamine synthetase, presumably by UDP-N-acetylglucosamine which would be formed in abnormally large amounts from these high concentrations of glucosamine. However, it is difficult to see how this mechanism could inhibit glycoprotein synthesis if the rate-limiting role of glucosamine synthetase depends on its supplying glucosamine for the synthesis of glycoprotein. Glucosamine synthetase might be inhibited by high concentrations of glucosamine but there should be sufficient glucosamine for a normal rate of glycoprotein synthesis.

McGarrahan and Maley (1962) showed that glucosamine was rapidly phosphorylated by rat liver to glucosamine-6-phosphate, which in turn was acetylated to N-acetylglucosamine-6-phosphate, and Lukie and Forstner (1972a) demonstrated that the synthesis from labelled exogenous glucosamine of N-acetylglucosamine-1-phosphate, UDP-N-acetylglucosamine, N-acetylneuraminic

acid and glycoprotein by rat intestinal mucosa in tissue culture was directly proportional to the glucosamine concentration of the culture medium, up to 18 mM glucosamine.

It would seem that the inhibitory effect of glucosamine on glycoprotein synthesis is not via the inhibition of glucosamine synthetase. It may be a general effect of high concentrations of hexosamine on the synthesis of all proteins.

CHAPTER 7

THE STUDY OF GLUCOSAMINE SYNTHETASE LEVELS

Glucosamine synthetase is assayed by measuring the hexosamine that is formed when tissue homogenates are incubated with fructose-6-phosphate and glutamine, usually at 37°. The concentrations of the substrates, the pH, ionic strength and composition of the homogenizing medium and of the incubating medium, the temperature and duration of the incubation, and the details of the preparation of the homogenate are all factors which can be varied. The choice of optimum incubation conditions is discussed in the next chapter. The hexosamine that is synthesized is acetylated and then acetylhexosamine is estimated by the Morgan-Elson reaction.

The Morgan-Elson reaction is about one-third as sensitive to acetylgalactosamine as it is to acetylglucosamine (Fig. 16) but, for the purposes of the assay of glucosamine synthetase, the hexosamine synthesized is expressed in terms of glucosamine as detected by this reaction. Glucosamine-6-phosphate gives about 80% of the colour of glucosamine (Kaufman et al, 1971). Usually this is ignored and the glucosamine-6-phosphate is estimated as glucosamine, but some workers apply the correction and express activity in terms of glucosamine-6-phosphate synthesized.

Units of glucosamine synthetase are moles of glucosamine or glucosamine-6-phosphate synthesized per unit time per unit of tissue. It is most frequently presented as units of enzyme per g or per mg of tissue protein but sometimes it is expressed as units per g wet weight of tissue. This point is discussed and investigated in Chapter 8.

In any assay of glucosamine synthetase, the units of the enzyme depend upon the conditions selected for the incubation (see Chapter 8) but within the range of conditions that have been used by the various workers in this field, the activity of the enzyme as determined under one set of conditions appears to bear a constant ratio to the activity as determined under a different set of conditions (see Table 2). Thus, provided the conditions are standardized in a particular study, the levels of glucosamine synthetase can be compared in a given tissue of a single species in different physiological and pathological situations. Whether the conditions of the assay of the enzyme in one organ of an animal allow comparison with other organs of the same animal can be debated. Generally it has been assumed that glucosamine synthetase is chemically similar in all parts of a single animal, although this may not be so (see Chapter 6 and Appendix B). In this thesis, it has been assumed for the purposes of miniaturization of the assay that glucosamine synthetase in all parts of the human gastrointestinal mucosa is an identical molecule, but the conclusions derived from the measurements of the levels of glucosamine synthetase in different parts of the gastrointestinal tract do not depend on this assumption.

In this thesis, the standard conditions which were selected (Chapter 8) were fructose-6-phosphate 20 mM, glutamine 8 mM, EDTA 1 mM, and pH 7.0, with a 3-hour incubation at 37°. The results are expressed as μmoles glucosamine synthesized/h/g wet wt or as μmoles synthesized/h/g protein.

GLUCOSAMINE SYNTHETASE IN MAMMALIAN TISSUES

Glucosamine synthetase was demonstrated in rat liver by Pogell an 1956. Kikuchi et al (1971) and Kaufman et al (1971) have given data for the distribution of glucosamine synthetase in the rat (Table 2). Although the incubation conditions vary in these and other studies, the results have been expressed in this Table in the units adopted in this thesis.

The apparently low levels of glucosamine synthetase in the small intestine (Table 2) are due to the measurement of the enzyme in homogenates of the full thickness of the intestine, including the submucosa and the muscle layers.

Bovine tracheal scrapings were shown by Ellis and Sommar (1972) to have a glucosamine synthetase activity of 300-600 μmoles glucosamine-6-phosphate

TABLE 2. Glucosamine synthetase levels in various tissues in the rat

Author	Incubation conditions	Organ	Glucosamine synthetase activity	
Kikuchi et al (1971)	F-6-P 10 mM, glutamine 15 mM, EDTA 0.2 mM, pH 7.5, incubated for 1 h at 37°	Liver	24.6	µmoles glucosamine synthesized/h/g protein
		Stomach	8.5	
		Small intestine	1.9	
		Colon	0.5	
		Muscle	0	
Kaufman et al (1971)	F-6-P 10 mM, glutamine 12 mM, EDTA 1 mM, pH 7.7, incubated for 2 h at 37°	Liver	0.6-0.9	µmoles glucosamine synthesized/h/g wet wt
		Small intestine	0.13	
		Heart, Muscle	0.04	

synthesized/h/g protein at fructose-6-phosphate 10 mM, glutamine 2 mM and pH 7.0, incubated for 30 min at 37°.

Glucosamine synthetase is also found in connective tissue, where it supplies hexosamine for the glycosaminoglycans. Castellani and Zambotti (1956) made early measurements in homogenates of rabbit epiphyseal cartilage, with 1.7 µmoles hexosamine synthesized/h/g wet wt at glucose-6-phosphate 10 mM, glutamine 10 mM and pH 6.4, incubated for 3 h at 30°. Lesser amounts of hexosamine were synthesized by homogenates of rib cartilage, lung and liver, and no synthesis was detected in the heart, kidney or spleen.

Bollet and Shuster (1960) induced the formation of new connective tissue in rats by inserting polyvinyl sponges subcutaneously. Glucosamine synthetase was present at 4.2 µmoles glucosamine synthesized/h/g protein (at fructose-6-phosphate 10 mM, glutamine 12.5 mM, pH 7.7, incubated for 1 h at 37°) compared to 3.2 units in the liver. The enzyme was present at concentrations of 0.6–9.7 units in human synovial tissue in several disease states.

The enzyme was measured by Jacobson and Boström (1963) in bovine heart valves, in which the enzyme activity decreased with age.

Glucosamine synthetase has been assayed by Trujillo et al (1971) in bovine thyroid tissue following removal of the colloid by fractionation. The level was 30 µmoles glucosamine synthesized/h/g protein. Thyroglobulin is a glycoprotein.

Danishefsky and Deutsch (1968) measured the enzyme in mast cells in relation to heparin synthesis.

VARIATIONS IN GLUCOSAMINE SYNTHETASE LEVELS

There have been no published studies relating glucosamine synthetase levels in any tissue to the density of different cell types in that tissue. Such a study would best be performed in relation to pathological variations in cell densities.

One instance in which a relationship to cell densities has been demonstrated is in the case of rat tumours, in which Kaufman et al (1971) showed that the glucosamine synthetase activity per g of tissue was highest when there was least stroma and lowest when there was most stroma. Glucosamine synthetase levels in human tumour tissue are discussed in Chapter 12.

In experimental animals, there have been studies of the effects of drugs, particularly the ulcerogenic drugs, on glucosamine synthetase. These are discussed in Chapter 15. Glucosamine synthetase levels have been studied by Sander et al (1975) in restrained rats, many of which developed gastric ulceration.

There are no published reports of glucosamine synthetase measurements in human tissue.

Partial hepatectomy

Akamatsu and Maeda in 1971 performed partial hepatectomy on neonatal rats. The glucosamine synthetase levels in the livers of these animals doubled over the next three days, whereas sham-operated animals showed only a very slight rise in hepatic glucosamine synthetase levels (Fig. 12). This is probably the most important observation that has been made with regard to glucosamine synthetase activity in regenerating tissue and the findings reported in Chapter 9 of this thesis for patients recovering from ulcerative colitis are in line with the observation of Akamatsu and Maeda. If the cell types which contain glucosamine synthetase can be identified, then the glucosamine synthetase activity of such cells could be an index of regeneration.

This effect in neonatal rat liver has been studied further by Bley, Okubo and Chandler (Bley et al, 1973; Okubo and Chandler, 1974). This rise in glucosamine synthetase levels following partial hepatectomy started at about 18 hours and it began to wane at 2-3 days. These workers also measured the concentration of UDP-N-acetylhexosamine in the rat liver tissue. This also

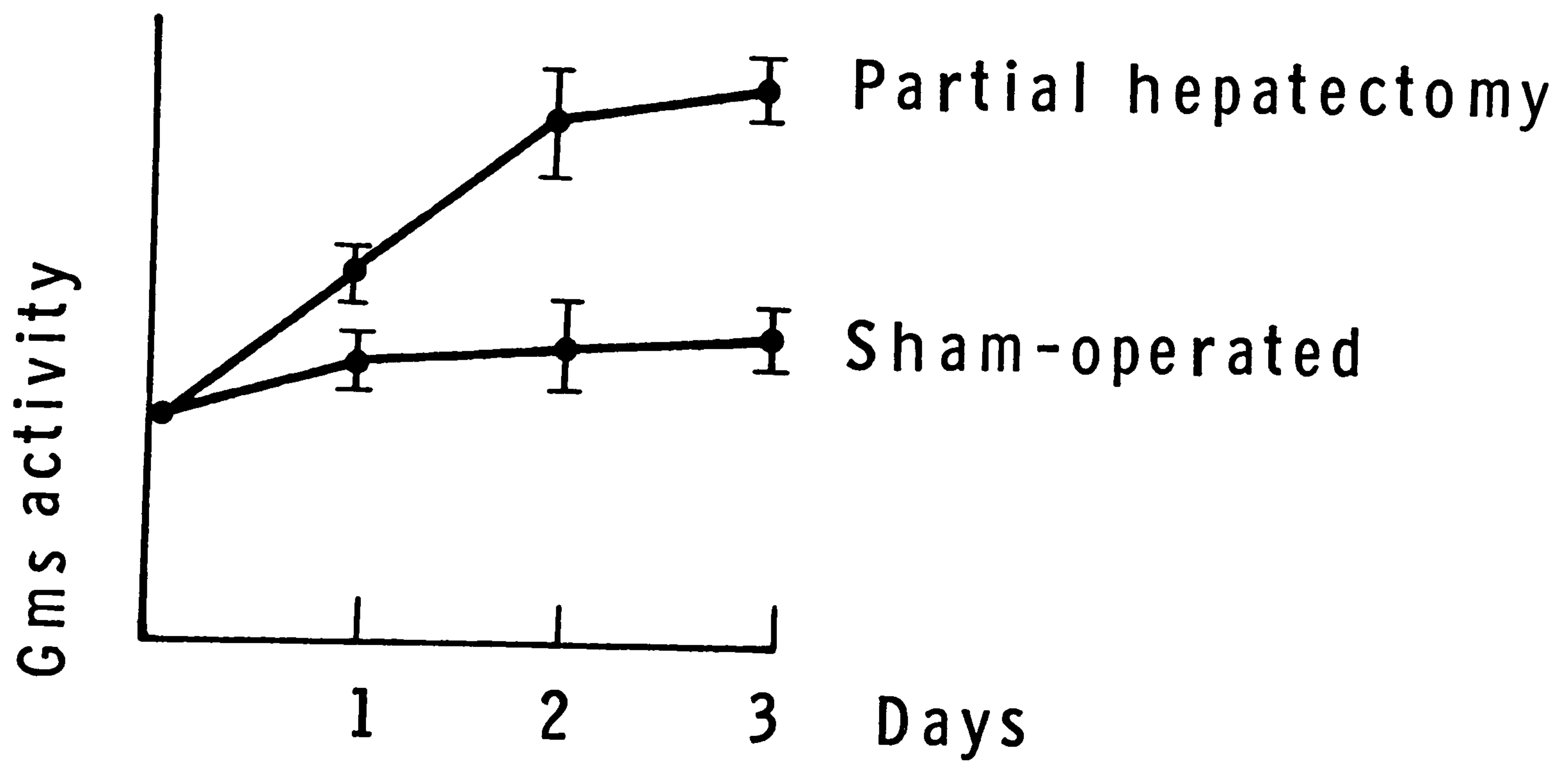


FIG. 12. Glucosamine synthetase (GmS) activity in neonatal rat liver following partial hepatectomy and following a sham operation. (From Akamatsu and Maeda, 1971.)

rose following partial hepatectomy but the rise was delayed for 2 days and it was preceded by a fall which began at the same time as the glucosamine synthetase levels started to rise. Whilst UDP-N-acetylhexosamine levels do represent the actual amounts of this amino sugar nucleotide in the tissue, the glucosamine synthetase levels do not represent the actual rate of synthesis of glucosamine because the synthetase levels are measured *in vitro* with substrates supplied greatly in excess of the physiological concentrations of fructose-6-phosphate and glutamine. Under such *in vitro* conditions, the concentrations of UDP-N-acetylhexosamine cannot be expected to be inhibitory to glucosamine synthetase to any significant extent. The explanation for these effects cannot therefore relate to inhibition by UDP-N-acetylglucosamine of glucosamine synthetase. A likely explanation is that glycoprotein synthesis increases in the hepatectomized livers about 18 hours after hepatectomy, the intracellular stores of UDP-N-acetylhexosamine are depleted initially in the formation of new glycoprotein, but increased quantities of glucosamine synthetase eventually regenerate the pool of UDP-N-acetylglucosamine.

Peritoneal injury

Okubo and Chandler (1974) observed a different effect in similar rats which were subjected to laparotomy with peritoneal injury. In these rats, the hepatic glucosamine synthetase levels began to rise at 6 hours, reached a peak at about 12 hours, and returned to normal by 18 hours. The UDP-N-acetyl-hexosamine levels showed a similar pattern. The stimulus to the rise in glucosamine synthetase levels occurred sooner than in the hepatectomized rats and there was no depletion of UDP-N-acetylhexosamine. It may have been an inflammatory stimulus, with the products of injured tissue being carried to the liver in the portal vein, which caused the rise in glucosamine synthetase levels.

Glucosamine synthetase levels and glycoprotein synthesis

The relationship to glycoprotein synthesis of such changes in glucosamine synthetase and UDP-N-acetylhexosamine levels can only be speculative in the absence of direct measurements of glycoprotein synthesis. Even the synthesis of hexosamine may not be related directly to glucosamine synthetase levels. The complex interactions of substrates, products and nucleotides with

glucosamine synthetase make predictions of the in vivo rate of glucosamine synthesis rather difficult. Nevertheless, the assay of glucosamine synthetase is relatively simple, and in this thesis it will be shown to be sufficiently sensitive to be applied to biopsy specimens of the human gastrointestinal tract. The measurement of glucosamine synthetase levels in human pathological conditions is important for the study of mucus synthesis even though these levels may give only a partial picture of the total situation.

SECTION II

THE METHOD

CHAPTER 8

THE DEVELOPMENT OF THE MICROASSAY FOR GLUCOSAMINE SYNTHETASE

Glucosamine synthetase is assayed by a chemical method. The tissue homogenate is incubated with fructose-6-phosphate and glutamine. After a certain length of time, the reaction is terminated and the hexosamine in the incubate is assayed by a colorimetric method. The exact conditions of the incubation have been different in the various papers in which glucosamine synthetase measurements have been reported. So long as a standard set of conditions is selected in any study, the results from different physiological and pathological conditions can be compared. The aim of the present study has been to measure glucosamine synthetase in biopsy specimens from the human gastrointestinal tract. These are very small samples of tissue. It has therefore been necessary to ensure the maximum sensitivity of the glucosamine synthetase assay by determining the optimum conditions for the greatest yield of glucosamine.

Before the study began, it was by no means certain that it would be possible to miniaturize the assay sufficiently for use with these biopsy specimens. At the outset, the object of the study was to investigate rectal biopsies from patients with ulcerative colitis and Crohn's disease of the colon, to see whether the histological differences in goblet cell density in the two diseases were reflected in any biochemical differences in glycoprotein biosynthesis. The method was therefore miniaturized for use with such biopsies. The subsequent application of the method to gastric, duodenal, jejunal and liver biopsies was a consequence of the success in miniaturizing the method for rectal biopsies.

MATERIALS

Acetic acid, glacial (Analar grade) was obtained from BDH Chemicals Ltd, Poole.

Acetic anhydride (Analar grade) was obtained from BDH Chemicals Ltd.

Acetone was obtained from BDH Chemicals Ltd.

Boric acid (Analar grade) was obtained from BDH Chemicals Ltd.

Bovine serum albumin (crystallized and lyophilized, Lot 52C-8090) was obtained from the Sigma Chemical Co, St Louis, Missouri.

Cupric sulphate, $CuSO_4.5H_2O$ (Analar grade) was obtained from BDH Chemicals Ltd.

4-Dimethylaminobenzaldehyde (DAB, Analar grade) was obtained from BDH Chemicals Ltd.

Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$ (Analar grade), was obtained from BDH Chemicals Ltd.

Ethylenediaminotetra-acetic acid disodium (EDTA), $[CH_2.N(CH_2COOH)-CH_2COONa]_2.2H_2O$ (Analar grade), was obtained from BDH Chemicals Ltd.

Folin and Ciocalteu's phenol reagent was obtained from BDH Chemicals Ltd.

Fructose-6-phosphate disodium was obtained from Boehringer Mannheim GmbH, West Germany. For a few months, Boehringer Mannheim were unable to supply the material and it was obtained instead from BDH Chemicals, Ltd. There was no difference in glucosamine synthetase activity when a single homogenate of colonic mucosa was assayed with equivalent amounts of fructose-6-phosphate from each source.

D(+)Galactosamine hydrochloride (grade 1) was obtained from the Sigma Chemical Co.

Gentamicin sulphate (Genticin Pure Powder) was obtained from Nicholas Laboratories, British Schering Ltd, Slough.

D(+)Glucosamine hydrochloride was obtained from the Sigma Chemical Co.

L-Glutamine was obtained from BDH Chemicals Ltd.

Hydrochloric acid, sp. gr. 1.18 (Analar grade), was obtained from BDH Chemicals Ltd.

Perchloric acid, sp. gr. 1.70 (Analar grade), was obtained from BDH Chemicals Ltd.

Potassium chloride (Analar grade) was obtained from BDH Chemicals, Ltd.

Potassium hydroxide (Analar grade) was obtained from BDH Chemicals, Ltd.

Sodium carbonate, anhydrous (Analar grade), was obtained from BDH Chemicals, Ltd.

Sodium dihydrogen phosphate, $NaH_2PO_4 . 2H_2O$ (Analar grade), was obtained from BDH Chemicals, Ltd.

Sodium hydroxide 0.1N (concentrated volumetric solution, Analar grade), was obtained from BDH Chemicals, Ltd.

Tris(hydroxymethyl)aminomethane (Analar grade) was obtained from BDH Chemicals, Ltd.

Fructose-6-phosphate disodium was hygroscopic and the material as supplied was found on dessication to contain 3-17% water. Samples of each batch used were desiccated from time to time to relate the observed weight of fructose-6-phosphate disodium to the water-free weight. The degree of hydration of single batches did not vary by more than 2-3% over several months.

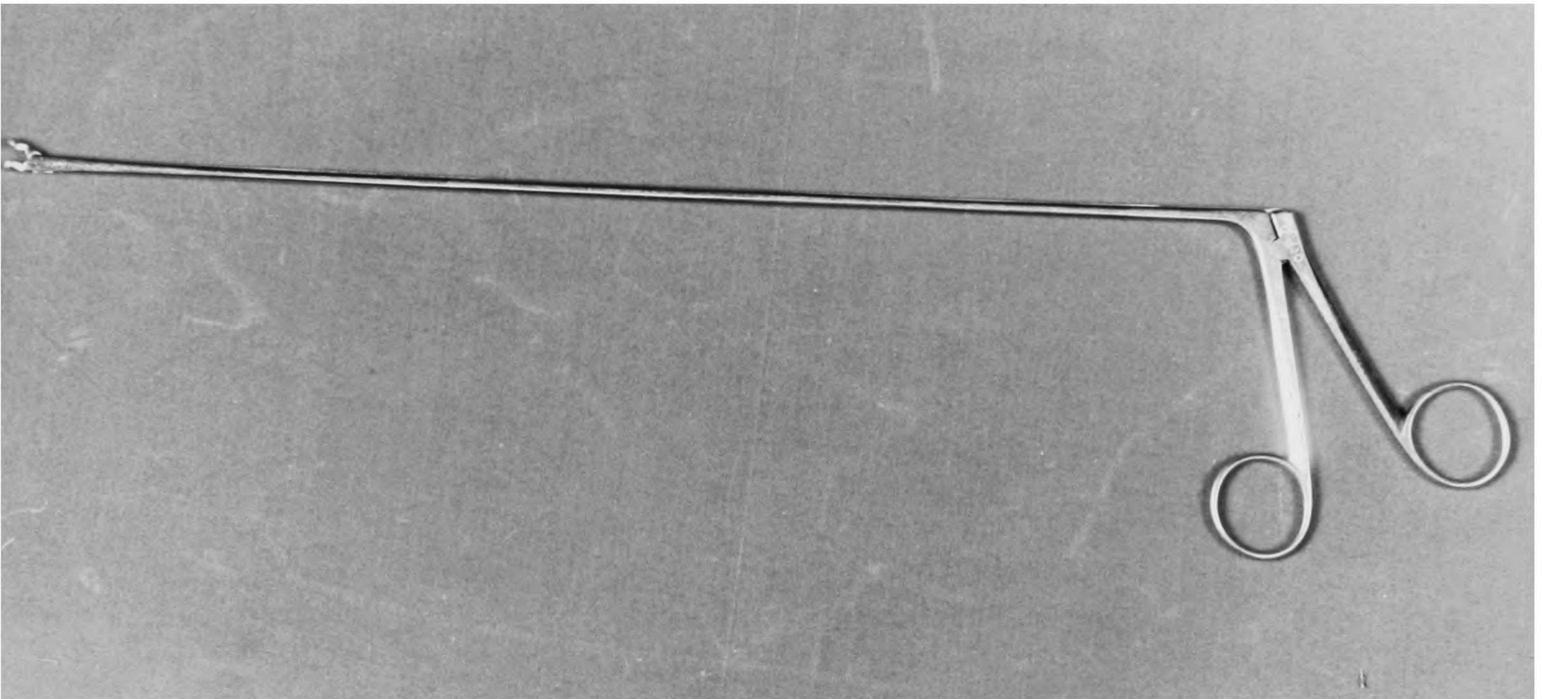
Bovine serum albumin was also hygroscopic and a sample was desiccated on each occasion on which the tissue protein concentrations were estimated.

L-Glutamine and glucosamine hydrochloride were non-hygroscopic and there was no detectable loss of weight on desiccation.

THE DEGREE OF MINIATURIZATION REQUIRED

Rectal biopsies are taken with cupped biopsy forceps (Fig. 13) through a rigid sigmoidoscope. This is a routine gastroenterological investigation. The biopsies are examined histologically for the purposes of diagnosis and management of the inflammatory diseases of the large intestine. In a sample of 19 rectal biopsies, the weight of each biopsy was between 4.8 and 27.0 mg; 15 of them weighed less than 15 mg.

Initially, it would have been considered satisfactory to miniaturize the method sufficiently to assay a single rectal biopsy. However, it proved possible to miniaturize the assay to a degree which allowed just over 4 mg of tissue to be used, which meant that only half of a single rectal biopsy was needed. This



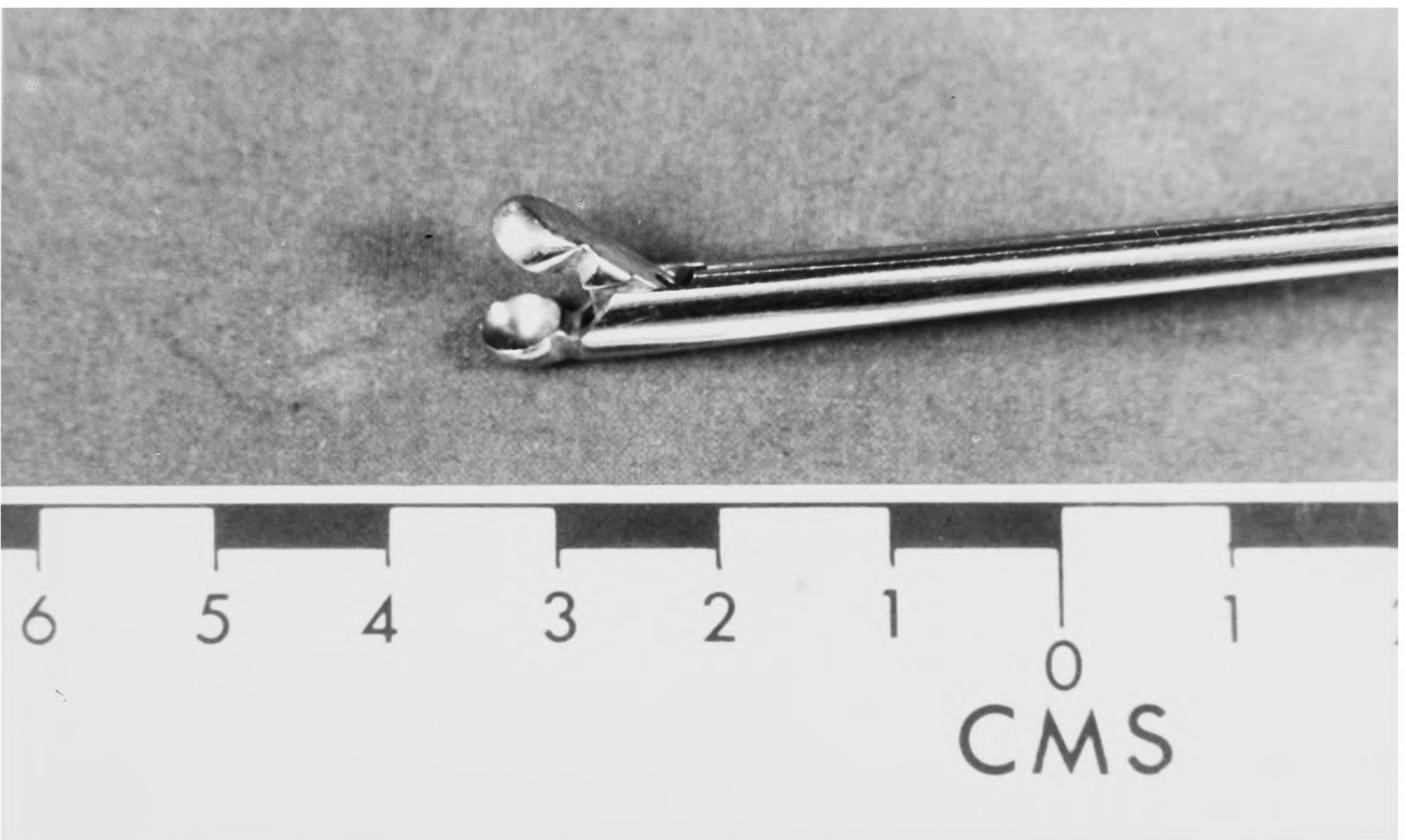


FIG. 13. The biopsy forceps used for taking a rectal biopsy through a rigid sigmoidoscope.

was fortunate because it meant that in patients from whom rectal biopsies were being taken for clinical diagnosis, there was no need to take an additional biopsy for the purposes of this study. Sufficient tissue was present in the remaining half of the biopsy for diagnostic histology.

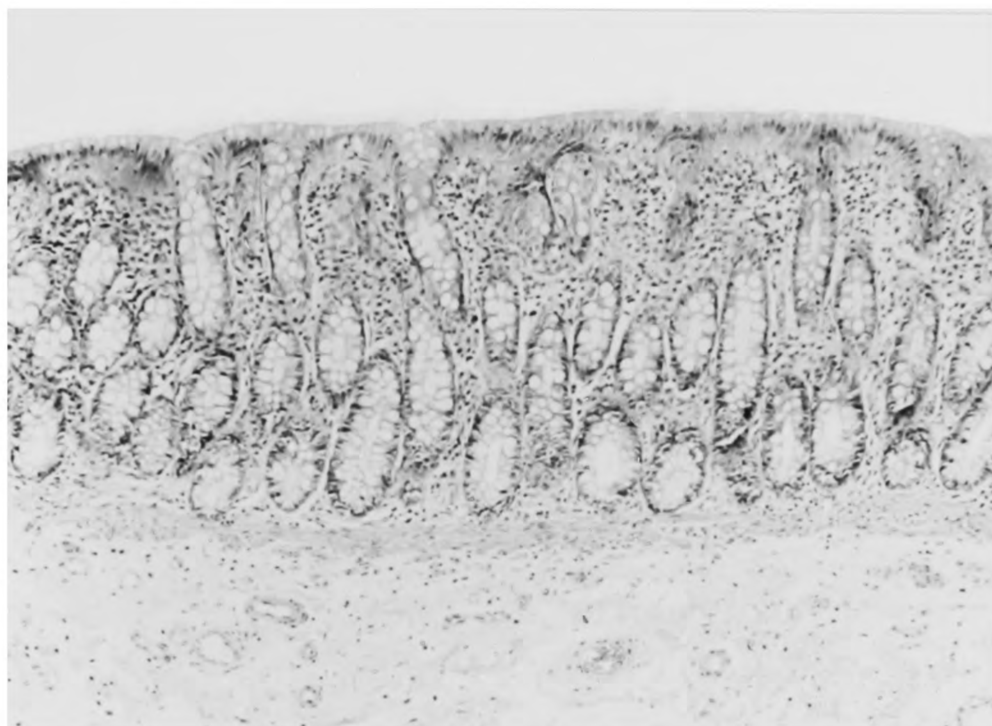
METHOD OF OBTAINING NORMAL COLONIC MUCOSA FOR THE MINIATURIZATION OF THE ASSAY

The optimum conditions of the assay were ascertained by studies on normal human colonic mucosa. This was obtained from the cut ends of surgical colectomy specimens of patients undergoing resection of part of the colon for carcinoma of the colon. There were also a few specimens from patients undergoing partial colectomy for localized areas of diverticulitis. Patients with a previous history of inflammatory bowel disease were excluded, as were patients with endocrine disorders (such as diabetes mellitus, thyrotoxicosis and myxoedema), patients taking hormonal preparations (including corticosteroids), and patients with chronic liver disease (as there might have been occult inflammatory bowel disease in such patients). Patients with peptic ulceration were not excluded.

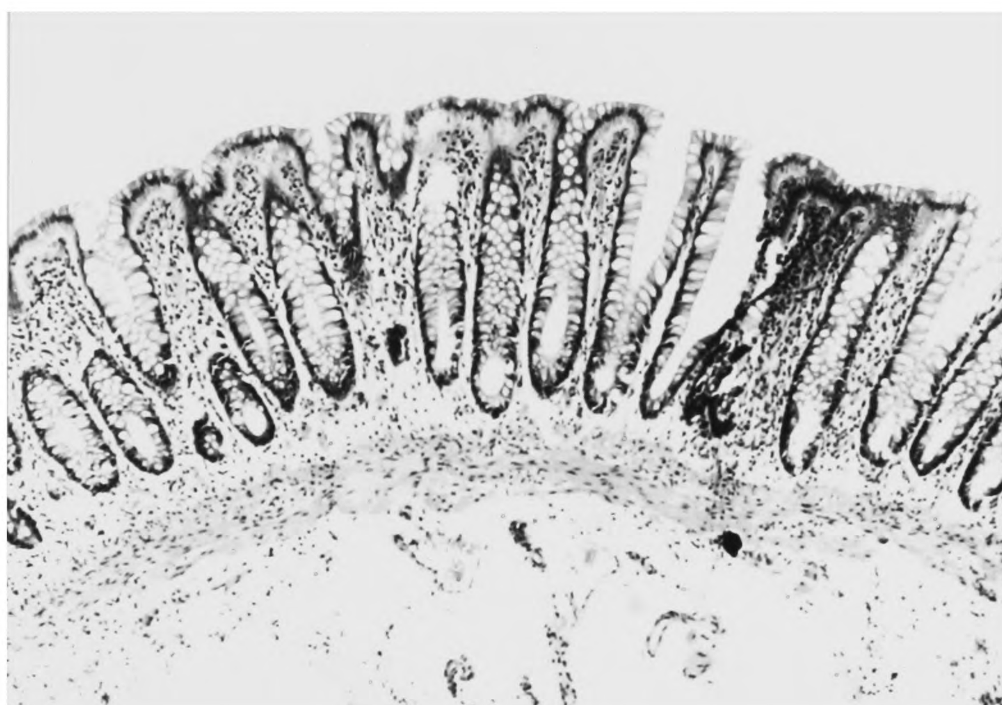
The cut ends of the colectomy specimens were ascertained on macroscopic examination and by histology to be free from tumour. In those cases in which the operation was performed for diverticulitis, the cut ends were free from inflammation when examined histologically.

The surgeon performed the colectomy operation in the normal way. The operative specimen, when removed, was placed in a dry container free from formaldehyde. The operating theatre technician contacted the laboratory as soon as the specimen was removed and it was brought immediately to the laboratory. The mucosa at the cut end was stripped off the submucosa by blunt dissection and the portion of mucosa which was to be used in the study was placed in the homogenizing buffer, which was kept at 4°. The remainder of the surgical specimen was taken to the Pathology Department, where it was placed in formol saline for routine histology to be carried out.

The mucosa and muscularis mucosae are obtained by this method. The depth of colonic mucosa so obtained corresponds to that of a rectal biopsy (Fig. 14).



(a)



(b)

FIG. 14. (a) Colonic mucosa obtained by dissection of surgical material. (b) Rectal biopsy for comparison.

THE METHOD ADOPTED INITIALLY

As a starting point in the determination of the optimum conditions for the assay, the method of Winterburn and Phelps (1971a) was followed, with some modifications.

The incubation medium of Winterburn and Phelps contained 50 mM tris buffer adjusted to pH 7.5 with HCl, EDTA 1 mM, fructose-6-phosphate 5 mM, glutamine 12 mM, and KCl to make the medium up to an ionic strength of 0.2. The homogenizing medium of Winterburn and Phelps contained 50 mM tris buffer adjusted to pH 7.8 with 2M HCl, EDTA 5 mM, glutathione 5 mM, and KCl 100 mM.

Large numbers of biopsy specimens were to be collected, homogenized and incubated and, for convenience, it was decided to use the same buffer for the collection, the homogenization and the incubation, although the substrates would be added at the time of incubation. This was most easily done by making stock "double buffer", which could be diluted with an equal volume of water to give "single buffer" for homogenization or to which aqueous solutions of the substrates in appropriate concentrations could be added to make the incubation medium. The composition of the "single buffer" in the initial experiments was tris-HCl 50 mM at pH 7.5, EDTA 1 mM and KCl 100 mM.

The colonic tissue was washed twice in fresh buffer, blotted gently on filter paper, weighed on a fine balance, and then homogenized in the "single buffer" using a Potter-Elvehjem homogenizer. 0.5 ml of the homogenate was added to 0.5 ml of substrate-buffer mixture. This substrate-buffer mixture was made up by adding aqueous solutions of fructose-6-phosphate and glutamine to "double buffer" to give concentrations of the substrates of fructose-6-phosphate 10 mM and glutamine 24 mM. Thus in 1.0 ml of incubate, the concentrations were fructose-6-phosphate 5 mM and glutamine 12 mM, as used by Winterburn and Phelps.

The incubate was placed in a 37° water-bath for 90 minutes. At the end of this time, 0.05 ml concentrated perchloric acid (71%, sp. gr. 1.70) was added (Winterburn and Phelps used 6% perchloric acid w/v in the final incubate). The homogenate was centrifuged at 2500 rpm for 15 minutes to precipitate the protein. 0.8 ml of the supernatant was taken. To this was added 0.05 ml 9M KOH,

a quantity which almost, but not quite, neutralized the perchloric acid. M KOH was then added until the pH was between 6 and 11, using Merck non-bleeding indicator strips.

The insoluble potassium perchlorate was precipitated by centrifugation at 2500 rpm for 5 minutes. 0.5 ml of the supernatant was taken for estimation of the glucosamine content by the Morgan-Elson reaction according to the method of Good and Bessman (1964).

THE MORGAN-ELSON REACTION

Morgan and Elson (1934) described a colorimetric method, now known as the Morgan-Elson reaction, for the determination of N-acetylglucosamine. The acetylhexosamine is boiled in alkali for 5 minutes and then cooled. Glacial acetic acid is added and then dimethylaminobenzaldehyde in hydrochloric acid. A magenta colour develops on standing after 45 minutes. This colour remains for about 1 hour and then fades.

The boiling with alkali converts the acetylhexosamine to pyrroles, which condense with DAB to give the magenta colour. Hydrochloric acid accelerates the development of the colour reaction although it also accelerates the decay of the colour (Morgan and Elson, 1934).

The Elson-Morgan reaction (Elson and Morgan, 1933) is a different colour reaction for hexosamines, in which acetylacetone converts the hexosamine to a pyrrole which forms a stable red colour with dimethylaminobenzaldehyde.

Whereas only pure preparations of DAB should be used in the Elson-Morgan reaction (Blix, 1948), Roseman and Daffner (1956) found that commercial preparations of DAB could be as effective as pure DAB in the Morgan-Elson reaction.

In the method of Good and Bessman for the Morgan-Elson reaction, which is only slightly modified from that of Levvy and McAllan (1959), glucosamine is acetylated and converted to pyrrole in a single step. In the method adopted in this study, to 0.5 ml glucosamine solution was added 0.5 ml potassium borate buffer (1.12M boric acid with 0.56M KOH, giving a pH of 9.2) and then 0.1 ml acetic anhydride 1.5% w/v in acetone, in screw-cap test-tubes with a teflon seal (made by Sovirel, France and supplied by V.A. Howe and Co). The

test-tubes were placed in a boiling water-bath for 3 minutes and then in an ice water-bath for 5 minutes. "Stock DAB" solution was made by dissolving 10 g DAB in glacial acetic acid and hydrochloric acid, the final volume being 100 ml and containing 87.5 ml glacial acetic acid and 12.5 ml concentrated hydrochloric acid. This was stored at 4° and was diluted 1 in 10 with glacial acetic acid immediately prior to use. 3.9 ml of the diluted DAB solution were added to the test-tubes containing the hexosamine and placed in a water-bath at 37° for 20 minutes, after which the tubes were placed in an ice water-bath. A magenta colour developed in the presence of acetylated glucosamine, and this was read in a spectrophotometer (Unicam SP 500) at 545 nm, using glass cuvettes of 1 cm light path. Good and Bessman read the colour at 570 nm but Winterburn and Phelps used 545 nm. This matter was put to the test and 545 nm gave 7% more colour than 570 nm (Fig. 15). 545 nm was the wavelength used in all estimations.

The borate buffer needs to be added to the glucosamine test solution before the acetic anhydride is added. Addition of the acetic anhydride to the glucosamine solution before the addition of the borate buffer failed to produce any colour in the Morgan-Elson reaction.

STUDIES ON THE COLORIMETRIC ASSAY FOR GLUCOSAMINE

Standard solutions

The standard solutions used throughout the work for this thesis were made up from glucosamine hydrochloride. A sample of glucosamine hydrochloride as supplied was desiccated for 24 hours in a vacuum desiccator. The loss of hygroscopic water was 0.0008 g in a sample of 0.6792 g, and 0.0006 g in another sample of 0.2359 g. This was considered to be insignificant.

2 mM glucosamine hydrochloride solution was made up by dissolving 0.2156 g glucosamine hydrochloride in 500 ml deionized water. More dilute standards were made by diluting the 2 mM solution.

Reading the colour

The colour formed in the Morgan-Elson reaction was read in a Unicam SP 500 spectrophotometer. Glass cuvettes were used and they were not completely matched. Matched cells were procured at one stage but with use they soon

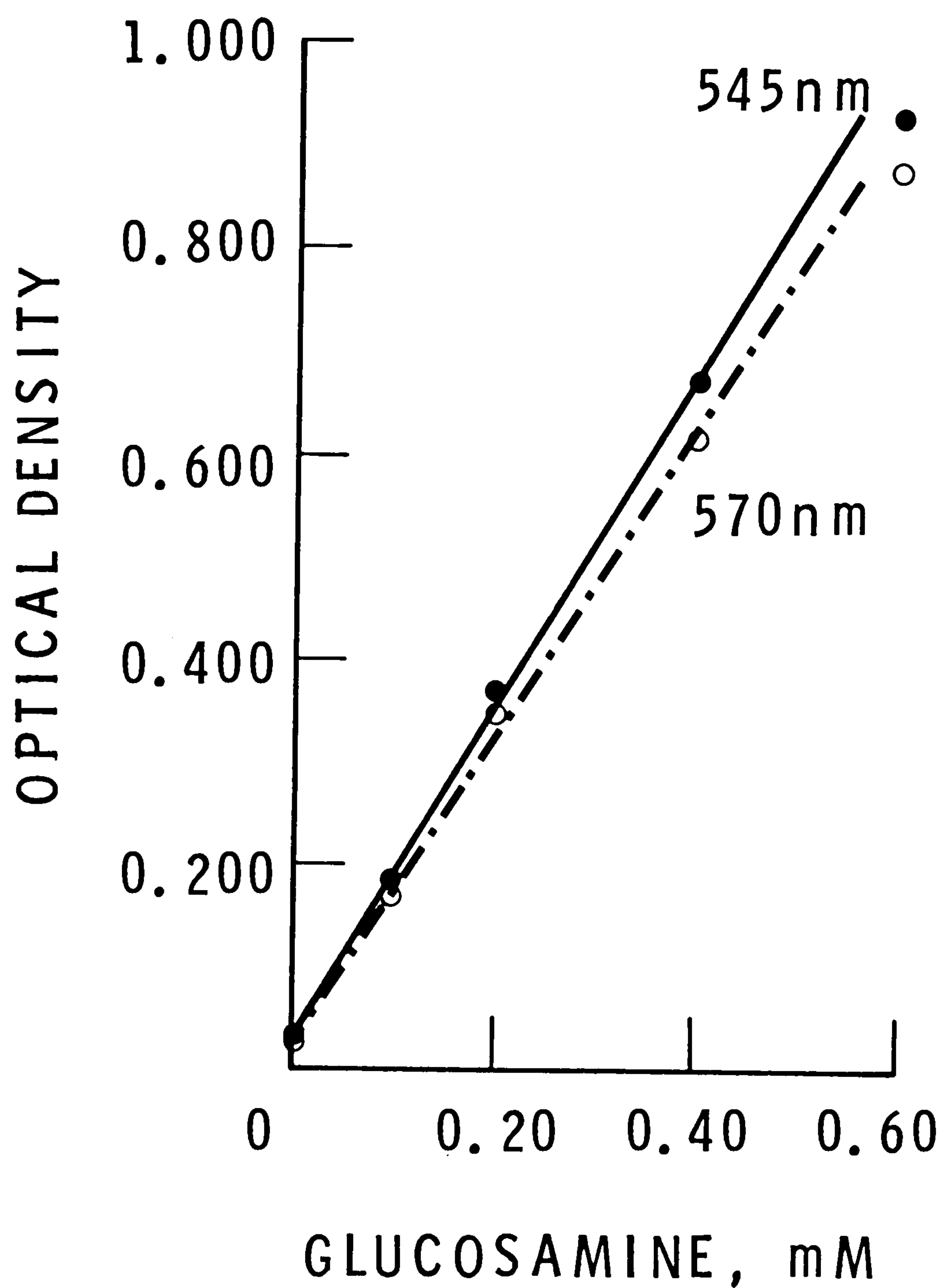


FIG. 15. Optical density of solutions of glucosamine hydrochloride assayed by the Morgan-Elson reaction with the colour read in a spectrophotometer at 545 nm (closed circles) and at 570 nm (open circles).

acquired slightly different optical densities. Therefore, whenever the optical density of glucosamine solutions was measured, the optical density of the cell, determined against a water-blank of lower O.D., was subtracted from the O.D. as read.

The pH of the borate buffer

The predicted pH of the borate buffer using the molarities of boric acid and KOH recommended by Good and Bessman (1964) was 9.2. The observed pH of the buffer as made up was usually between 9.3 and 9.5. Using a glucosamine standard of 0.20 mM, the O.D. was read in the Morgan-Elson reaction, using the same dilute DAB solution, with three different borate buffers. In two of these buffers, KOH was added to 1.12M boric acid to give an observed pH of 9.2 in one buffer and of 9.8 in the second buffer. In the third buffer, 0.56M KOH was used, as recommended, and the observed pH was 9.5. The best colour yield was obtained using the recommended amount of KOH, giving an observed pH of 9.5 (Table 3).

TABLE 3. Optical density of a solution of 0.20 mM glucosamine in the Morgan-Elson reaction, measured 3 times in the same batch with dilute DAB, but using borate buffer at different pH values

Observed pH	Predicted pH	O.D.
9.2		0.196
*9.5	9.2	0.280
9.8		0.248

The linearity of the glucosamine assay

This was confirmed for the range 0-0.40 mM glucosamine. Two aliquots each, of 0.5 ml each, of glucosamine standards of 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50 mM, were assayed by the Morgan-Elson reaction as used in this thesis. Two water-blank standards were also assayed. The results are shown in Fig. 16. It can be seen that the plot of O.D. against glucosamine concentration is quite linear up to 0.40 mM glucosamine although it is no longer linear at 0.50 mM.

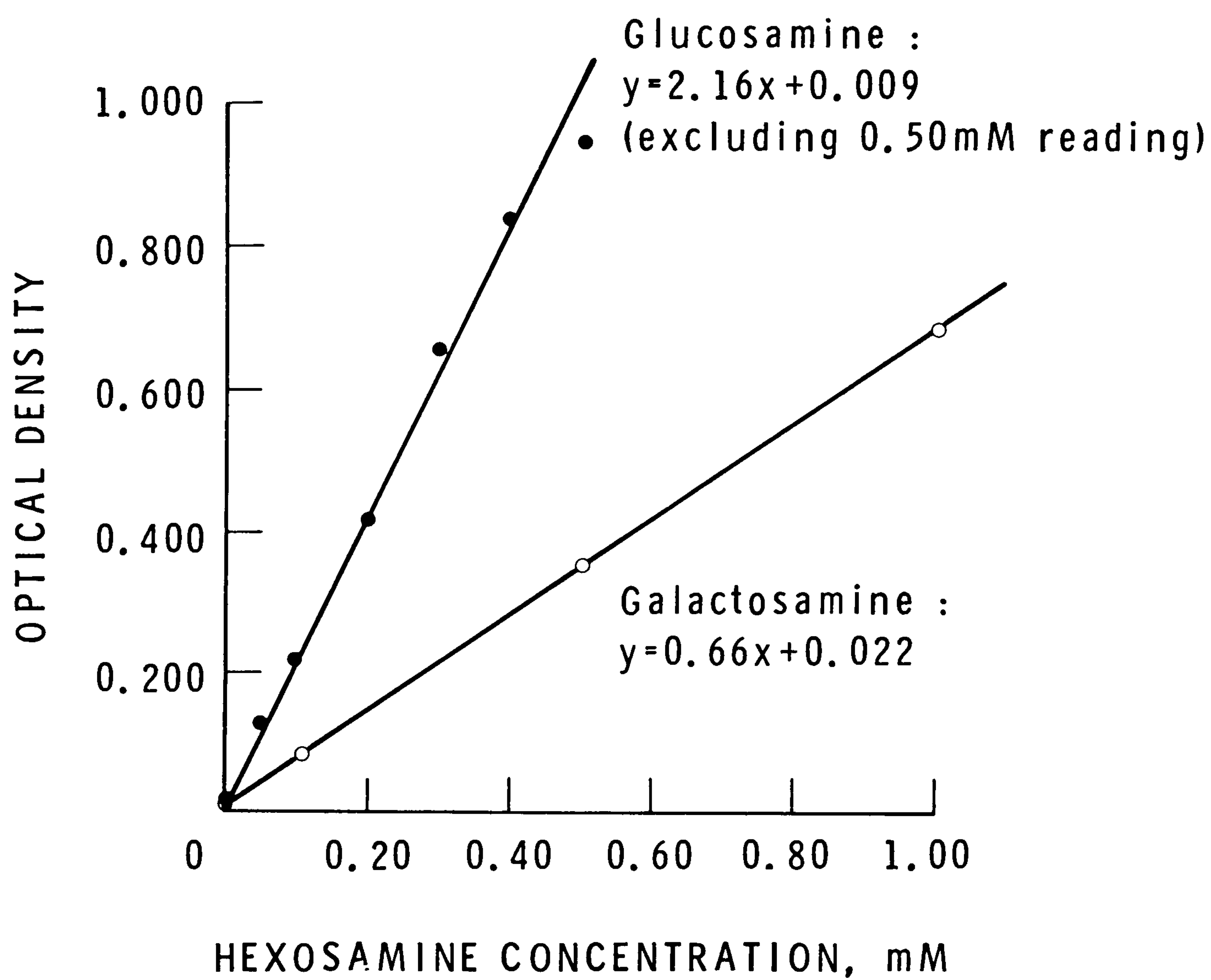


FIG. 16. The colour produced in the Morgan-Elson reaction by glucosamine hydrochloride (closed circles) and by galactosamine hydrochloride (open circles).

The reproducibility of the glucosamine standards

On one day, six separate standard solutions of glucosamine 0.20 mM were made up in the manner that was adopted in all the biopsy assays. 0.2156 g glucosamine hydrochloride was weighed and dissolved in 500 ml deionized water to make a 2 mM solution of glucosamine hydrochloride. 7.5 ml of this 2 mM solution were pipetted into a 50 ml volumetric flask and the volume made up to 50 ml with deionized water, to give 0.30 mM glucosamine, from which the other standards were made by dilution. The 0.20 mM standard was made by taking 8.0 ml 0.30 mM glucosamine and adding 4.0 ml deionized water.

Two 0.5 ml volumes of each standard solution were assayed by the Morgan-Elson reaction in the same batch with the same solution of dilute DAB. The mean O.D. was 0.326 and the standard deviation was 0.010 (3.1%). Taking the six mean values of O.D. for the pairs of readings taken from each standard solution, the mean of these was, of course, also 0.326 and the S.D. was 0.007 or 2.0%.

The decay of glucosamine standard solutions

In the same batch as the freshly prepared 0.20 mM glucosamine standards were assayed in the previous experiment, two 0.5 ml volumes were also assayed of 0.20 mM glucosamine made on the day of the experiment by dilution of a 2 mM glucosamine solution which had been made up 17 days previously. The O.D.'s of these two samples were 0.321 and 0.319, giving a mean of 0.320, which was within 2% or 1 standard deviation of the mean of the 6 pairs of samples from freshly prepared glucosamine standards.

In another experiment, 0.40 mM glucosamine standards were made up by dilution of 2 mM solutions. One such standard solution was made up freshly on the day of the assay, a second solution had been made one week previously and kept in a refrigerator, and a third solution was made up by dilution on the day of the experiment of a 2 mM glucosamine solution that had been made up 1 month previously and stored in the refrigerator. Two 0.5 ml volumes of each solution were assayed by the Morgan-Elson reaction in the same batch. Considerable differences were observed (Table 4).

From the results, it was considered necessary always to use freshly diluted glucosamine standards. It was considered satisfactory to store the 2 mM glucosamine stock solutions, from which the standards were diluted, in a

TABLE 4. Optical densities of 3 solutions of glucosamine hydrochloride 0.40 mM, each assayed twice in the same batch with dilute DAB. Each made from stock 2 mM solution

Date of preparation	O.D. of samples	Mean
Fresh	0.592 0.588	0.590
1 week previously	0.653 0.655	0.654
Stock 2 mM made 1 month previously. Freshly diluted	0.650 0.666	0.657

refrigerator for up to 1 week.

Decay of the colour

The colour produced by the Morgan-Elson reaction begins to decay within 1 hour (Morgan and Elson, 1934). By recommending the immediate reading of the colour, Good and Bessman (1964) avoided having to take account of the colour decay. Winterburn and Phelps (1971a) read the colour density twice and extrapolated back to zero time to determine the true reading. Winterburn and Phelps noted a colour decay of 14% per hour.

The colour decay was investigated at room temperature and in an ice water-bath. Standards of 0.10, 0.20 and 0.40 mM glucosamine, of 0.5 ml, and water-blanks, were assayed for glucosamine and the colour was read immediately after incubation at 37° and again 1 hour later after standing at room temperature. The colour decay was 12.3, 12.4 and 11.8% respectively for the 0.10, 0.20 and 0.40 mM standards.

Two standards each of 0.05, 0.20 and 0.60 mM glucosamine, of 0.5 ml, and water-blanks, were assayed for glucosamine and the colour read immediately after incubation and at approximately 20, 40, 60, 90 and 120 minutes later after standing in an ice water-bath (Fig. 17). The decay for the 0.05, 0.20 and 0.60 mM standards was 3.8, 8.0 and 8.5% respectively for the 2-hour period, equivalent to 2-4% per hour. During this period, the O.D. of the two water-blanks rose from 0.019 to 0.025.

Thus, if the colour was read within 15 minutes of the end of the 37° incubation, with the tubes kept in an ice water-bath from the end of the incubation until

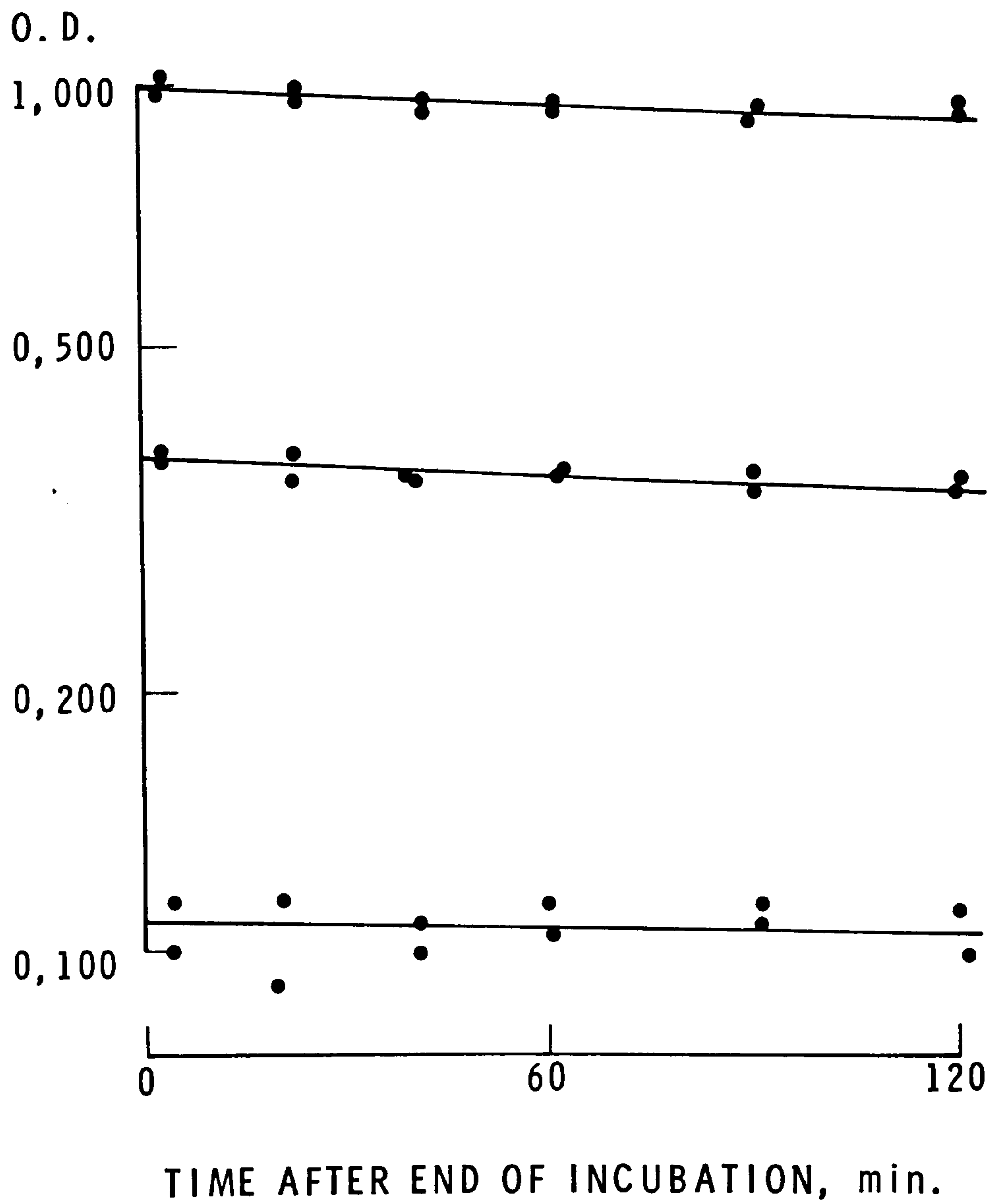


FIG. 17. Decay, at 2°, of the colour produced in the Morgan-Elson reaction by two samples each of glucosamine hydrochloride 0.05, 0.20 and 0.60 mM.

the reading of the colour, the error would be no more than 1%.

The error due to the colour decay was minimized by keeping to the following procedure in performing the glucosamine assay. Batches of no more than 24 tubes were read together. After boiling with alkali and acetic anhydride and cooling in an ice water-bath, dilute DAB was added to 3 tubes at a time, at 2-minute intervals, and the 3 tubes were then placed in the 37° water-bath. Each set of 3 tubes was removed from the 37° water-bath after 20 minutes in the water-bath and placed in an ice water-bath. All the tubes in the batch were taken to the room in which the spectrophotometer was situated and the colour in the tubes was read in sets of 3 tubes, again at 2-minute intervals, in the same sequence as in the 37° incubation. By this means, each tube remained in the ice water-bath for the same length of time and the error due to the colour decay was the same in all tubes. When test solutions of glucosamine were read against standards, this error, being constant, was thus eliminated. With 24 tubes, the delay in reading the colour of each tube was about 15 minutes.

During humid weather, condensation quickly formed on the spectrophotometer cuvettes when the test solutions were taken directly from the ice water-bath into the cuvettes. During such conditions, the test-tubes were removed from the ice water-bath 2 minutes before they were to be read in the spectrophotometer.

Decay of activity of DAB solutions

"Stock DAB" solution was kept in a refrigerator and fresh "dilute DAB" solution was prepared freshly for each batch of readings by diluting 1 volume of the stock solution with 9 volumes of glacial acetic acid. The chromogenic activity of freshly prepared dilute DAB solutions slowly diminished the longer the period that the stock DAB solution had been kept since being made up itself. This loss of activity was about 25% per month. Hence it was decided to discard unused stock DAB after 4 weeks and to make up a fresh supply.

The activity of the dilute DAB solutions decayed at room temperature after they had been made up from the stock DAB. This was investigated as follows. Stock DAB was freshly prepared and dilute DAB was freshly made up from this. Several tubes were prepared containing glucosamine 0.15 mM standard solution, 0.5 ml, to which were added borate buffer, 0.5 ml, and 1.5% acetic anhydride in acetone, 0.1 ml. The tubes were boiled and cooled, with their screw-caps

in place. Two tubes were assayed for glucosamine using the freshly prepared dilute DAB solution. Portions of the dilute DAB solution were kept on the bench at room temperature, in a refrigerator at 4°, and in an ice water-bath at 37°. Two hours after preparation of the dilute DAB, two tubes containing the glucosamine 0.15 mM standard were assayed using the dilute DAB on the bench, at 27°, and two tubes were assayed using the dilute DAB from the 37° water-bath. At 5 hours, two tubes of the glucosamine standard were assayed with the dilute DAB on the bench, now at 34° on a hot day, two tubes with the dilute DAB from the 37° water-bath, and two tubes with the dilute DAB from the refrigerator.

The results are shown in Fig. 18. After 2 hours on the bench at 27°, the chromogenic activity of dilute DAB had decayed by 15%, After 5 hours at 4°, the chromogenic activity had also decayed by 15%. Therefore freshly diluted DAB was always used for each batch of tubes to be read. Between the first and last set of 3 tubes in a batch of 24 tubes, when dilute DAB was being added to one set of 3 tubes every 2 minutes, the delay was 14 minutes. This meant that at 27° the activity of the dilute DAB decayed by about 2% during that period. This was considered acceptable. Care was taken not to allow the temperature of the laboratory to rise above 27°. It did not prove helpful to keep the dilute DAB in ice during the addition to the test-tubes on hot days as these were generally humid days and water would condense on the inside of the container of the dilute DAB.

Galactosamine as assayed by the Morgan-Elson reaction

Good and Bessman (1964) devised a colorimetric means of determining separately the concentrations of glucosamine and galactosamine in mixtures of the two hexosamines. This relies on the fact that in the Morgan-Elson reaction, glucosamine gives a stronger colour than equimolar concentrations of galactosamine, whereas in the Elson-Morgan reaction galactosamine gives a stronger red colour with DAB following treatment with acetylacetone than does glucosamine.

In the Morgan-Elson reaction, Good and Bessman found that 0.6 mM galactosamine gave the same amount of colour as 0.3 mM glucosamine. This was investigated in the present study, comparing solutions of 0.10, 0.50, 1.00 and 2.00 mM galactosamine with 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50 mM

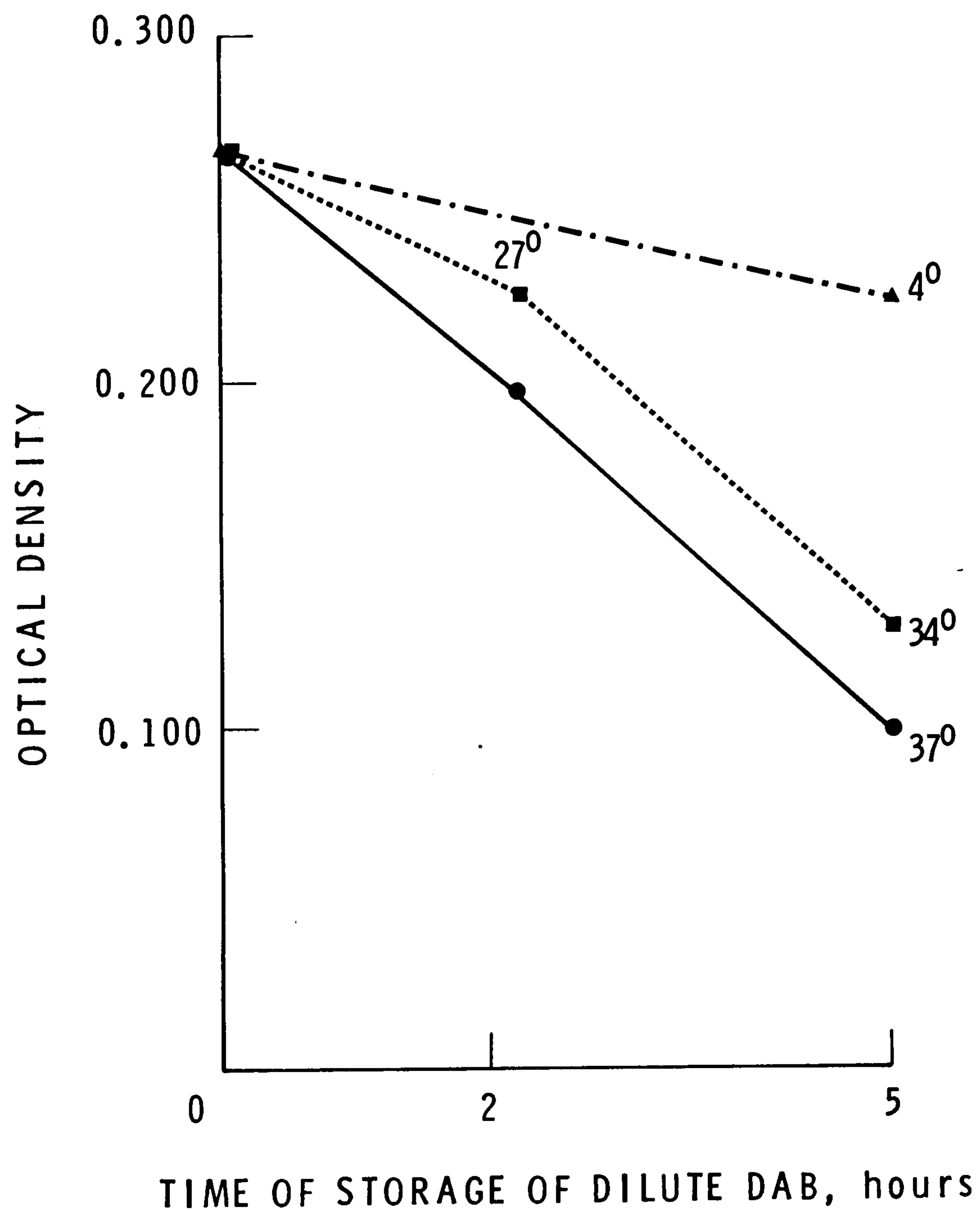


FIG. 18. Decay of the chromogenic activity of dilute DAB. O.D. of the colour produced by a standard solution of 0.15 mM glucosamine with a freshly made solution of dilute DAB and following storage of that solution at different temperatures for up to 5 hours.

glucosamine. The gradient of the linear plot $\frac{\text{O.D.}}{\text{mM hexosamine}}$ was 2.16 for glucosamine and 0.66 for galactosamine (Fig. 16). Thus 1 mM galactosamine is detected by the Morgan-Elson reaction as equivalent to 0.32 mM glucosamine.

The presence of galactosamine as distinct from glucosamine was ignored in the present studies. Any galactosamine present would be detected as one-third of its molarity of glucosamine.

THE NEUTRALIZATION OF THE PERCHLORIC ACID

The test solutions were boiled with a potassium borate buffer, 1.12M, at pH 9.2 in the first stage of the Morgan-Elson reaction. Therefore it was sufficient to neutralize the perchloric acid to within a range of pH 6-11 prior to boiling with the potassium borate buffer. The tissue was homogenized and incubated in a buffered solution, which aided the accuracy of the neutralization of the perchloric acid. (Initially this was a 50 mM tris-HCl buffer at pH 7.5 although subsequently a sodium-phosphate buffer at pH 7.0 was used, see below.)

As it was important to keep the volumes as small as possible, the perchloric acid in the supernatant from the centrifugation of the protein precipitate was neutralized first with 0.05 ml 9M KOH, which was found to neutralize the perchloric acid almost but not completely, and then a further small volume of M KOH was added slowly to a pH of between 6 and 11 using Merck non-bleeding indicator strips. The volume of M KOH used for neutralization was recorded to the nearest 0.005 ml. In a typical batch of 19 test samples, this volume varied between 0.01 and 0.14 ml, with a mean of 0.08 ml.

THE DETERMINATION OF GLUCOSAMINE SYNTHETASE ACTIVITY IN TISSUE HOMOGENATES

The units of glucosamine synthetase activity used in this thesis are μmoles glucosamine synthesized per hour either per gram wet weight of tissue or per gram of tissue protein. The question of the denominator of the units of activity, whether it should be g wet weight or g tissue protein, is discussed and studied later in this chapter. In the development of the microassay, the optimum

conditions of incubation were determined by comparing the effects of different sets of conditions on aliquots of the same tissue homogenate. Hence tissue concentration was unimportant in these studies except insofar as it was desirable to keep the tissue concentration in the homogenate in the range to be used for the biopsy studies. For that purpose it was sufficient to study the enzyme activity per g wet weight.

The calculation of the glucosamine synthetase activity

Let the concentration of tissue in the homogenate be c mg/ml and let the weight of the blotted tissue sample be w mg. This sample was dissolved in $\left[\frac{w}{c} - \frac{w}{1000}\right]$ ml homogenizing solution.

0.5 ml homogenate was added to 0.5 ml substrate-buffer mixture to give an incubate of 1.0 ml with a tissue concentration of $\frac{c}{2}$ mg/ml.

At the end of the incubation period, 0.05 ml perchloric acid was added. This volume of 1.05 ml therefore contained $\frac{c}{2}$ mg tissue.

Suppose the glucosamine synthetase activity of the tissue was x μmoles glucosamine synthesized/h/g wet wt and the concentration of glucosamine in the 1.0 ml incubated solution immediately prior to the addition of the perchloric acid was y mM. Then y μmoles glucosamine were synthesized by $\frac{c}{2}$ mg tissue.

If the incubation period was t hours, then $\frac{c}{2}$ mg tissue synthesized y μmoles glucosamine in t hours.

Therefore 1 mg tissue synthesized $\frac{2y}{c}$ μmoles glucosamine in t hours, equivalent to $\frac{2y}{ct}$ μmoles in 1 hour. Therefore 1 g tissue synthesized $\frac{2000y}{ct}$ μmoles glucosamine per hour, that is,

$$x = \frac{2000y}{ct}.$$

Following the addition of 0.05 ml perchloric acid, the glucosamine concentration became $\frac{y}{1.05}$ mM.

After precipitation of the protein by centrifugation, 0.8 ml of the supernatant was taken. This had a glucosamine concentration of $\frac{y}{1.05}$ mM and to it was added 0.05 ml 9M KOH and then a recorded volume, say p ml, of M KOH to neutrality.

The glucosamine concentration now became $\frac{0.8\,y}{1.05\,(0.85 + p)}$ mM.

This was the concentration of glucosamine that was read against glucosamine standards in the Morgan-Elson reaction. Let this concentration be M mM. Then

$$M = \frac{0.8\,y}{1.05\,(0.85 + p)}$$

$$= \frac{y}{1.12\,(1 + 1.18\,p)}.$$

Now,

$$x = \frac{2000\,y}{ct},$$

therefore,

$$y = \frac{xct}{2000}$$

Therefore,

$$M = \frac{0.8}{1.05\,(0.85 + p)} \cdot \frac{xct}{2000}.$$

Therefore,

$$x = \frac{2000 \cdot 1.05\,M\,(0.85 + p)}{0.8\,ct}$$

$$= \frac{2000 \cdot 1.05 \cdot 0.85\,M\,(1 + 1.18\,p)}{0.8\,ct}$$

$$= \frac{2231\,M\,(1 + 1.18\,p)}{ct}$$

Internal standards: simplification of the calculation

Instead of comparing the glucosamine in the Morgan-Elson reaction directly with standard solutions of glucosamine in that reaction, the standard solutions were treated with perchloric acid and KOH in the same manner as the incubated homogenates. These were not true internal standards as they did not contain tissue, nor substrates, nor EDTA, KCl or buffer. Comparison of these acid-and-alkali treated standards with true internal standards is considered later.

Suppose 1.0 ml glucosamine standard of y_s mM was taken. 0.05 ml perchloric acid was added. The glucosamine concentration was now $\frac{y_s}{1.05}$ mM.

Suppose 0.8 ml of this solution was neutralized with 0.05 ml 9M KOH and p_s ml M KOH. If the concentration of glucosamine in the neutralized standard solution was M_s mM, then,

$$M_s = \frac{0.8\, y_s}{1.05\,(0.85 + p_s)}$$

$$= \frac{y_s}{1.12\,(1 + 1.18\, p_s)}$$

The standards of glucosamine used were between 0 and 0.30 mM. It was convenient to convert these to units of 0.01 mM, designating y_s as A_s in such units, so that

$$A_s = 100\, y_s$$

and therefore,

$$M_s = \frac{A_s}{112\,(1 + 1.18\, p_s)}.$$

Similarly, y was expressed in the same units, as A, so that

$$A = 100\, y,$$

therefore,

$$M = \frac{A}{112\,(1 + 1.18\, p)}.$$

Let D equal 1000 times the optical density of the colour produced by the test solution and let D_s equal 1000 times the O.D. of the internal standard solution.

D is directly proportional to M. Therefore,

$$D = jM + k,$$

where j and k are constants, and

$$D_s = jM_s + k.$$

Now,

$$M = \frac{A}{112\,(1 + 1.18\, p)},$$

therefore,

$$D = \frac{jA}{112\,(1 + 1.18\,p)} + k.$$

Therefore,

$$\frac{A}{1 + 1.18\,p} = \frac{112\,D}{j} - \frac{112\,k}{j}$$

$$= k_1 D + k_2,$$

where k_1 and k_2 are constants. Similarly,

$$\frac{A_s}{1 + 1.18\,p_s} = k_1 D_s + k_2.$$

Let

$$B = \frac{A}{1 + 1.18\,p},$$

then

$$B = k_1 D + k_2$$

and

$$B_s = k_1 D_s + k_2$$

Thus B can be determined by plotting measured values of D_s for the internal standards against B_s, calculated from the known concentrations, A_s, by the formula

$$B_s = \frac{A_s}{1 + 1.18\,p_s},$$

and reading the value of B for a test solution corresponding to the measured value of D, according to the standard curve so obtained.

To calculate x, the glucosamine synthetase concentration of the tissue, from B,

$$x = \frac{2000\,y}{ct}$$

$$= \frac{20\,A}{ct},$$

and

$$A = B\,(1 + 1.18\,p),$$

therefore,

$$x = \frac{20\ B\ (1 + 1.18\ p)}{ct}.$$

In practice, following the addition of 0.05 ml perchloric acid to 1.0 ml of the glucosamine standard, the neutralization was performed on the 1.05 ml of standard + acid without taking an 0.8 ml aliquot. Instead of adding 0.05 ml 9M KOH, 0.065 ml was added ($0.05 \times \frac{1.05}{0.8} = 0.0656$). The volume of M KOH to neutralize was designated q ml.

Instead of

$$M_s = \frac{0.8\ y_s}{1.05\ (0.85 + p_s)},$$

we have

$$M_s = \frac{1.0\ y_s}{1.115 + q}$$

$$= \frac{A_s}{100\ (1.115 + q)}$$

$$= \frac{A_s}{111.5\ (1 + 0.90\ q)}$$

$$\simeq \frac{A_s}{112\ (1 + 0.90\ q)}.$$

Therefore,

$$B_s = \frac{A_s}{1 + 0.90\ q}.$$

The need for internal standards

The use of acid-and-alkali treated standards simplified the calculation of glucosamine synthetase activity. The results obtained by treating the standards in this way were compared with those obtained by calculating the glucosamine synthetase activity by comparison of the test solutions with untreated glucosamine standards.

Two 0.5 ml volumes each of 0.05 and 0.15 mM glucosamine and two water-blanks were assayed directly for glucosamine, in the same batch as two standards each of 0.05, 0.10, 0.15 and 0.25 glucosamine which had been treated with perchloric acid and KOH. From the O.D. of the untreated standards, M_s was plotted against O.D. for the untreated solutions of

glucosamine. From this curve, values of M for the treated standards were derived. These values were compared with the predicted values of M, M_{calc}, for each treated standard, from the formula

$$M_{calc} = \frac{y_s}{1.12\ (1 + 0.90\ q)}.$$

The predicted and the observed values of M were plotted against each other and there was a close correlation (Fig. 19).

Acid-and-alkali treated standards were considered preferable to directly estimated standards because they took into account the volumetric errors resulting from the assumption that strong solutions of perchloric acid and potassium hydroxide, when mixed to precipitate potassium perchlorate, result in a solution equal in volume to the combined volumes of acid and alkali. At the stage of $KClO_4$ precipitation, the concentration of perchlorate was $71 \times \frac{0.05}{1.05} = 3.4\%$ w/v. To this was added, assuming a mean value for p of 0.08 ml, 0.05 ml 9M KOH (containing 0.45 mmole) and 0.08 ml M KOH (containing 0.08 mmole), equivalent to 0.13 ml containing 0.53 mmole, i.e., 4M KOH, or 224 g/l, or 22.4% w/v.

Although the use of treated standards made the calculations simpler, it would have been possible to plot accurately the relationship between treated and untreated glucosamine standards and to use untreated standards to plot B versus D using a correction factor so derived. This would have eliminated the need for the addition of acid and alkali to each standard in each batch. However, it was not done and all readings were made with acid-and-alkali treated standards.

True internal standards

The acid-and-alkali treated standards were aqueous solutions of glucosamine which did not contain tissue, nor substrates, nor the solutes of the homogenizing buffer.

Two 1.0 ml volumes each of 0.05 and 0.15 mM glucosamine were treated with acid and alkali and from these and two water-blanks a curve for B versus D was plotted. From the same stock of 0.60 mM glucosamine, 0.25 ml of 0.20 and 0.60 mM glucosamine, two samples of each, and two 0.25 ml samples of water were taken and to each was added 0.25 ml of an homogenate of human colonic mucosa, 40 mg/ml, in a buffer containing double the usual concentrations of constituents (i.e., containing tris-HCl 100 mM at pH 7.5,

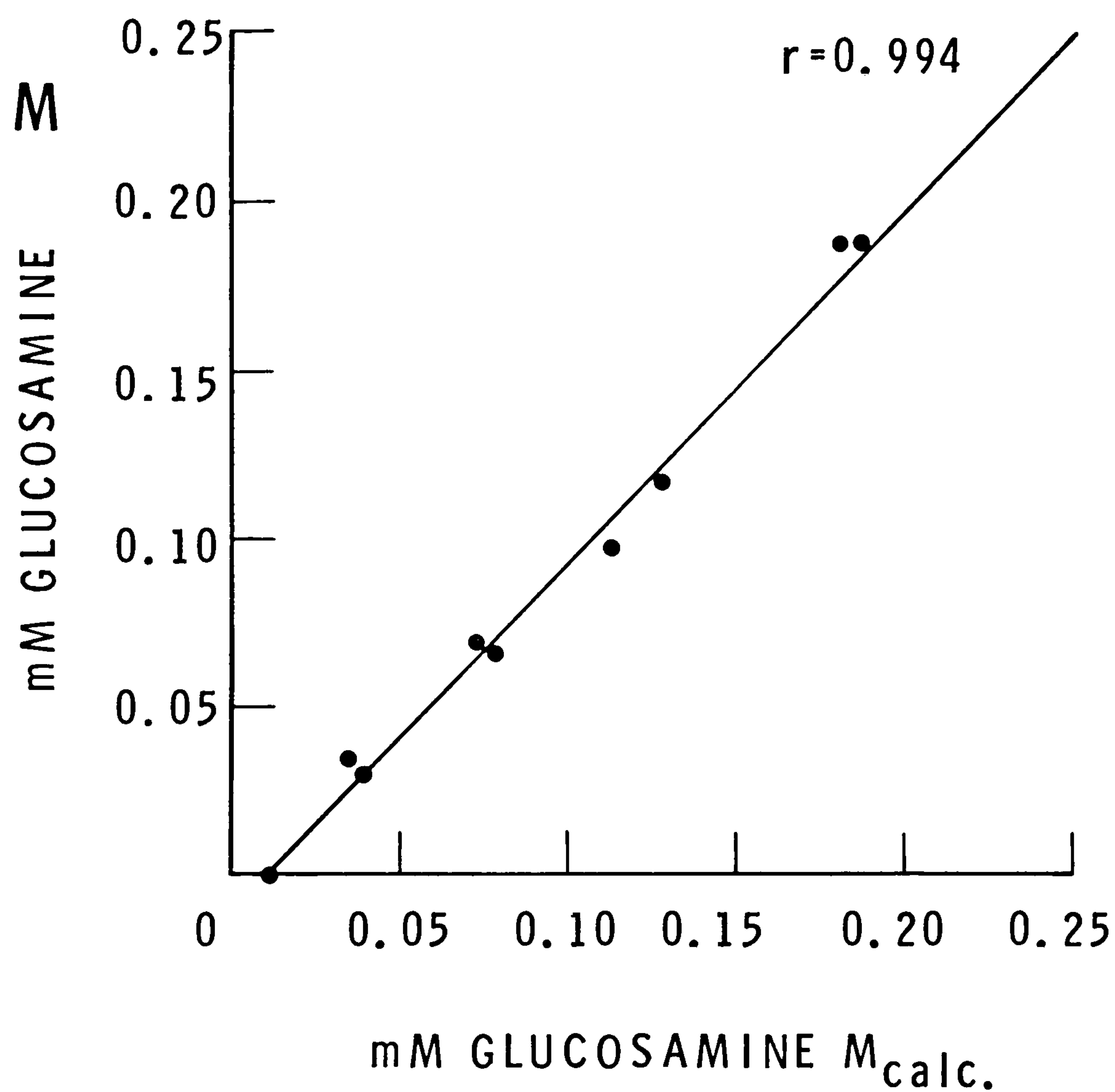


FIG. 19. Comparison of glucosamine standards directly assayed by the Morgan-Elson reaction with standards treated with perchloric acid and KOH. From a standard curve of directly assayed standards, the glucosamine concentration of the treated standards (M) was derived and compared with $M_{calc.}$, the value of M predicted from the known values of dilution with acid and alkali.

EDTA 2 mM and KCl 200 mM) and 0.5 ml of buffer of normal strength (tris-HCl 50 mM, EDTA 1 mm, KCl 100 mM) containing fructose-6-phosphate 10 mM and glutamine 8 mM. This resulted in 1.0 ml volumes of normal-strength buffer containing 10 mg homogenized colonic mucosa, 5 mM fructose-6-phosphate, 4 mM glutamine, and either water or 0.05 or 0.15 mM glucosamine (two 1.0 ml volumes of each). These true internal standards were treated with perchloric acid and KOH in the same manner as the acid-and-alkali treated aqueous standards, except that it was necessary to precipitate the protein by centrifugation and to take 0.8 ml of the supernatant for KOH neutralization so that p ml M KOH was added for neutralization instead of q ml for the aqueous standards. D was measured for true internal standards in the same batch as the acid-and-alkali treated aqueous standards, and B was calculated. These points were plotted on the same graph as the plot for the treated aqueous standards (Fig. 20). They fell close to the curve for the aqueous standards. It was concluded that true internal standards were not required and that aqueous standards, treated with perchloric acid and KOH to simplify the calculation, were satisfactory.

The choice of standards

In the Morgan-Elson reaction, the optical density was shown to bear a linear relationship to the glucosamine concentration up to 0.40 mM glucosamine (Fig. 16). This corresponded to an O.D. of approximately 0.900. It was decided to keep the O.D. readings below 0.500 and so it was decided to use standards up to about 0.30 mM.

In most of the preliminary studies in the determination of the optimal conditions for the microassay and in all the biopsy studies, the standards used were 0.05, 0.10, 0.20 and 0.30 mM glucosamine, plus a water-blank. These gave values for A_s of 5.0, 10.0, 20.0 and 30.0, and 0 for the water-blank. The values of B_s varied according to q (the volume of M KOH used to neutralize the perchloric acid). An average value of q was 0.11, giving a value for B_s equal to $\frac{A_s}{1.1}$. Average values for B_s were thus 0, 4.5, 9.1, 18.2 and 27.3.

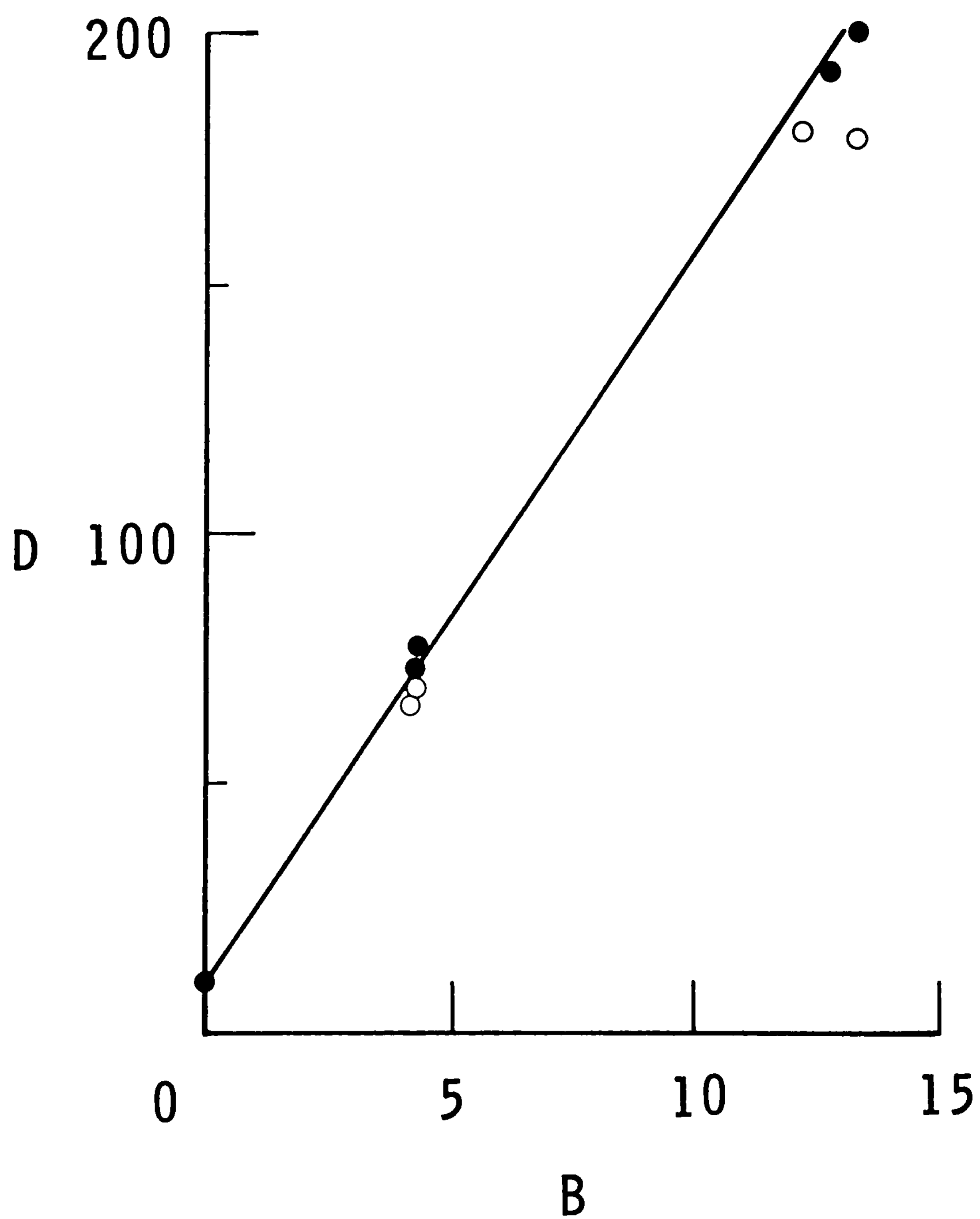


FIG. 20. Plot of B against D for acid-and-alkali treated aqueous glucosamine standards (closed circles) and for similar standards containing homogenate and substrates (open circles).

The linearity of the standard curve

The five points for B_s between 0 and approximately 27 gave a linear curve when plotted against D_s. This was drawn visually. A typical curve is shown in Fig. 21.

The linearity of the standard curve and the error due to the use of visually drawn curves is discussed in Appendix A.

THE DETERMINATION OF THE OPTIMAL CONDITIONS FOR GLUCOSAMINE SYNTHESIS

In order to miniaturize the assay of glucosamine synthetase, it was necessary to determine the incubation conditions which gave the best yield of glucosamine. This was done by studying the effects of varying the following factors:—

(a) The requirement for EDTA.
(b) The tissue concentration in the homogenate.
(c) The duration of the incubation.
(d) The pH.
(e) The ionic strength of the incubate.
(f) The fructose-6-phosphate concentration.
(g) The glutamine concentration.

The requirement for homogeneity of the homogenate was also studied.

Each of these variables was investigated separately, apart from the concentrations of the two substrates, which were studied together. In each study, all factors other than the one under consideration were kept constant. 1.0 ml volumes were prepared, containing the homogenized tissue and the substrates dissolved in buffer, using two such volumes for each of the selected values of the factor that was being studied. The same tissue homogenate was used to prepare all the 1.0 ml volumes for a given study.

Temperature

The Morgan-Elson reaction required incubation at 37° and it was convenient to use the same water-bath for the incubation of the glucosamine synthetase reaction. 37° is a physiological temperature and it was used by Winterburn and Phelps (1971a) and by nearly all other workers, although a slightly better yield (2-3%) was obtained at 40° (Appendix A, Fig. 105).

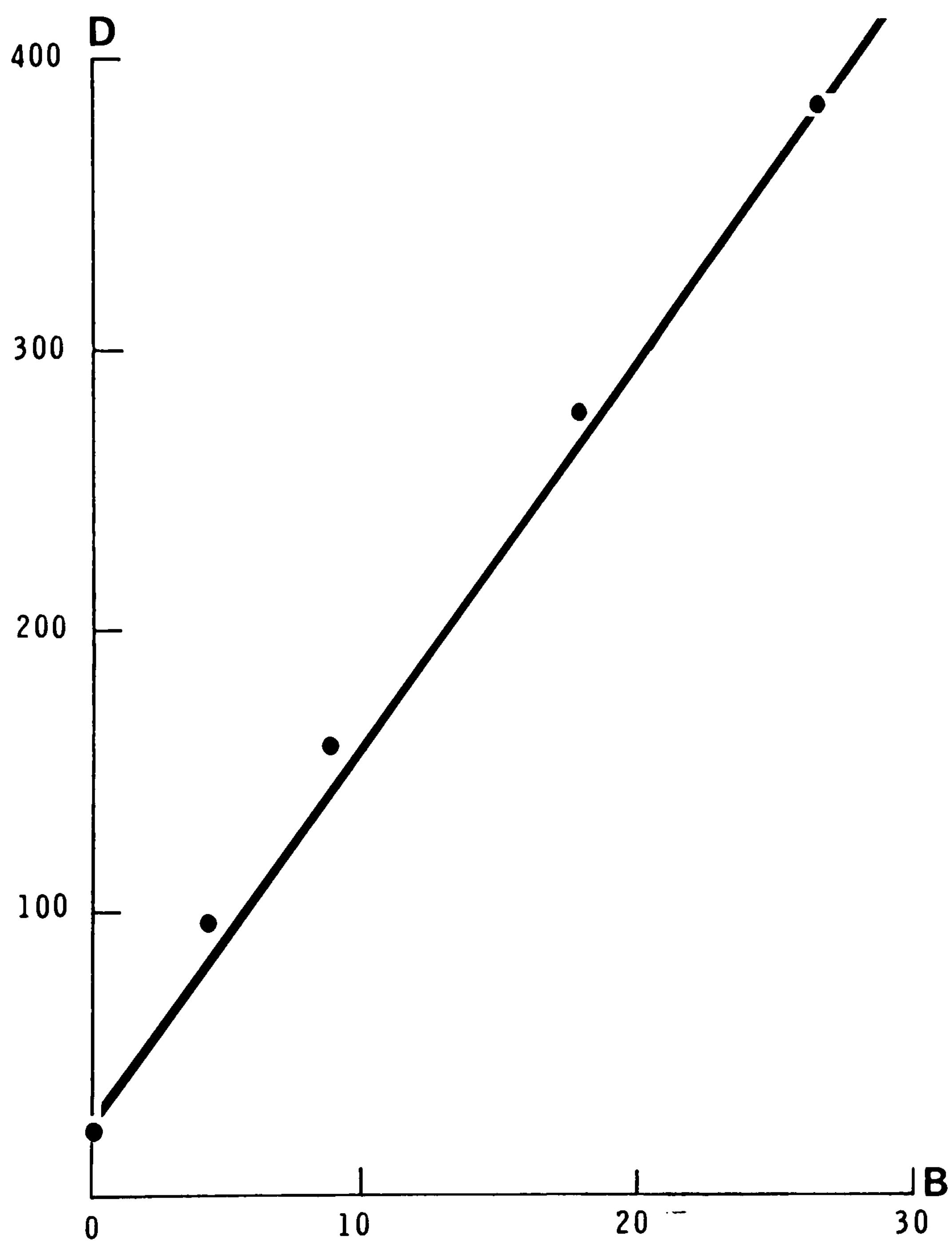


FIG. 21. A typical standard curve for B against D using acid-and-alkali treated standards as used in the 10 mg assay for glucosamine synthetase.

The homogeneity of the homogenate and the need for centrifugation prior to incubation with the substrates

Glucosamine synthetase is a cytoplasmic enzyme. As such, the first stage in its assay has always been the centrifugation of the homogenate, with glucosamine synthetase being estimated in the supernatant. However, it was conceivable that a greater yield of enzyme activity from a given piece of tissue could be obtained by incubating with the substrates the uncentrifuged homogenate. There would be no wastage of material in the deposit and in the supernatant lost in the transfer to another test-tube.

The uncentrifuged homogenate contained visible debris, which tended to settle at the bottom of the test-tube during the substrate incubation. The homogenate was thus visibly non-homogeneous and the requirement for a shaking water-bath for the substrate incubation was investigated as follows.

Two homogenates of normal colonic mucosa were prepared from two separate colectomy specimens, at 20 mg tissue per ml homogenate (in tris-HCl 50 mM at pH 7.5, EDTA 1 mM, and KCl 100 mM). From the first homogenate, two aliquots of 0.5 ml were taken, to each of which was added 0.5 ml substrate-buffer mixture to give final concentrations of fructose-6-phosphate 5 mM and glutamine 4 mM. These two samples were incubated for 90 min at 37° in a shaking water-bath. A third aliquot of 0.5 ml of the first homogenate was treated similarly but was incubated in a non-shaking water-bath. Two further aliquots of 1.0 ml of each homogenate were taken, to each of which was added 1.0 ml substrate-buffer mixture. Each 2.0 ml sample was incubated in a non-shaking water-bath, with the debris being allowed to sediment. After 15 min, the upper layer of 1.0 ml of each sample was carefully pipetted into a separate tube and the incubation of both the upper and the lower layers was continued for a further 75 min. The results of the several samples are compared in Table 5.

These results indicated that the homogenate was homogeneous to a degree which was satisfactory for the estimation of glucosamine synthetase.

The homogenate compared to the supernatant

In one experiment, two portions of an homogenate of colonic mucosa, 10 mg/ml, were centrifuged at 2500 rpm for 15 min. Three of each of the following samples were assayed for glucosamine synthetase under identical

TABLE 5. Glucosamine synthetase activity of 2 homogenates of colonic mucosa assayed under various conditions to demonstrate the need for homogeneity of the homogenate

Conditions of assay		Glucosamine synthetase activity (units/g wet wt) 1st homogenate	2nd homogenate
1.0 ml, 90 min in still water-bath		17.8	
1.0 ml, 90 min in shaking water-bath		16.4 15.8	
2.0 ml, 15 min in still water-bath	1.0 ml upper layer placed in another tube in a still water-bath for a further 75 min	15.2 14.5	6.4 7.1
	1.0 ml lower layer incubated for a further 75 min	15.4 14.7	7.4 7.5

conditions, with the same batch of dilute DAB and standards: the uncentrifuged homogenate; the supernatant of the centrifuged homogenate; and the other centrifuged homogenate which was re-homogenized by shaking. There was no difference in enzyme activity between the supernatant and the sample of homogenate similarly centrifuged and then re-homogenized, but the activity in these samples was less than in the uncentrifuged homogenate (Table 6). There may have been some loss of enzyme activity as a result of heating in the centrifugation. It certainly appeared justifiable to omit the centrifugation of the homogenate and it was possibly advantageous to do so.

TABLE 6. Glucosamine synthetase activity in an homogenate of colonic mucosa compared to the supernatant of the homogenate following centrifugation

Treatment of homogenate	Glucosamine synthetase activity (units/g wet wt)
Uncentrifuged	8.9
Centrifuged, supernatant	7.2
Centrifuged then rehomogenized	7.3

The requirement for EDTA

Clarke and Pasternak (1962) showed that EDTA activated glucosamine synthetase in B. subtilis and all workers have used EDTA in the incubation medium in the estimation of glucosamine synthetase. Free Ca^{++} must be inhibitory to the enzyme reaction. The requirement for EDTA was investigated in this study as follows.

Two adjacent pieces of colonic mucosa were homogenized in a buffer containing tris-HCl 50 mM at pH 7.5 and KCl 100 mM, but no EDTA, at 10 mg tissue per ml buffer. Two 0.5 ml aliquots of each homogenate were incubated for 3 hours with 0.5 ml volumes of a similar buffer containing fructose-6-phosphate, glutamine, gentamicin and EDTA giving final volumes of 1.0 ml containing fructose-6-phosphate 5 mM, glutamine 2 mM, gentamicin 100 μg/ml and EDTA 1 mM (for discussion of the 3-hour incubation with gentamicin, see below). Two further 0.5 ml aliquots of each homogenate were incubated with a substrate-buffer mixture which was similar except for the omission of EDTA. The synthesis of glucosamine with and without EDTA is shown in Table 7. In the absence of EDTA, the yield of glucosamine was only 15-30% of the yield in the presence of 1 mM EDTA.

TABLE 7: Glucosamine synthetase activity in 2 homogenates of colonic mucosa incubated with 1 mM EDTA and without EDTA

	Glucosamine synthetase activity (units/g wet wt)	
Homogenate No.	EDTA 1 mM	No EDTA
1	6.6	2.0
	6.6	0.1
2	4.4	1.2
	4.0	1.6

In a second experiment, one piece of colonic mucosa was homogenized in the same homogenizing buffer as in the previous experiment. An adjacent piece of mucosa was homogenized in a buffer which was similar except that it contained 1 mM EDTA. Two 0.5 ml aliquots of each homogenate where incubated with substrate-buffer mixture as in the previous experiment, except that EDTA was adjusted to be 1 mM in all the final 1.0 ml volumes. For the homogenate

prepared with EDTA, glucosamine synthesis was 4.7 μmoles/h/g wet wt (mean of 4.9 and 4.5) and for the homogenate prepared without EDTA but with EDTA in the incubate the glucosamine synthesis was 4.75 μmoles/h/g wet wt (mean of 4.4 and 5.1).

From the second experiment, it appeared that EDTA was not needed in the homogenizing buffer provided it was supplied in the incubate. However, for simplicity and because it made no difference, EDTA 1 mM was always included in the homogenizing and incubating buffers.

Tissue concentration in the homogenate

0.5 ml volumes of the homogenate were incubated with 0.5 ml of substrate-buffer mixture for the glucosamine synthetase reaction. The smaller the volume, the greater would be the error in pipetting. To use different proportions of homogenate and substrate-buffer mixture would have meant using a smaller volume of one of the components than of the other and the increase in the error due to the use of the smaller volume would be greater than the lessening of the error due to the use of the larger volume of the other component. Therefore, equal volumes of homogenate and substrate-buffer were used.

In a preliminary study, an homogenate containing 41 mg tissue per ml buffer was incubated for 90 min in an incubate containing tris-HCl 50 mM at pH 7.5, EDTA 1 mM, KCl 100 mM, fructose-6-phosphate 5 mM and glutamine 12 mM, which were the incubation conditions of Winterburn and Phelps (1971a). The assay of the glucosamine synthesized in this incubation gave an O.D. with the Morgan-Elson reaction of 0.421. Acid-and-alkali treated glucosamine standards were used and the O.D. corresponded to 0.245 mM glucosamine.

It was thought best to have homogenates of normal mucosa giving final optical densities of approximately 0.200, so that the range of O.D. for specimens with a glucosamine synthetase activity of between 50% and 200% of normal would be 0.100-0.400.

Therefore in the initial experiments the homogenate was made with a tissue concentration of 20 mg/ml. (This corresponded to a concentration of 10 mg tissue per ml in the incubate.) This was for a 90 min incubation. With the doubling of the incubation period to 180 min (see next section), the tissue concentration in the homogenate was reduced to 10 mg/ml. This concentration was used in all the studies reported in subsequent chapters of this thesis.

The volume of the tissue was taken into account, at 0.001 ml per mg tissue, so that the tissue was homogenized in 0.0099 ml homogenizing buffer per 0.1 mg tissue.

The duration of the incubation

Winterburn and Phelps (1971a) incubated their preparations of glucosamine synthetase with fructose-6-phosphate and glutamine for 90 min. Other workers have used periods of between 30 min (Ellis and Sommar, 1971) and 3 hours (Kizer and McCoy, 1959; Sander et al, 1975). The relationship between the duration of the incubation and the synthesis of glucosamine was investigated as follows.

Three homogenates of normal colonic mucosa were used in the study, two of normal mucosa and one of Crohn's disease mucosa, at 10 mg tissue per ml homogenate. 0.5 ml aliquots were incubated at 37° with 0.5 ml volumes of substrate-buffer mixture, giving 1.0 ml volumes containing sodium-phosphate buffer at pH 7.0 and gentamicin 100 μg/ml (the use of a buffer at pH 7.0 is discussed below). The substrate concentrations were fructose-6-phosphate 5 mM and glutamine 2 mM in the first experiment (Fig. 22,a) and fructose-6-phosphate 10 mM and glutamine 4 mM in the other two experiments (Fig. 22,b). With the first homogenate, of normal mucosa, the incubation was terminated with perchloric acid in two tubes at each of the following time intervals: 0, 30, 60, 90, 120 and 180 min. In the other two homogenates, one of normal mucosa and one of inflamed Crohn's disease mucosa, the reaction was terminated, in two tubes each, at 0, 60, 120 and 180 min. The glucosamine formed was assayed.

Fig. 23 shows the combined results for the three experiments, taking the glucosamine synthesized at 60 min as 100 arbitrary units. The mean glucosamine synthesis of each pair of samples, including the 30 and 90 min samples in the first experiment, was quoted in terms of these arbitrary units. The rate of glucosamine synthesis in the second hour was 77% of the rate in the first hour, and in the third hour it was 68% of that in the first hour.

In the experiments in which the fructose-6-phosphate concentration was 10 mM and the glutamine concentration was 4 mM, the rates of glucosamine synthesis in the second and third hours were 88% and 84% respectively of that in the first hour, compared to 58% and 38% respectively in the experiment in which fructose-6-phosphate was 5 mM and glutamine was 2 mM. This suggests

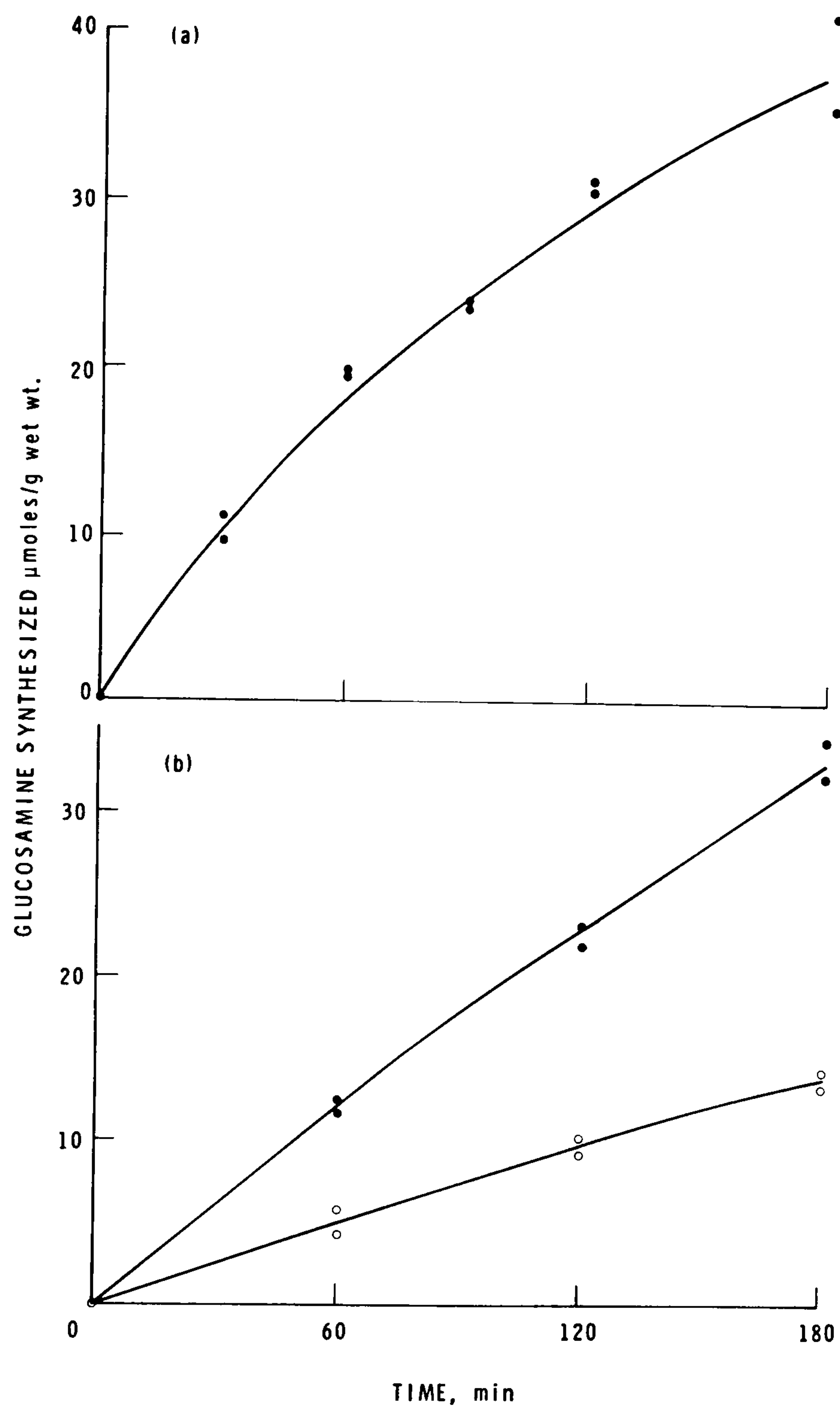


FIG. 22. Synthesis of glucosamine over 3 hours by three homogenates of colonic mucosa. (a) Fructose-6-phosphate 5 mM, glutamine 2 mM. (b) Fructose-6-phosphate 10 mM, glutamine 4 mM. (Closed circles = normal mucosa, open circles = Crohn's disease mucosa.)

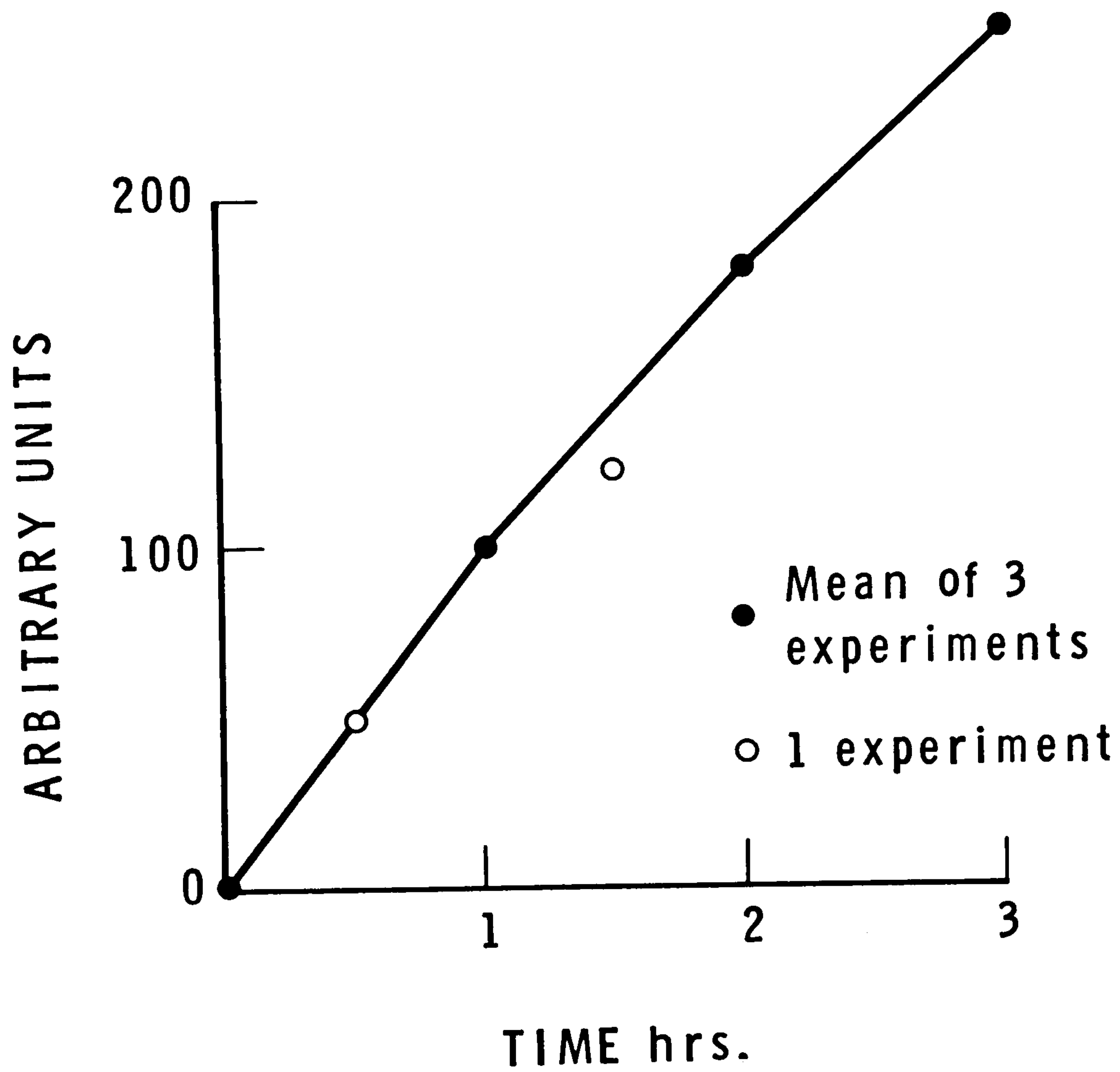


FIG. 23. The three experiments of Fig. 22 taken together. Arbitrary units: 100 = glucosamine synthesis at 60 minutes in each experiment.

that the velocity of the enzyme reaction is maintained for longer in the presence of higher substrate concentrations (see below for a discussion of substrate concentrations). In the experiment with the lower substrate concentrations, the rate of glucosamine synthesis in the first hour was 9.9 μmoles/h/g wet wt. In 1.0 ml of the incubate, there were 5 μmoles of fructose-6-phosphate and 2 μmoles of glutamine, but only 0.099 μmole of glucosamine was synthesized, so there was no significant depletion of the substrates by the enzymic reaction. On the other hand, one of the two homogenates with less decay with time came from a mucosa with Crohn's disease which had an abnormally low enzyme concentration.

As a result of these studies, it was decided to use an incubation period of 3 hours for the biopsy assays, which would allow the tissue concentration in the homogenate to be halved from 20 mg/ml for a 90 min incubation to 10 mg/ml, with only a 10-15% loss of glucosamine yield.

The use of gentamicin

The glucosamine synthetase assay was to be used with rectal biopsies. After 3 hours at 37°, it was possible that there would be growth of faecal bacteria. E. coli, for instance, has significant glucosamine synthetase activity (Ghosh et al, 1960). It was therefore decided to add to the incubate a broad-spectrum antibiotic, gentamicin.

In preliminary work in which the tissue culture of rectal biopsies was being attempted, pieces of normal human colonic mucosa, washed and blotted and weighing between 400 and 800 mg, were incubated in Warburg flasks at 37° in Krebs III medium (Krebs, 1950), which is a physiological saline low in phosphate, bicarbonate and CO_2 and which contains metabolic substrates. The oxygen consumption was measured in Warburg manometers and plotted against time for 4 hours.

In one experiment, two pieces of mucosa were incubated with no antibiotic, two pieces were incubated with gentamicin 100 μg/ml, and two pieces were incubated with gentamicin 1000 μg/ml. In a second experiment, two pieces were incubated without antibiotic and one piece was incubated with gentamicin 100 μg/ml. In the flasks in which there was no antibiotic, the oxygen consumption remained steady from 1 to 3 hours and then rose by 50-150% in the fourth hour. In the flasks to which gentamicin had been added, there was no rise in

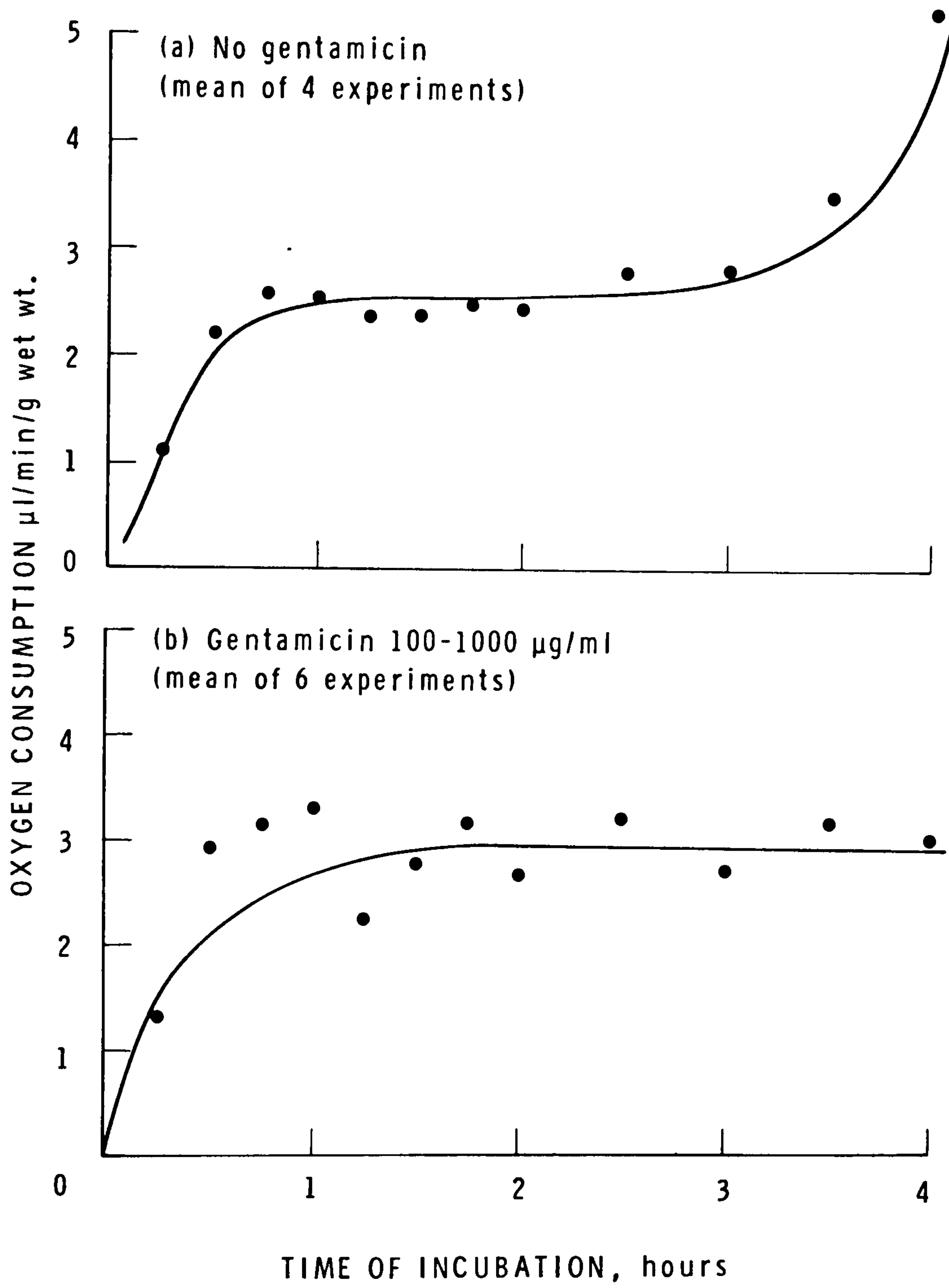


FIG. 24. Oxygen consumption by pieces of rectal mucosa incubated at 37° in Krebs III medium, (a) without added antibiotics (mean of 4 samples), and (b) with gentamicin 100-1000 µg/ml (mean of 6 samples).

oxygen consumption in the fourth hour (Fig. 24).

In the first experiment, culture of a flask without antibiotic gave a heavy growth of faecal streptococci and of Bacteroides; culture of a flask containing gentamicin 100 μg/ml gave a heavy growth of faecal streptococci but no Bacteroides. In the second experiment, there was a heavy growth of E. coli (more than 10^5 organisms per ml) from a flask with no antibiotic, whereas there was no growth of organisms after 48 hours incubation of a culture taken from a flask containing gentamicin.

These experiments were not performed in the same incubation medium as was used in the glucosamine synthetase assays but in view of the influence of bacterial metabolism on the oxygen consumption in the fourth hour of incubation, it was felt that a 3-hour incubation for glucosamine synthetase would introduce the possibility of bacterial contamination and that this should be prevented by the addition of gentamicin, provided that the antibiotic did not interfere with the assay.

Gentamicin, although an oligosaccharide, gave no colour reading in the Morgan-Elson reaction at concentrations of up to 10 mg/ml (10 000 μg/ml). Standard solutions of 0.05, 0.10 and 0.25 mM glucosamine were prepared, containing gentamicin in concentrations of 1000 and 2000 μg/ml. When these solutions were assayed by the Morgan-Elson reaction, there were no significant differences in O.D. from similar standards containing no gentamicin.

0.5 ml aliquots of an homogenate of colonic mucosa, 10 mg/ml, were incubated for 3 hours with 0.5 ml volumes of substrate-buffer mixture with final concentrations of tris-HCl 50 mM at pH 7.5, EDTA 1 mM, KCl 100 mM, fructose-6-phosphate 5 mM, and glutamine 2 mM. Two of the 1.0 ml volumes were incubated with no gentamicin, two were prepared containing gentamicin 5.9 μg/ml, two contained gentamicin 59 μg/ml, and two contained 590 μg/ml. A preparation of gentamicin sulphate was used containing 59% of gentamicin base. The experiment was repeated with a second sample of colonic mucosa from the same patient, with two samples without gentamicin and two samples containing 590 μg/ml. Gentamicin did not interfere with the estimation of glucosamine synthetase (Table 8).

The minimum inhibitory concentration for gentamicin against Streptococcus faecalis is 8 μg/ml (Garrod and O'Grady, 1971). It was therefore decided to use gentamicin 100 μg/ml in all 3-hour incubation in the glucosamine synthetase assay.

TABLE 8. Effect of gentamicin on glucosamine synthetase activity

Homogenate No.	Glucosamine synthetase activity (μmoles glucosamine synthesized/h/g wet wt at F-6-P 5mM, glutamine 2 mM, pH 7.5, 3 h incubation at 37°)			
	No gentamicin	Gentamicin added		
		5.9 μg/ml	59 μg/ml	590 μg/ml
1	11.1	10.9	11.2	12.7
	11.5	10.7	11.1	11.8
2	9.1	9.6	8.9	9.0
	8.1	11.4	9.3	9.1

The pH of the incubation

Winterburn and Phelps (1971a) found that the optimum pH for glucosamine synthetase prepared from rat liver was 7.5 (Fig. 25). Pogell in 1956 had found that the optimum pH was between 7.4 and 8.0 for the enzyme in rat liver. On the other hand, Ellis and Sommar (1972) found that the optimum pH for glucosamine synthetase from the bovine tracheal mucosa was on a broad range from 6.5 to 7.5.

To determine the optimum pH for glucosamine synthetase in human colonic mucosa, 11 homogenates were studied using colonic mucosa from 5 different patients. The mucosa was homgenized in a buffer in the centre of the pH range to be studied. Substrate-buffer mixtures were made up containing components of the buffer in proportions which gave the desired pH when 0.5 ml of the substrate-buffer mixture was added to 0.5 ml of the homogenate. For all the experiments, the tissue concentration in the homogenate was 10 mg/ml, the incubation time was 3 hours, and in the incubates the fructose-6-phosphate concentration was 5 mM, glutamine was 2 mM and gentamicin was included at 100 μg/ml.

Tris-HCl 50 mM was the buffer used by Winterburn and Phelps. This was only suitable for studying the pH between 7.2 and 7.8. When it became apparent that the optimum pH might lie below 7.2, it was necessary to use a buffer which was suitable for a lower pH range. Sodium-phosphate buffer, Na_2HPO_4 / NaH_2PO_4, was used, at 50 mM, to study the pH range 6.4-7.2.

Of the 11 homogenates, 4 were homogenized in tris-HCl buffer at pH 7.5 and incubated at pH values of 7.2, 7.5 and 7.8; 2 were homogenized in

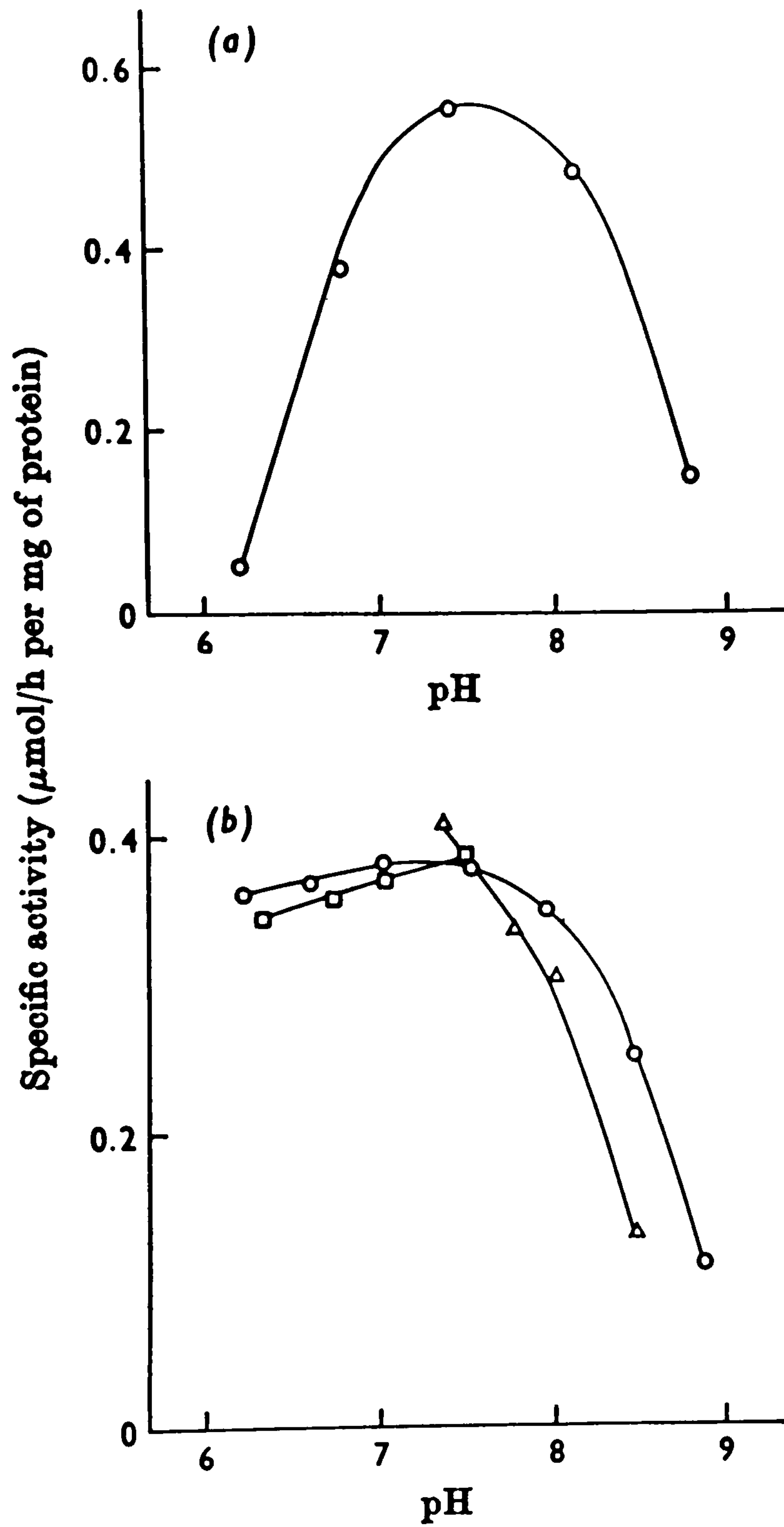


Fig. 25. The pH dependence of rat liver glucosamine synthetase, according to Winterburn and Phelps (1971a): (a) the purified enzyme, (b) the enzyme purified in the presence of 1 mM fructose-6-phosphate.

tris-HCl at pH 7.5 and incubated at 7.2 and 7.5 only; one was homogenized in sodium-phosphate buffer at pH 7.5 and incubated at 6.8, 7.0, 7.2 and 7.5; and 4 were homogenized in sodium-phosphate buffer at pH 6.8 and incubated at 6.4, 6.6, 6.8, 7.0 and 7.2. Two 0.5 ml aliquots of each homogenate were incubated with 0.5 ml volumes of the appropriate substrate-buffer mixture at each pH.

For each homogenate, the mean of the two readings for the glucosamine synthetase activity at pH 7.2 was taken as 100 arbitrary units and the readings at other pH values were expressed in these arbitrary units. The totals of the values so expressed for all the homogenates at each pH tested were added together and their mean values were calculated, giving a mean percentage at each pH compared to the activity at pH 7.2 (Fig. 26).

The pH optimum was shown to lie on a flat part of the curve between 6.5 and 7.5. For subsequent experiments and for the biopsy assay, a pH of 7.0 was chosen, using Na_2HPO_4/NaH_2PO_4 buffer, 50 mM.

This pattern of pH dependence corresponds to that found by Ellis and Sommar (1972) in bovine tracheal mucosa, rather than to the curve for rat liver glucosamine synthetase (Fig. 25).

The effect of the nature of the buffer

Changing the buffer from tris-HCl to Na_2HPO_4/NaH_2PO_4 resulted in a change of ionic strength. Using the formula

$$I = \tfrac{1}{2}\Sigma\ mz^2,$$

in which I is the ionic strength, m the molar concentration of each ion, and z the valency of the ion, the ionic strength of the incubate was 0.15 when tris-HCl was used at pH 7.5. 50 mM sodium-phosphate buffer at the same pH gave an ionic strength of the homogenizing buffer of 0.21.

Homogenates of colonic mucosa incubated at pH 7.2 with tris-HCl buffer were compared with homogenates of adjacent mucosa incubated at the same pH with sodium-phosphate buffer. One sample of colonic mucosa was homogenized in tris-HCl buffer at pH 7.5, in a concentration of 40 mg tissue per ml. One portion of this homogenate was diluted in the same buffer to 10 mg/ml and then incubated with substrates at pH 7.5 in tris-HCl buffer. Another portion of the same concentrated homogenate was diluted to 10 mg/ml with sodium-phosphate

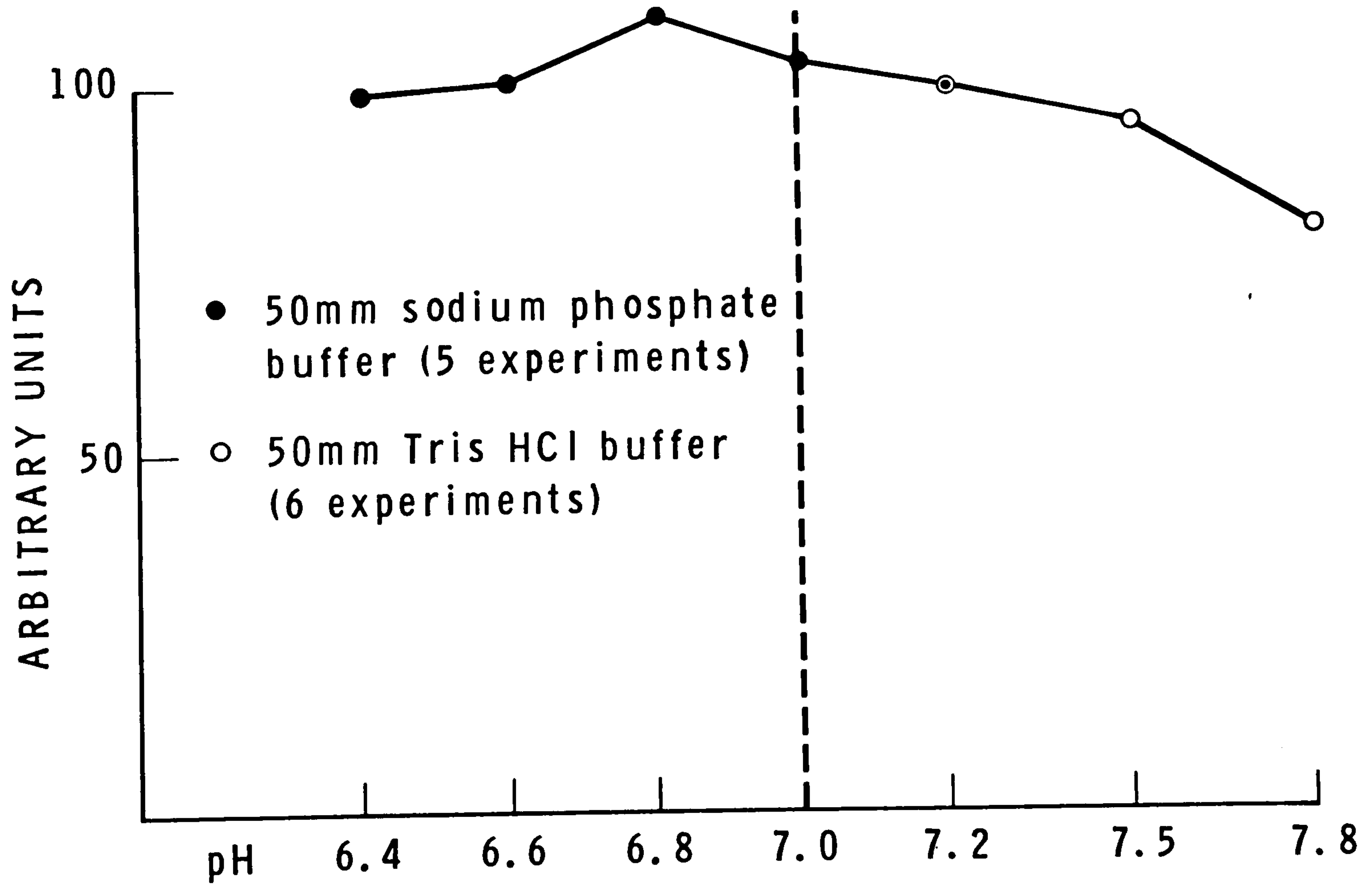


FIG. 26. Glucosamine synthetase activity in normal human colonic mucosa in relation to pH. Otherwise standard conditions. Arbitrary units: 100 = glucosamine synthesis at pH 7.2.

buffer at pH 7.5, so that this incubate contained $\frac{7}{8}$ sodium-phosphate buffer and $\frac{1}{8}$ tris-HCl, at pH 7.5. The results are compared in Table 9.

TABLE 9. Glucosamine synthesized by adjacent pieces of 3 colectomy specimens homogenized and incubated with fructose-6-phosphate and glutamine in different buffers at the same pH (μmoles glucosamine synthesized/h/g wet wt). Fructose-6-phosphate 5 mM, glutamine 2 mM, 50 mM buffer, 3 h incubation at 37°. Each result is a mean of 2 readings

Colectomy specimen No.	pH	Glucosamine synthetase activity		
		Tris-HCl buffer	Na-PO_4 buffer	$\frac{7}{8}$ $NaPO_4$ + $\frac{1}{8}$ tris-HCl
1	7.5	3.1	6.4	2.5
	7.2	3.2	8.5	
2	7.2	6.9	7.9	
			5.2	
3	7.2	9.4	12.4	
			8.0	

There appeared to be differences between tris-HCl and sodium-phosphate buffers when used at 50 mM at the same pH but the differences were not consistent.

The ionic strength

The effects on glucosamine synthetase activity of variations in the ionic strength were studied by varying the KCl concentration.

An homogenate of colonic mucosa, 10 mg/ml, was prepared in 50 mM sodium-phosphate buffer at pH 7.0, containing EDTA 1 mM and KCl 40 mM, which was the concentration of KCl calculated to keep the ionic strength at 0.15, as it was with 100 mM KCl in tris-HCl 50 mM buffer at pH 7.5. Two 0.5 ml aliquots of the homogenate were incubated with 0.5 ml volumes of each of four substrate-buffer mixtures, of composition such that the KCl concentrations in the 1.0 ml incubates were 40, 100 and 150 mM respectively. The substrate concentrations were fructose-6-phosphate 5 mM and glutamine 2 mM and gentamicin was added at 100 μg/ml., in a 3-hour incubation. The KCl concentration did not affect the glucosamine synthetase activity (Table 10).

TABLE 10. Effect of KCl concentration on glucosamine synthetase activity. (a) Results from a single homogenate, each a mean of 2 readings. (b) Homogenate of an adjacent piece of the same mucosa. (μMoles glucosamine synthesized/h/g wet wt at fructose-6-phosphate 5 mM, glutamine 2 mM, 50 mM sodium phosphate buffer at pH 7.0, EDTA 1 mM, incubated for 3 h at 37°.)

Homogenate	KCl mM	Ionic strength	Glucosamine synthetase activity
(a)	40	0.15	16.0
	100	0.21	15.9
	150	0.26	16.2
(b)	0	0.11	13.7

An adjacent piece of mucosa was homogenized in a buffer which was similar but contained no KCl and was incubated under similar conditions but, again, with no KCl. The glucosamine synthetase activity in the absence of KCl was slightly less than the activity measured in the homogenate of the adjacent mucosa in the presence of KCl but it was within the expected sample variation (see Table 12).

Thus the requirement for KCl can be disputed and glucosamine synthetase does not seem to be greatly influenced by the ionic strength of the incubation medium, up to 0.26. To maintain a physiological concentration of cations of 152 mEq and to conform with other workers, it was decided to use 100 mM KCl in the buffer and in the incubate in subsequent work.

(The effects on glucosamine synthetase activity of varying the ionic strength with sodium chloride are discussed in Appendix B, Fig. 108.)

The substrate concentrations

Glucosamine synthetase has been estimated using fructose-6-phosphate concentrations of between 5 mM (Winterburn and Phelps, 1971a) and 20 mM (Danishefsky and Deutsch, 1968) and glutamine concentrations of between 2 mM (Ellis and Sommar, 1972) and 20 mM (Danishefsky and Deutsch, 1968). The concentrations most commonly used have been 10 mM for fructose-6-phosphate and 12 mM for glutamine.

Ellis and Sommar (1971) showed that glutamine in concentrations of greater than 5 mM was inhibitory to glucosamine synthetase in bovine tracheal mucosa but glutamine was not inhibitory in rat liver even at 20 mM.

The influence of the substrate concentrations on glucosamine synthetase activity in the human colon was investigated as follows.

Four homogenates of normal human colonic mucosa were prepared, 10 mg tissue per ml homogenizing buffer. Two of the homogenates were from adjacent pieces of mucosa from the same patient and the other two homogenates were from two other patients. 0.5 ml aliquots were incubated with 0.5 ml of various substrate-buffer mixtures made up to give final substrate concentrations of 5, 10, 20, 40 and 80 mM fructose-6-phosphate and 2, 4, 8, 16 and 32 mM glutamine, with duplicates for each pair of substrate concentrations. All the incubations were for 3 hours in sodium-phosphate buffer 50 mM at pH 7.0 and with gentamicin 100 μg/ml. With one of the homogenates, the volumes used were 0.2 ml homogenate with 0.2 ml of substrate-buffer mixture (see below for the 4 mg assay).

Not all the homogenates were incubated at each pair of substrate concentrations in the matrix but all of them were incubated at fructose-6-phosphate 20 mM and glutamine 4 mM and the mean of the duplicated readings of glucosamine synthesis at these concentrations was taken as 100 arbitrary units. The mean values of the readings at the other pairs of substrate concentrations were expressed in the same arbitrary units (Table 11). The greatest yield of glucosamine was at fructose-6-phosphate 20 mM and glutamine 8 mM, with a mean of 108 arbitrary units for the readings from the 4 homogenates. Higher concentrations of either substrate were found to be inhibitory (Fig. 27).

The incubations of surgical and biopsy material in the clinical studies were therefore carried out at fructose-6-phosphate 20 mM and glutamine 8 mM.

<u>The improved yield of glucosamine achieved as a result of these studies</u>

Compared to the method of Winterburn and Phelps (1971a) which was used initially, these studies increased the yield of glucosamine synthesis by an homogenate of a given concentration by the following estimated amounts:—

(a) Duration of the incubation. The 3-hour incubation gave a 70% greater yield of glucosamine than the 90 min incubation of Winterburn and Phelps.

(b) pH. Incubation at pH 7.0 gave an 8% greater yield than incubation at pH 7.5.

(c) Substrate concentrations. The use of fructose-6-phosphate 20 mM and glutamine 8 mM gave a 70% greater yield of glucosamine than fructose-6-phosphate 5 mM and glutamine 2 mM which was used by Winterburn and Phelps.

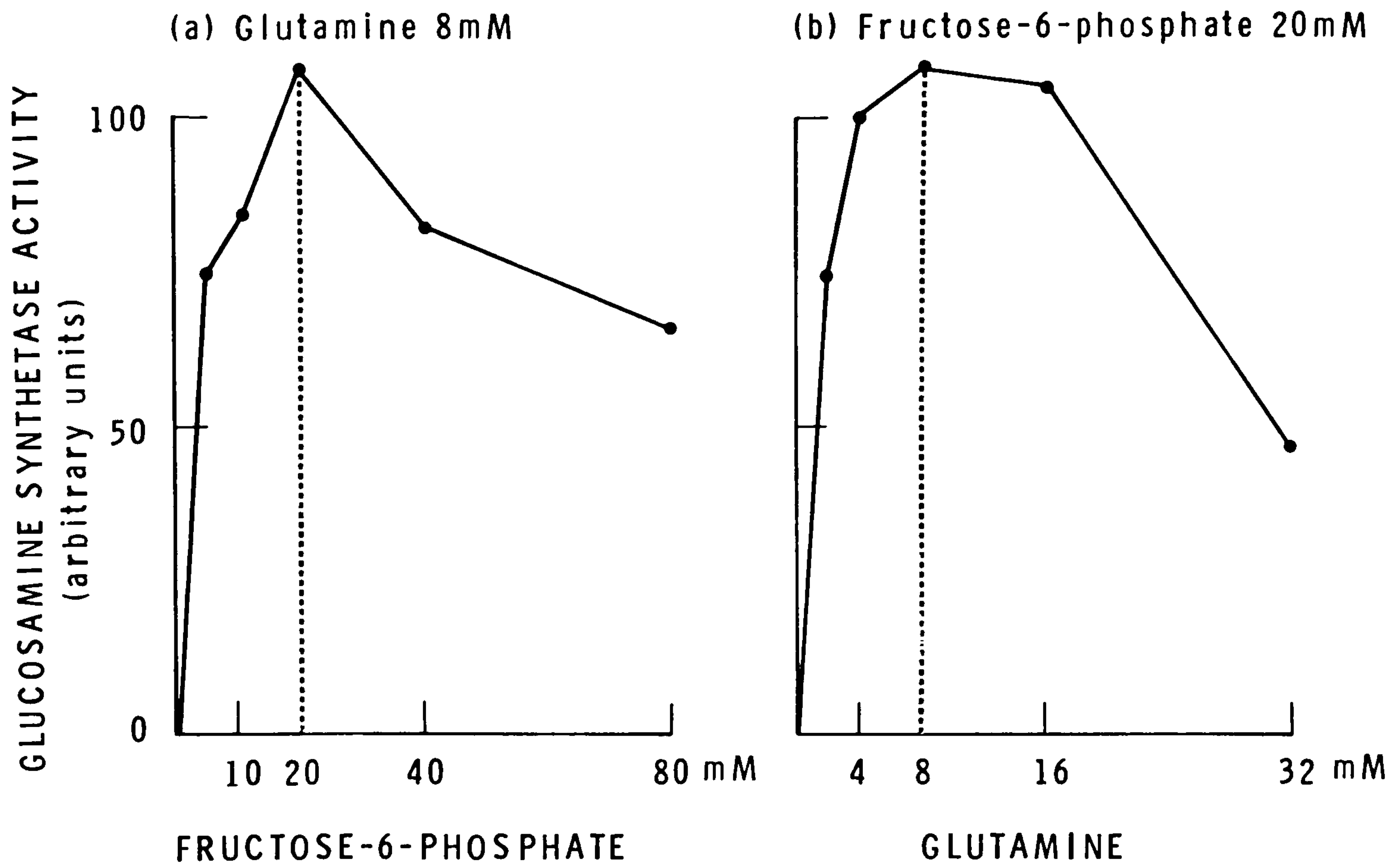


FIG. 27. Glucosamine synthetase activity in relation to substrate concentration.
(a) Fructose-6-phosphate concentration variable, glutamine 8 mM. (b) Glutamine concentration variable, fructose-6-phosphate 20 mM. Arbitrary units: 100 = activity at F-6-P 20 mM, glutamine 4 mM. Data from Table 11.

TABLE 11. Glucosamine synthetase activity in the presence of varying concentrations of substrates. Four homogenates incubated at pH 7.0 for 3 h at 37° at several different substrate concentrations. Two readings at each pair of substrate concentrations. (Mean activity in arbitrary units: 100 = activity at fructose-6-phosphate 20 mM, glutamine 4 mM.)

	Fructose-6-phosphate (mM)									
	5		10		20		40		80	
Glutamine (mM)	Mean activity	No. of expts	Mean activity	No. of expts	Mean activity	No. of expts	Mean activity	No. of expts	Mean activity	No. of expts
2	64	(1)	76	(1)	75	(1)				
4	73	(1)	87	(3)	100	(4)	88	(2)	62	(1)
8	75	(1)	84	(3)	108	(4)	83	(2)	66	(1)
16			87	(2)	105	(2)	76	(2)	60	(1)
32			56	(1)	47	(1)	55	(1)	53	(1)

Under these conditions, the yield of glucosamine was increased by a factor of 3, enabling one-third of the weight of tissue to be used.

In addition, the use of the uncentrifuged homogenate rather than the supernatant allowed a smaller volume of homogenate to be used, equivalent to further miniaturization by about 20%.

This enabled the tissue concentration in the homogenate to be reduced to 10 mg/ml. In order to take duplicate readings using 0.5 ml volumes of the homogenate, the amount of tissue required for the assay was thus 10 mg plus a small amount for wastage in the homogenizer.

THE ASSAY OF GLUCOSAMINE SYNTHETASE UNDER OPTICAL CONDITIONS

Duplicate readings

In the studies of the variables of the glucosamine synthetase reaction, all readings of a single variable including duplicates were made with the same batch of standards and dilute DAB. The object was to make accurate comparisons rather than to obtain accurate absolute values for glucosamine synthetase.

In the clinical studies of glucosamine synthetase activity, with the standard conditions of the incubation now established, it was considered that duplicate readings were best assayed in separate batches using dilute DAB made up freshly for each batch.

As the standard deviation of six separately prepared standard solutions of 0.20 mM glucosamine was only 2% (see above), it was decided for convenience not to make up separate standards for each duplicate batch if the batches were read on the same day or on consecutive days. If the second batch was read 2 or more days after the first batch, then fresh standards were made up.

The calculation of glucosamine synthetase activity under these standard conditions

The glucosamine synthetase activity, x, in μmoles glucosamine synthesized per h per g wet wt, is expressed by the formula

$$x = \frac{2000\,y}{ct} ,$$

in which y is the concentration in mM of glucosamine in the incubate at the end of the incubation, c is the concentration in mg/ml of the homogenate, and t is the duration of the incubation in hours. Comparison with acid-and-alkali treated standards gives a value A, equal to 100 y, which is calculated from B and p, B being determined by reference to the standard curve for B against D (D is 1000 times the O.D. in the Morgan-Elson reaction) and p being the volume of M KOH, in ml, used in neutralizing the perchloric acid:

$$A = B\,(1 + 1.18\,p) .$$

Since

$$A = 100\,y ,$$

then

$$x = \frac{20\,A}{ct} .$$

With the standard conditions adopted, c = 10 mg/ml and t = 3 hours, therefore,

$$x = \frac{20\,A}{30} = \frac{2}{3}A .$$

Taking the two duplicate readings of A as A_1 and A_2,

$$x = \frac{A_1 + A_2}{3} .$$

The linearity of the assay

It was important to confirm that the assay gave readings for glucosamine synthetase activity that bore a linear relationship to the amount of glucosamine synthetase in the tissue.

Two homogenates of normal colon were made up with 20 mg tissue per ml homogenate. Each was serially diluted to make homogenates containing 10, 5, 2.5, 1.25 and 0.625 mg tissue per ml. Two 0.5 ml aliquots of each dilution of each homogenate were assayed for glucosamine synthetase by the standard method that had been devised. One of the duplicate pairs of each dilution was taken and these were assayed in one batch with dilute DAB; the other duplicates were assayed in a second batch.

The relationship between the concentration of tissue in the homogenate and the glucosamine synthetase activity of the homogenate was linear for both homogenates, r = 0.9994 and 0.9990 respectively (Fig. 28). This confirmed that the method gave readings for glucosamine synthetase activity that were directly proportional to the amount of the enzyme in a diluted homogenate.

Other tissue factors which might interfere with the assay were also diluted out in these experiments but this drawback was not considered sufficient to invalidate the observed linearity of the relationship between the glucosamine synthetase concentration in a tissue and its activity as measured by this assay.

The assay of unincubated homogenate

In the study of the duration of the incubation, readings of glucosamine synthetase activity at zero time were made for the three homogenates of normal colonic mucosa containing 10 mg tissue per ml. The 1.0 ml volumes contained sodium-phosphate buffer 50 mM at pH 7.0, fructose-6-phosphate 5 mM or 10 mM, glutamine 2 mM or 4 mM, and gentamicin 100 μg/ml. The readings of O.D. in the Morgan-Elson reaction had a mean of 0.009 (range 0.007-0.014 for the six readings) and gave readings equivalent to a glucosamine synthetase activity of 0.5-1.0 μmole glucosamine synthesized/h/g wet wt in the 3-hour incubation used in this work. This indicated that control readings of glucosamine concentration in unincubated homogenates were unnecessary, at least in normal tissue.

A few control readings of glucosamine in homogenates of pathological tissue were made and they are reported in Chapter 9.

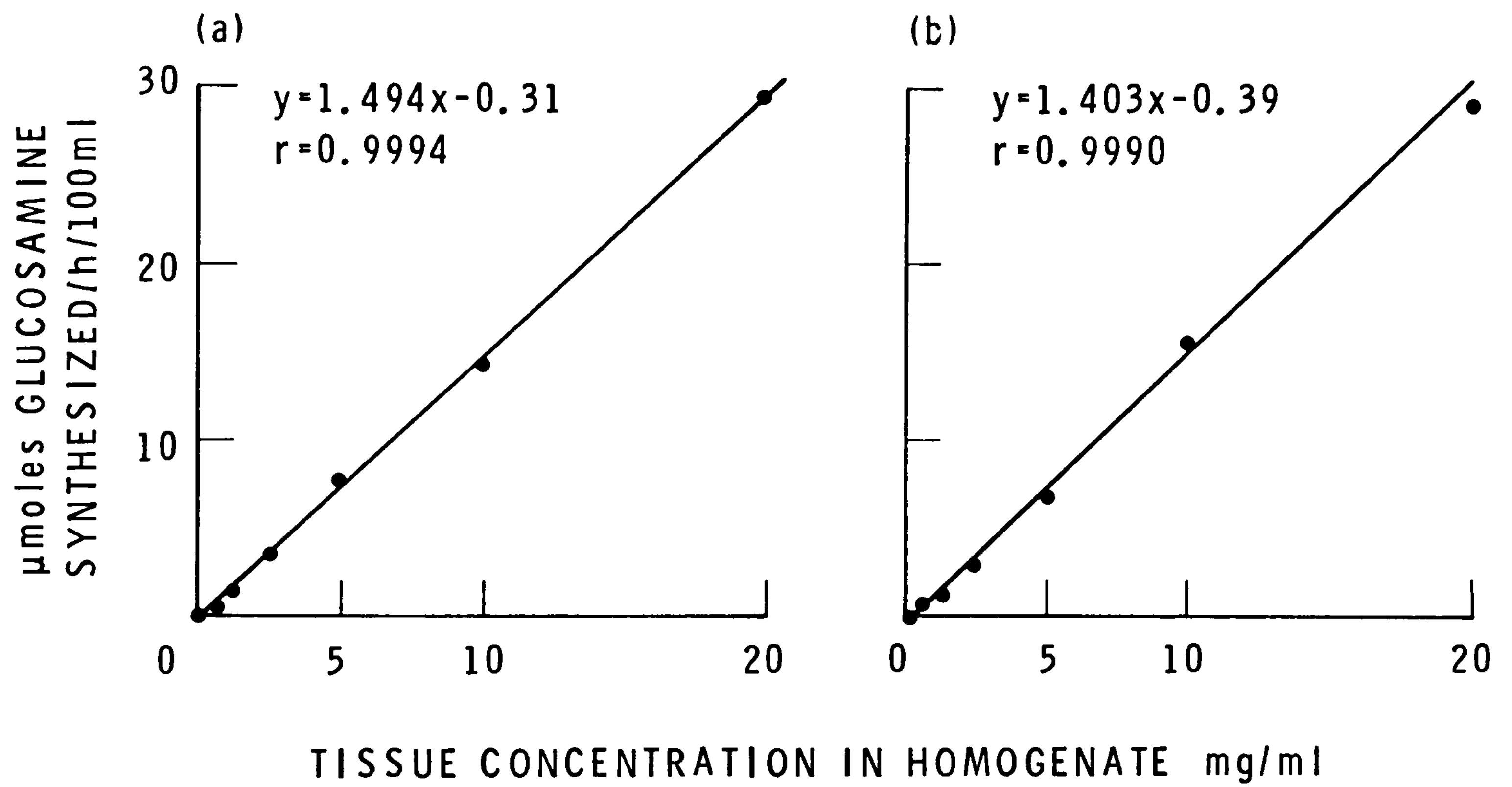


FIG. 28. Glucosamine synthetase activity as measured by the standard 10 mg assay versus tissue concentration of the homogenate, as diluted from two homogenates of normal colonic mucosa made at 20 mg/ml.

FURTHER MINIATURIZATION

The requirement for at least 10 mg tissue meant that some rectal biopsies would be too small to provide enough tissue for the assay and that the same biopsy specimen could not be used for the glucosamine synthetase estimation and for diagnostic histology.

By scaling down the method by a factor of 0.4, it was possible to perform duplicate readings with just over 4 mg of tissue.

The tissue was homogenized in buffer, to make 0.1 ml homogenate per mg tissue. 0.2 ml of the homogenate was incubated with 0.2 ml of the substrate-buffer mixture. The reaction was terminated by the addition of 0.02 ml perchloric acid. The protein was precipitated by centrifugation and 0.3 ml of the supernatant was pipetted into another test-tube. To this was added 0.02 ml 8M KOH (9M KOH often resulted in the solution being too alkaline) and then M KOH to neutrality. $KClO_4$ was precipitated by centrifugation and 0.2 ml of the supernatant was pipetted into a screw-cap tube for the assay of glucosamine.

To 0.2 ml of this solution was added 0.2 ml borate buffer and then 0.05 ml acetic anhydride in acetone. After boiling and cooling, 1.6 ml dilute DAB was added and the tubes were incubated at 37° for 20 minutes. The total volume was 2.05 ml, instead of 5.0 ml when 0.5 ml volumes of glucosamine solution were used. The colour was read using the same glass cuvettes of 1 cm light path but the slit of the spectrophotometer was used at half the usual height and was focussed onto the photocell using the lens in the instrument.

Standards were 0.5 ml volumes of glucosamine solutions. They were treated with 0.025 ml perchloric acid, then 0.035 ml 8M KOH and then M KOH to neutrality. $KClO_4$ was precipitated and 0.2 ml of the supernatant was used in the Morgan-Elson reaction.

This assay of glucosamine synthetase using 0.2 ml volumes was called the "4 mg assay" because it required 4 mg tissue for duplicate readings. The assay using 0.5 ml volumes was called the "10 mg assay".

The calculation of glucosamine synthetase activity with the 4 mg assay

The plot of B against D was made in the same way as with the 10 mg assay but adjustments had to be made to the volumes p and q, the volumes of M KOH used to neutralize the perchloric acid in the test solutions and in

the standard solutions respectively. These were designated p' for the homogenates and q' for the standards.

In the case of the homogenates, the concentration of glucosamine in the solutions assayed in the Morgan-Elson reaction, M, was related to the glucosamine concentration in the incubate, y, as follows.

Following the addition of perchloric acid, the glucosamine concentration became $\frac{0.4\,y}{0.42}$. To 0.3 ml of this was added 0.02 ml 8M KOH plus p' ml M KOH, giving a solution of glucosamine concentration $\frac{0.3}{(0.32 + p')} \cdot \frac{0.4\,y}{0.42}$, which was M. Therefore,

$$M = \frac{0.12\,y}{0.42\,(0.32 + p')}$$

$$= \frac{0.12\,y}{0.42 \cdot 0.32\,(1 + 3.1\,p')}$$

$$= \frac{y}{1.12\,(1 + 3.1\,p')}.$$

For the standards, to 0.5 ml of standard of concentration y_s were added 0.025 ml $HClO_4$, 0.035 ml 8M KOH, and q' ml M KOH. Therefore,

$$M_s = \frac{0.5\,y_s}{0.56 + q'}$$

$$= \frac{y_s}{1.12\,(1 + 1.8\,q')}.$$

Thus A and B were related by the formula

$$A = B\,(1 + 3.1\,p')$$

and A_s and B_s were related by the formula

$$A_s = B_s\,(1 + 1.8\,q').$$

Since $A = 100\,y$ and $A_s = 100\,y_s$, and since B_s could be calculated from y_s for the known standards, A was derived from reading D on the standard curve of B_s against D_s, and the glucosamine synthetase activity, x, was calculated by the formula

$$x = \frac{2}{3}A,$$

or, for two duplicates of A which were A_1 and A_2,

$$x = \frac{A_1 + A_2}{3}.$$

The use of smaller volumes introduced a greater error. A typical standard curve is shown in Fig. 29 and it is not as linear as a standard curve for the 10 mg assay (Fig. 21). The errors in the standard curves are discussed in Appendix A.

Pipettes

Eppendorf pipettes rather than glass pipettes were used most of the time. Eppendorf pipettes of 100 μl and 500 μl were shown to be more reproducible in repeated weighed pipettings than were glass pipettes. This was when the same Eppendorf pipette was used for the repeated readings. This was a valid comparison because the same 50 μl, 100 μl, 200 μl, 500 μl and 1000 μl Eppendorf pipettes were used for most of the glucosamine synthetase assays. For 0.3 ml and 0.8 ml volumes, a Finpipette was used with an Eppendorf tip. Perchloric acid, KOH and DAB were pipetted in glass pipettes.

New Eppendorf tips were used for each solution pipetted. Used and washed tips were found to be rather inaccurate (Appendix A).

STORAGE

The storage of incubates

All specimens of tissue were homogenized and the incubation with substrates commenced within 2 hours of removal from the body (see below for discussion on the storage of tissue). It was convenient to store the incubated solutions, after the addition of perchloric acid, in order to collect 19 samples to assay for glucosamine in a single batch with one set of standards.

Initial experiments showed that there was no marked loss of glucosamine activity in incubated solutions stored at -20°. The stability of the glucosamine activity was investigated as follows.

Two homogenates of normal colonic mucosa were used. From each homogenate, 20 samples were incubated using the 10 mg method and 20 samples were incubated using the 4 mg method. All the samples from each homogenate were incubated at the same time. After the addition of perchloric acid at the end of the incubation, all the samples were placed in a deep freeze at -20°.

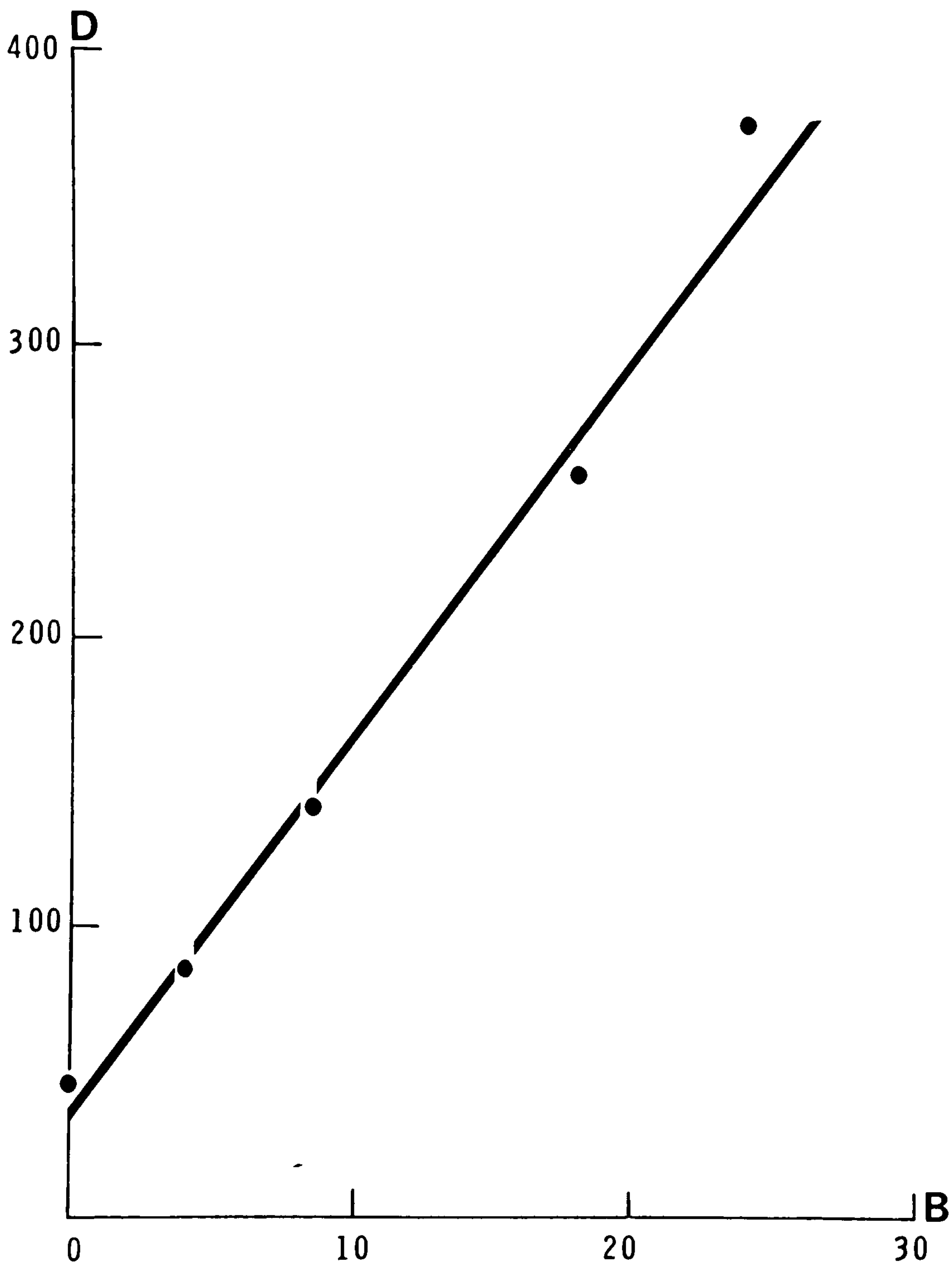


FIG. 29. Typical standard curve for B against D, using acid-and-alkali treated standards, in the 4 mg assay for glucosamine synthetase.

Pairs of samples were assayed for glucosamine at various times over the ensuing 6 months. There was a considerable scatter of results (Fig. 30).

With each homogenate, and with each of the two methods, the means of the duplicated results of the last 5 pairs of samples to be assayed were considerably less than the means of the first 5 pairs of samples. This showed that there was some loss of glucosamine activity with storage at -20°.

The decay factor

Although glucosamine activity did decay on storage, it was still convenient to be able to store the incubated samples for assay in batches. The decay was analysed in greater detail.

The two methods with the two homogenates gave four sets of 10 readings of glucosamine synthetase activity. The 10 readings were means of duplicates, read in separate batches within 24 hours of each other. Each group of 10 readings was arranged in chronological order of the assay and the readings were numbered from 1 to 10. Means were taken of the readings 1-4, 3-6, 5-8 and 7-10, and the mean date of assay of each of these groups of four readings was also calculated. For each of the four sets of 10 readings, the mean date of readings 1-4 was taken as day 0 and the mean glucosamine synthetase value of these readings was taken as 100 arbitrary units. The mean dates of the other groups of four readings were expressed in days from the mean date of readings 1-4 and their mean glucosamine synthetase values were expressed as percentages of the mean of readings 1-4.

It was assumed that the decay of glucosamine activity was logarithmic. For each of the groups of readings 3-6, 5-8 and 7-10 from each of the four sets of readings, the loss of activity was expressed logarithmically and an average loss per day was calculated. For the 12 groups of four readings, the mean loss of activity was 0.00169 in $\log_{10}$ units per day, equivalent to a loss of 0.39% per day or 32% in 100 days (Fig. 31).

Correction factors could be applied when the incubate containing synthesized glucosamine was stored at -20° prior to the assay of the glucosamine. The correction factor was $10^{0.00169}$ per day of storage. At 30 days, this was 1.12. It was considered that correction factors of between 1.00 and 1.12 would not contribute any great error to the glucosamine synthetase estimation. It was therefore considered justifiable to store the incubates at -20° for up to 30 days

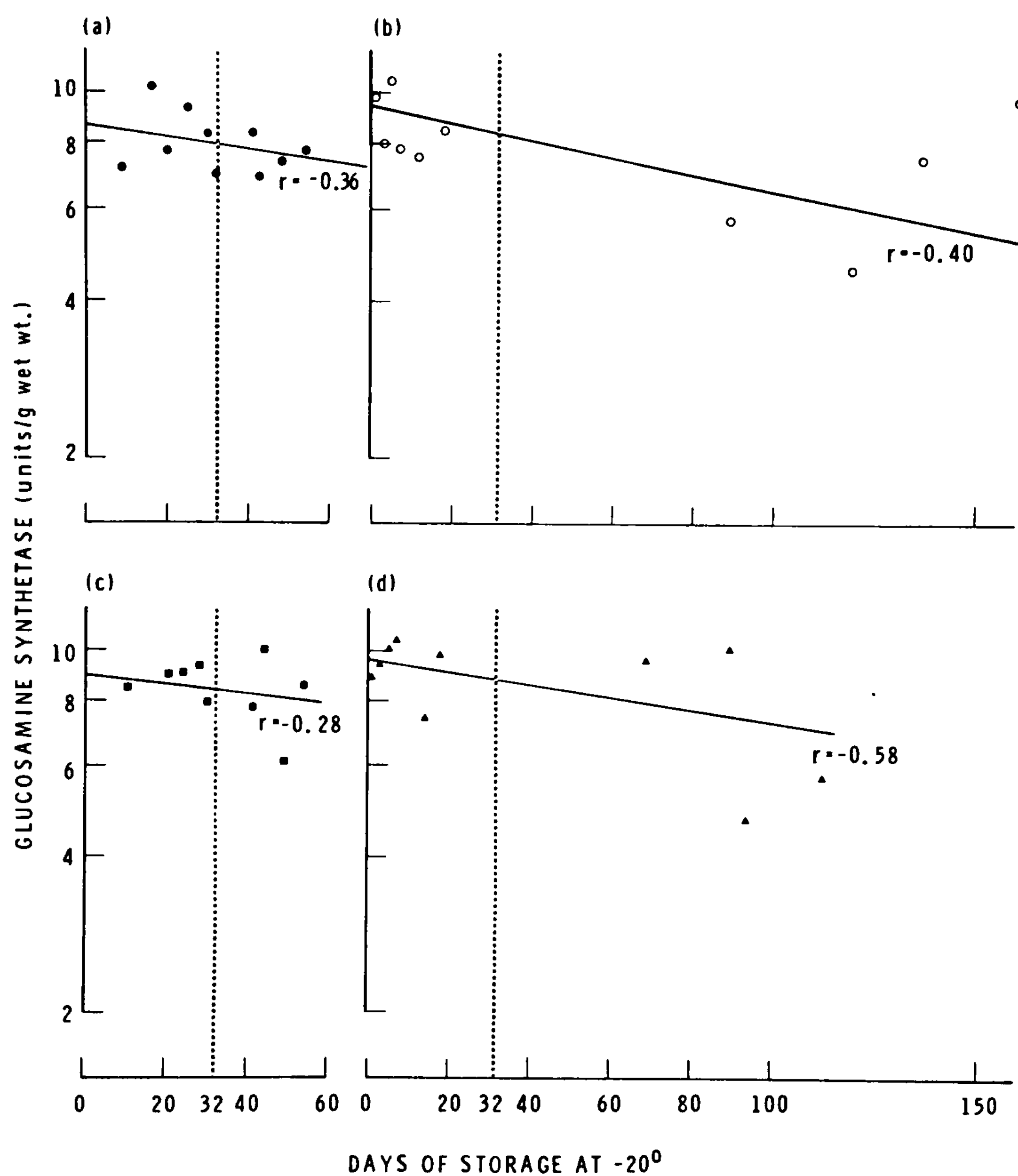


FIG. 30. Decay of glucosamine activity in incubates from the glucosamine synthetase reaction in colonic mucosa, stored at - 20°. (a), (b) Two homogenates assayed by the 10 mg method. (c), (d) The same two homogenates assayed by the 4 mg method. Each point is a mean of 2 readings. All readings from each homogenate were assayed with a different batch of dilute DAB.

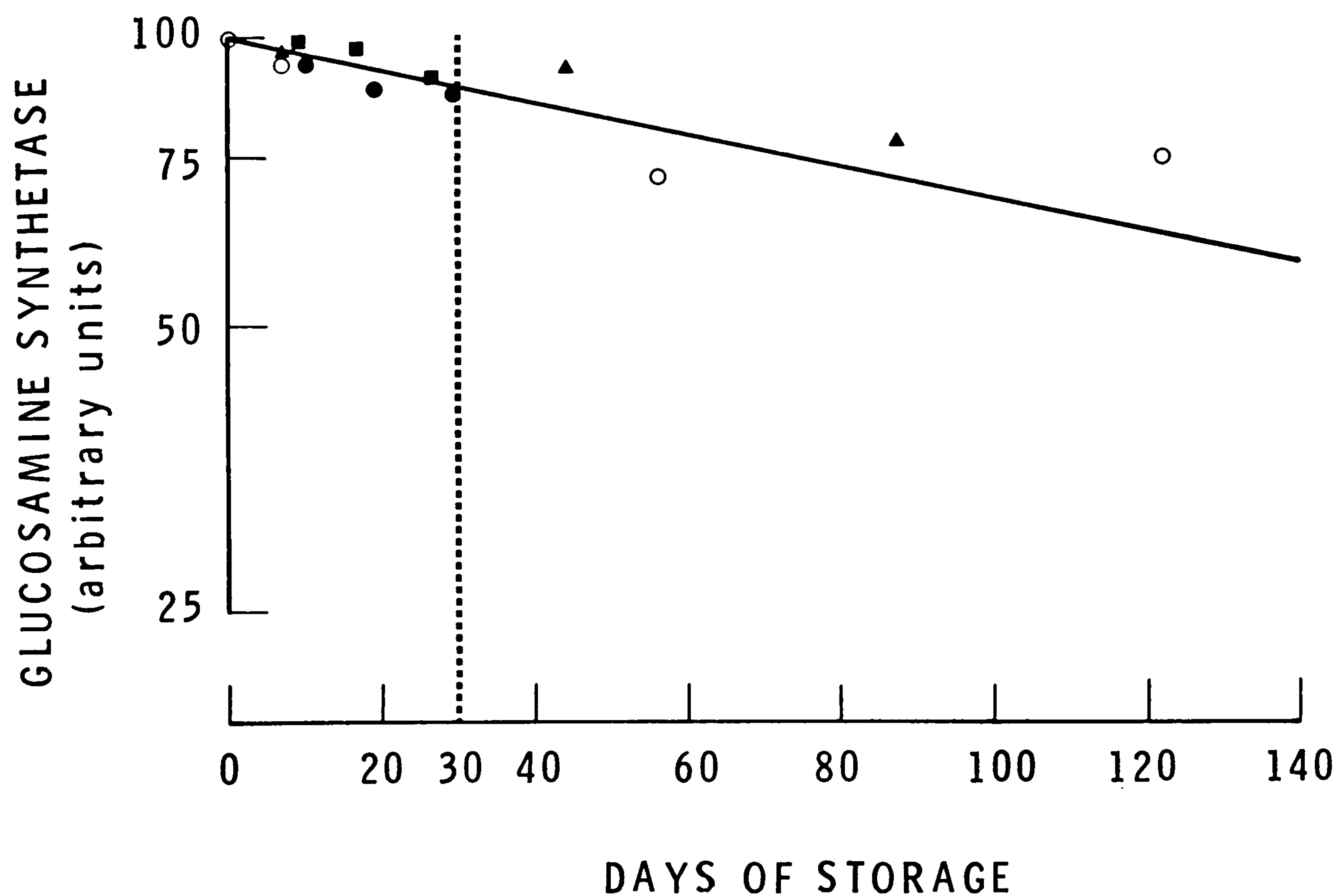


FIG. 31. The decay of activity of glucosamine in incubates from the glucosamine synthetase reaction. Derivation of the decay factor. Each point represents a mean of 4 consecutive assays from Fig. 30. Assays taken in each case: 1-4, 3-6, 5-8, 7-10. Mean of assays 1-4 taken as day 0, 100 arbitrary units.

for reading in batches, and the correction factor for glucosamine decay was applied to each result.

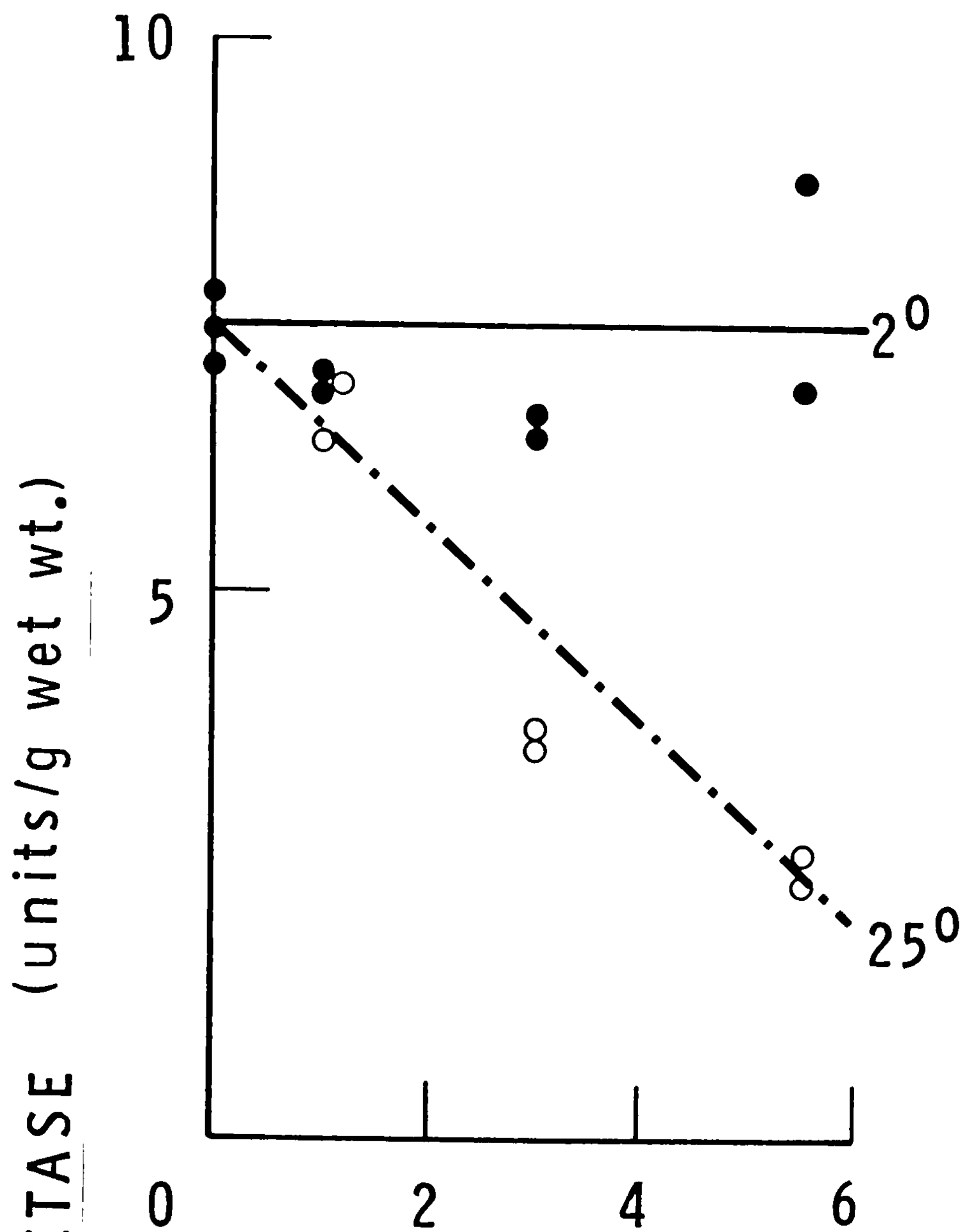
The decay was of glucosamine-6-phosphate as assayed by the Morgan-Elson reaction. The cause of the decay was not investigated. The incubated solutions contained substrates and other non-protein components of the tissue homogenate and the precipitated protein was not removed prior to storage. In particular, the glucosamine-6-phosphate synthesized by glucosamine synthetase was stored in 0.34M (3.4%) perchloric acid, which might have been able to oxidize glucosamine-6-phosphate and perhaps also glucosamine hydrochloride. Experiments to test this, by storing solutions of glucosamine-6-phosphate and glucosamine hydrochloride with and without perchloric acid, were not performed.

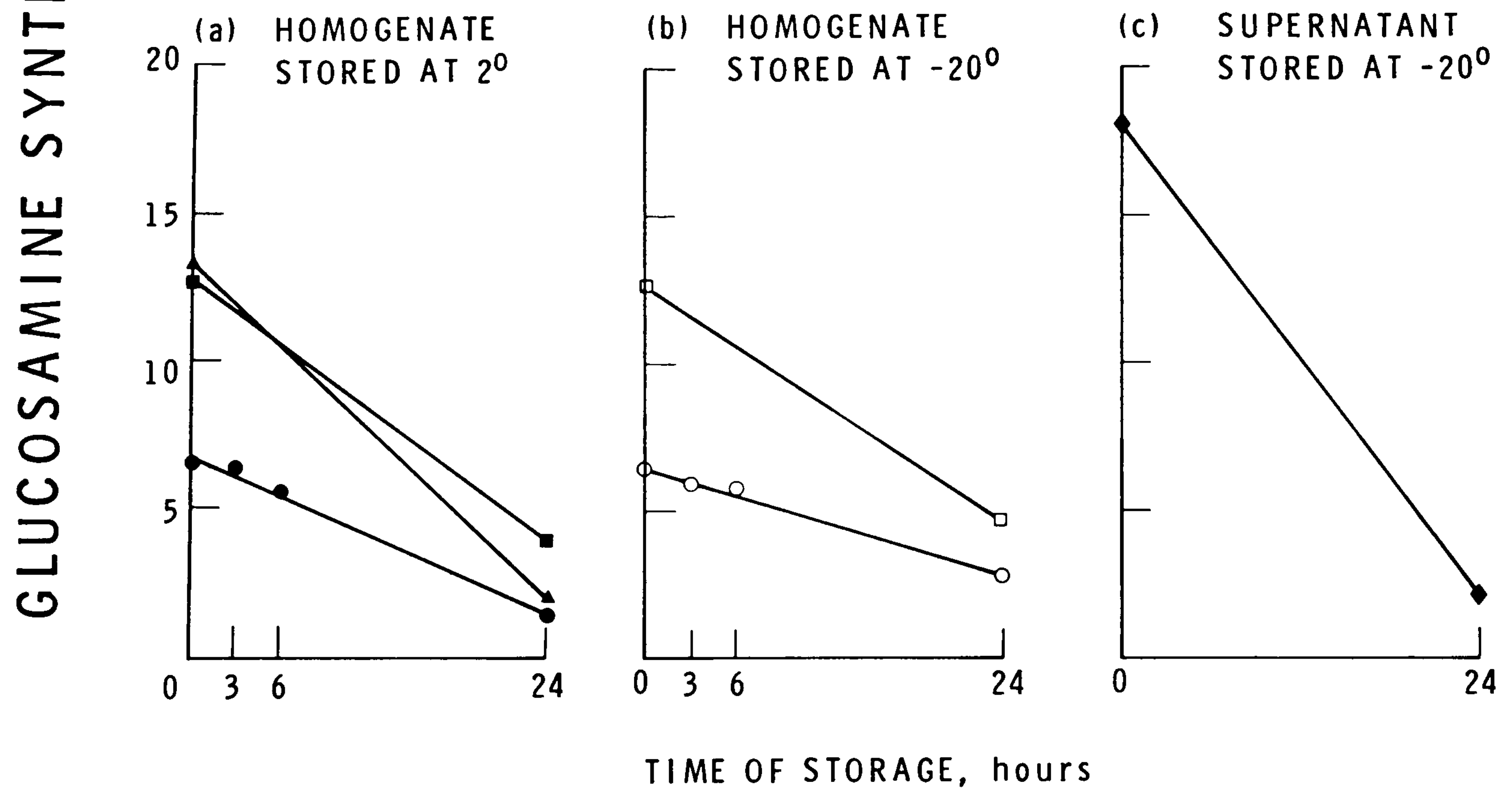
The storage of the homogenate

Winterburn and Phelps (1971a) found that the 50% inactivation time of purified preparations of glucosamine synthetase from rat liver was 15-20 hours at 4°, with no appreciable improvement in stability with storage at -15°. The presence of fructose-6-phosphate increased the 50% inactivation time to 8 days, which was probably the reason why Winterburn and Phelps found that in the 100 000 g supernatant of the homogenate, before further purification, there was only a slight loss of activity in the first 100 hours.

One homogenate of normal colonic mucosa was prepared using sufficient tissue for several estimations of the glucosamine synthetase activity. Two 0.5 ml aliquots were incubated with 0.5 ml volumes of substrate-buffer mixture with the incubation commencing immediately after homogenization, which was within 1 hour of removal of the tissue from the patient. One portion of the homogenate was kept in ice at 2° and another portion on the bench on a warm day at 25°. Two 0.5 ml volumes of each portion of the homogenate were incubated starting at 1, 3 and $5\frac{1}{2}$ hours after homogenization. There was no significant loss of glucosamine synthetase activity in the homogenate kept at 2° but in the homogenate kept at 25° there was a loss of activity of 65% when the incubation was delayed by $5\frac{1}{2}$ hours (Fig. 32).

The experiment was repeated with another homogenate, keeping one portion in ice at 2° and another portion in the deep freeze at -20°. The incubations were commenced immediately after homogenization and at 3, 6 and 24 hours after homogenization. Two more homogenates were studied similarly, with





FIGS. 32, 33. Decay of glucosamine synthetase activity in homogenized colonic mucosa.
FIG. 32.(above): Storage of an homogenate at 2° and 25° for up to 6 hours.
FIG. 33 (below): (a), (b) Storage of homogenates at 2° and -20°; (c) storage at -20° of a supernatant of a centrifuged homogenate.

incubations commencing immediately after homogenization and after 24 hours storage at 2° and at -20° with one homogenate and at 2° with the other homogenate (Fig. 33a,b). There was a slight decay in activity 6 hours after homogenization in these experiments, even with storage at -20°, and after 24 hours the loss of activity was approximately 80% at 2° and 70% at -20°. The effect was the same when the supernatant rather than the crude homogenate was stored at -20° (Fig. 33,c).

As a result of these studies, the homogenate was kept for no longer than 1 hour before commencement of the incubation.

The storage of unhomogenized tissue

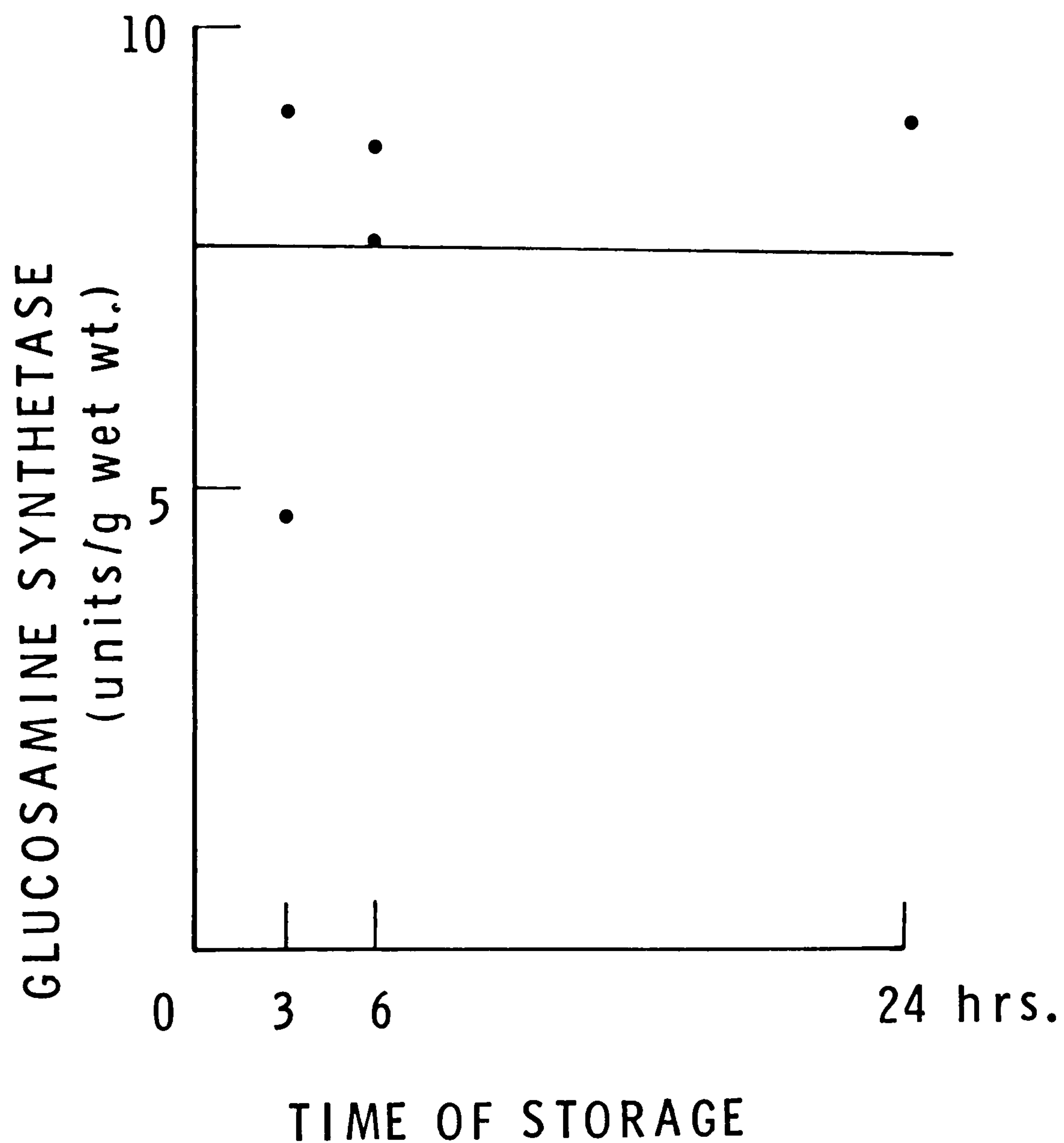
There was much less loss of enzyme activity in unhomogenized tissue.

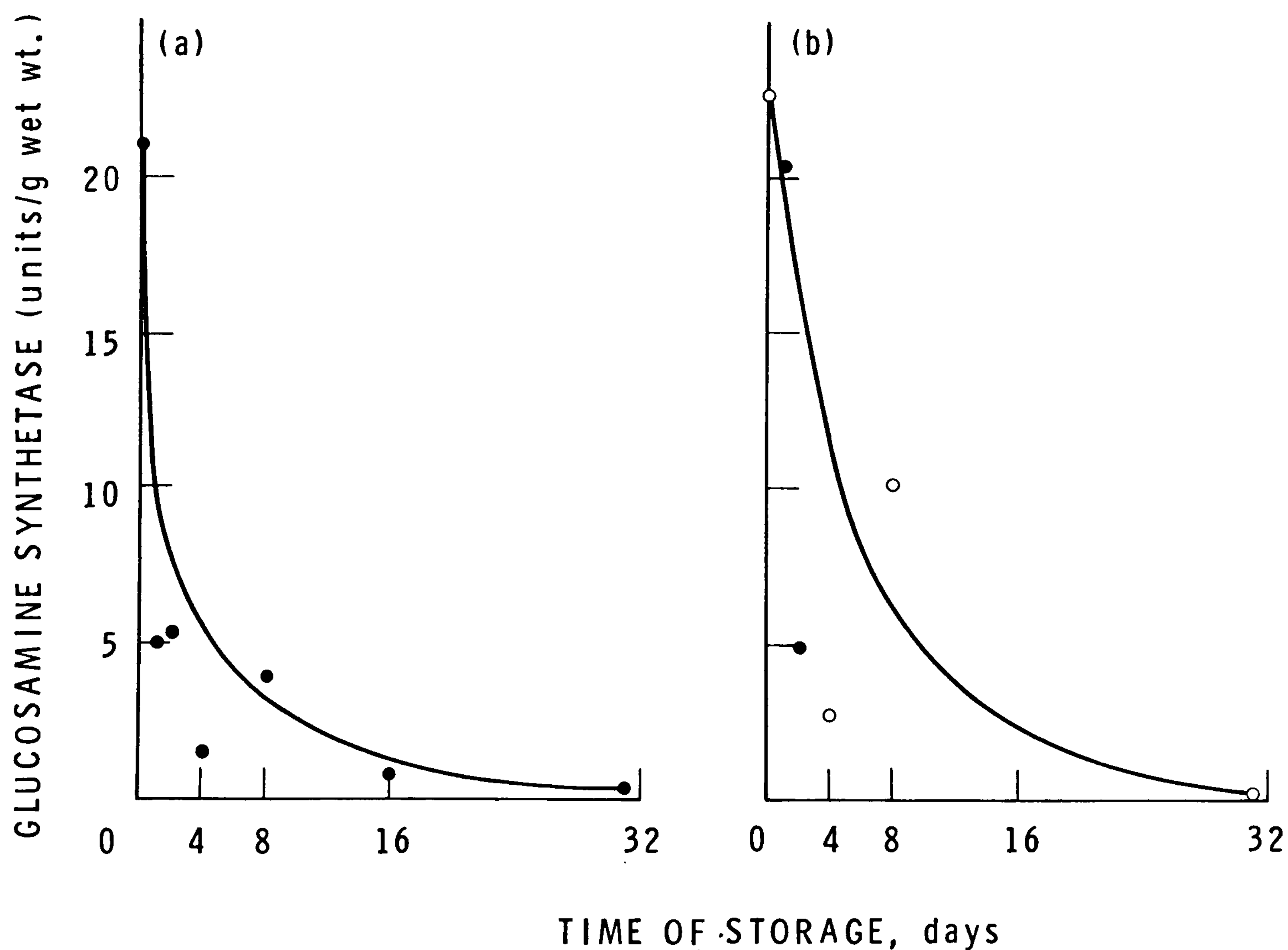
One piece of normal colonic mucosa was assayed by the 10 mg method with the incubation commencing within 1 hour of removal of the tissue from the patient. Adjacent pieces of the same mucosa were stored at -20° in parafilm and two pieces each were homogenized and the incubation commenced at 3, 6 and 24 hours after the homogenization of the first sample (Fig. 34). No loss of activity was detectable in 24 hours storage at -20° but the variability of enzyme activity between different samples of the mucosa meant that the results could not exclude some loss of enzyme activity.

The experiment was repeated with adjacent samples of two other specimens of normal colonic mucosa, with pieces of each mucosa stored at -20° for 1, 2, 4, 8, 16 and 31 days (Fig. 35). There was 50% loss of activity after 1-4 days, compared to 50% loss of activity at 12-16 hours for homogenized mucosa (Fig. 33).

THE NEED FOR DUPLICATE READINGS

Duplicate readings of glucosamine synthetase activity by the 10 mg method when the two readings were assayed in the same batch of dilute DAB and standards gave a mean variation of 8% (derived from 20 duplicated pairs) in the difference between the readings as a proportion of their mean. When 20 duplicated pairs were read with different batches of dilute DAB and standards, but within 24 hours of each other to eliminate the decay correction, the mean variation was 15%.





FIGS. 34, 35. Decay of glucosamine synthetase activity in adjacent samples of unhomogenized colonic mucosa stored at - 20°.

FIG. 34 (above): Samples from one patient stored for up to 24 hours.

FIG. 35 (below): Samples from two patients (a, b) stored for up to 1 month. [Open circles indicate unduplicated readings.]

This increased difference between the duplicate pairs using a different batch of dilute DAB for each reading in the pair was presumably due to variations in the reading of the standards and in the construction of the standard curve. As this was an inherent error of the method, it was considered that the duplicates should be assayed in separate batches in order to minimize this error. The glucosamine synthetase activity was, of course, derived from the mean of the two readings. Duplicate readings for A, namely A_1 and A_2, gave the glucosamine synthetase activity, x, by the formula

$$x = \frac{A_1 + A_2}{3}.$$

THE DENOMINATOR OF ENZYME ACTIVITY—WET WEIGHT OR TISSUE PROTEIN?

For the study of biopsies, it was impracticable to assay glucosamine synthetase activity per g tissue protein as this would mean taking additional tissue purely for the estimation of the protein concentration of the homogenate.

In many of the specimens of mucosa taken at colectomy, the protein concentration of the homogenate was measured and the glucosamine synthetase activity was then expressed in μmoles glucosamine synthesized per h per g protein.

The estimation of tissue protein

The method used was that of Lowry et al (1951):

Reagent A was 2% sodium carbonate in 0.1N sodium hydroxide. 500 ml reagent A was made up by using volumetric concentrates of NaOH.

Reagent B was 0.5% $CuSO_4 . 5H_2O$ in 1% aqueous sodium citrate.

Reagent C was made up freshly by adding 10 ml reagent B to 500 ml reagent A.

Reagent E was Folin-Ciocalteu reagent, supplied at approximately 2N and the exact normality then determined at pH 7. The reagent was diluted with water to 1N.

0.5 ml of the tissue homogenate, 10 mg/ml in buffer, was diluted 1 in 8 with 3.5 ml water.

To 0.5 ml of the diluted homogenate, 5 ml reagent C were added. After 10-60 minutes, 0.5 ml reagent E was added and the test-tube stirred vigorously

with a vortex stirrer. After 40 minutes, the blue colour which formed was read in a spectrophotometer at 750 nm.

Bovine serum albumin (BSA) was used as a protein standard. The same stock of BSA was used for all estimations. The BSA was hygroscopic and a portion of it was desiccated for 24 hours at the time of each estimation to determine the proportion of protein in the weighed hydrated BSA.

Two water-blanks were used and two samples of each of five standards of approximately 0.05, 0.10, 0.15, 0.20 and 0.25 mg protein per ml, the exact protein concentration of the standard being calculated from the weight of protein used and the observed degree of hydration. The standard curve was linear in this range (Fig. 36).

With a tissue protein concentration of approximately 0.1 mg protein per mg tissue, and with the diluted homogenate containing 1.25 mg tissue per ml, the concentration of protein in the diluted homogenate was approximately 0.08 mg/ml.

Two samples of each diluted homogenate were assayed for protein, the result being expressed as g protein per g tissue. The glucosamine synthetase activity per g protein was thus calculated from the activity per g wet wt and the protein concentration in the tissue.

The accuracy of the method for tissue protein

Sometimes the duplicated assays gave widely varying values for tissue protein concentration. Some of these readings were considered to be grossly erroneous. Out of 149 pairs of readings for the protein concentration in the tissue, a histogram was constructed of the differences between the two readings of each homogenate (Fig. 37). Differences of between 0 and 0.039 g protein/g tissue appeared to have a Gaussian distribution, which was assumed to represent the statistical error of the method of assay. Differences of 0.040 or more seemed to be beyond the Gaussian curve and they were therefore considered erroneous and not taken as reliable estimates of the protein concentration. The protein concentrations of these diluted homogenates were assayed again on another occasion.

Even excluding the pairs of duplicates with differences of 0.040 or more, the standard deviation of the remaining 134 pairs of readings was 0.015 g protein/g tissue or approximately 15%, so that the paired duplicate readings

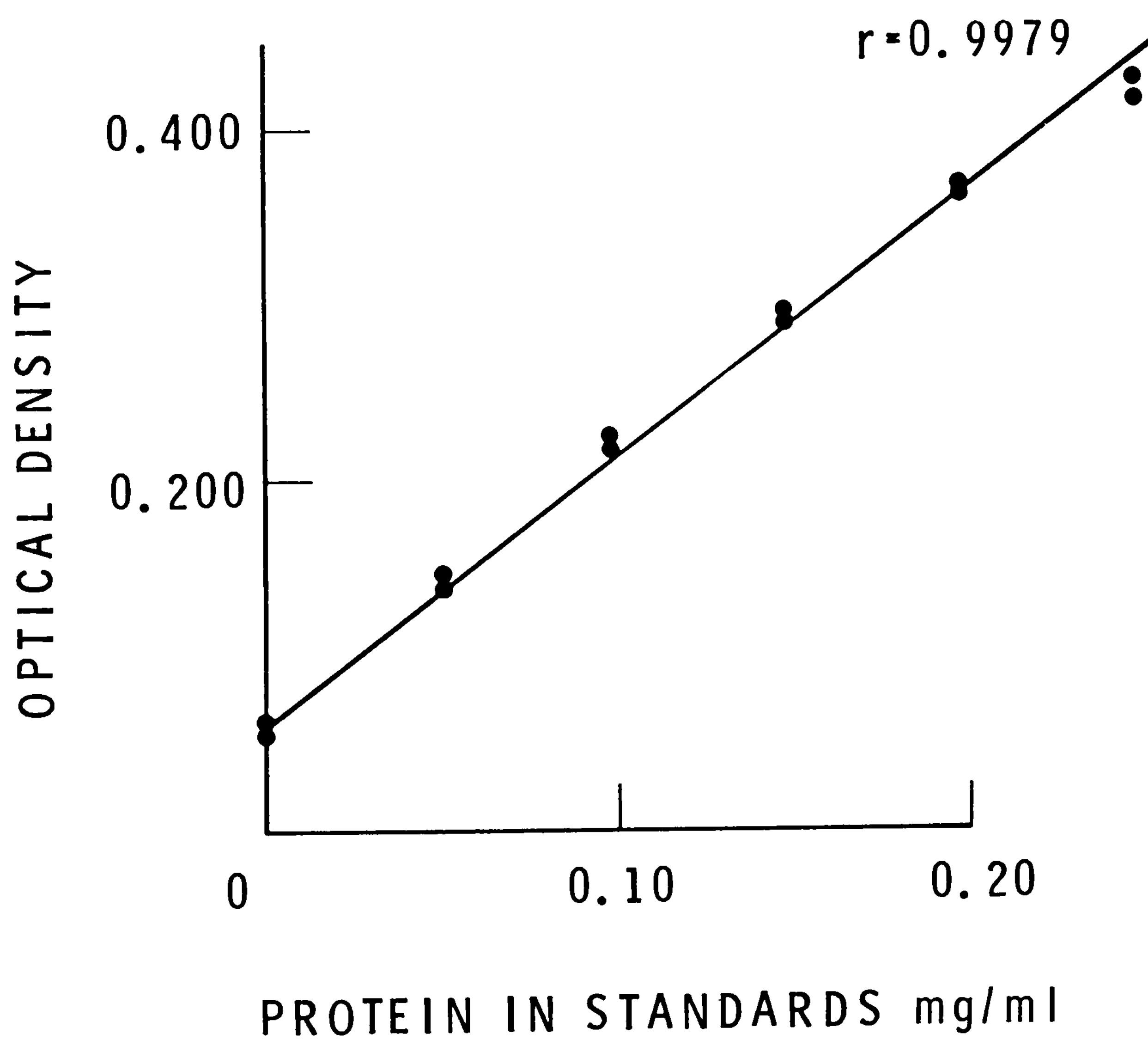


FIG. 36. Standard curve of bovine serum albumin in the Lowry protein estimation.

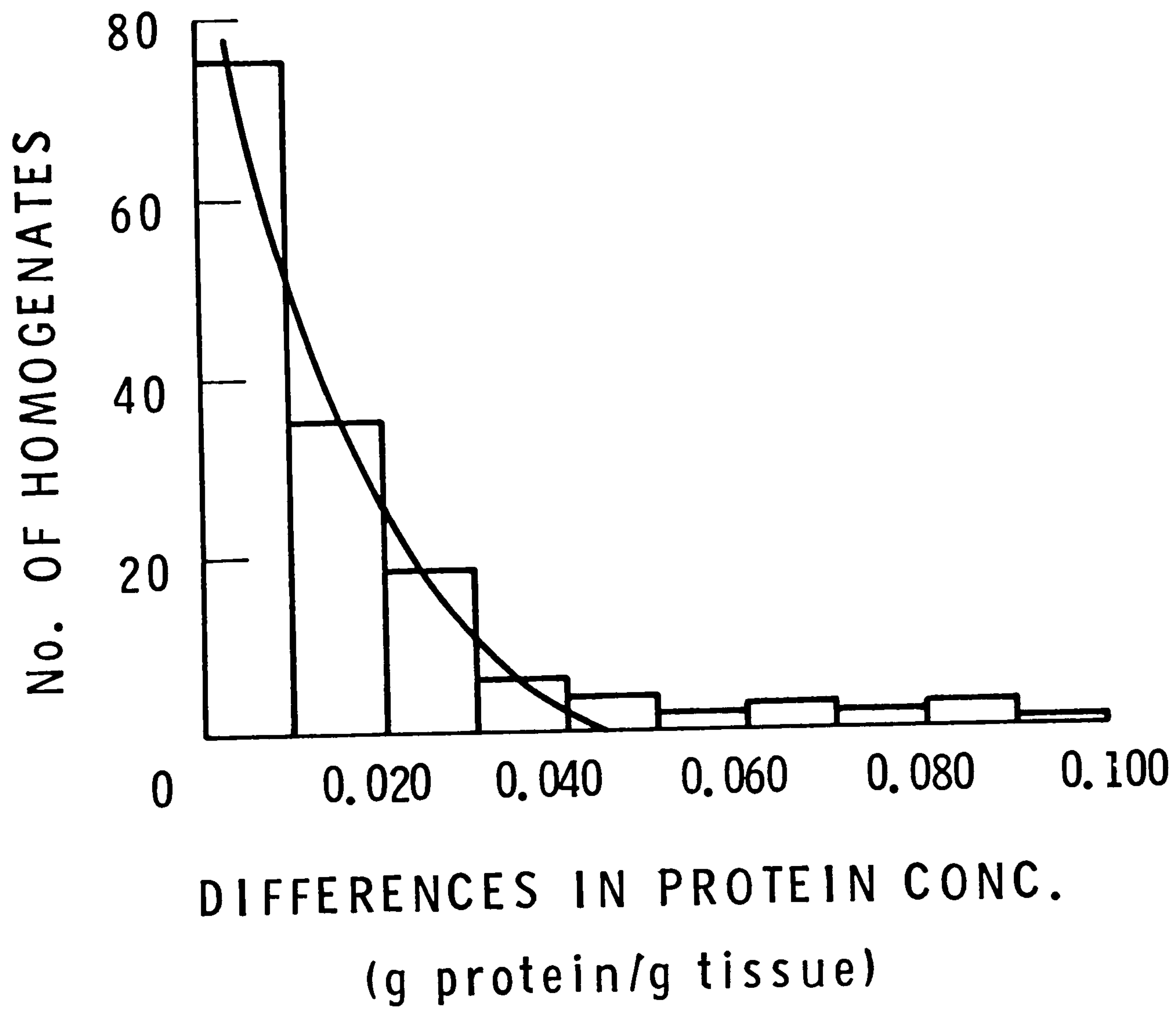


FIG. 37. Differences in protein concentration between pairs of readings in the Lowry protein estimation of homogenates of colonic mucosa of 1.25 mg/ml.

varied from their means with an S.D. of 7-10%.

Ten 0.5 ml samples of each of two homogenates, containing 10 mg tissue per ml, were assayed for their protein concentration using separate BSA standards and fresh reagents for each of the 10 samples from each homogenate. For one of the homogenates, the mean tissue protein concentration was 0.081 g protein/g wet wt, with an S.D. of 0.007, or 9%. With the other homogenate, three of the 10 pairs of readings were discounted because they varied by more than 0.039. The remaining 7 pairs of readings gave a mean of 0.074 g protein/g wet wt, with an S.D. of 0.005, or 7%.

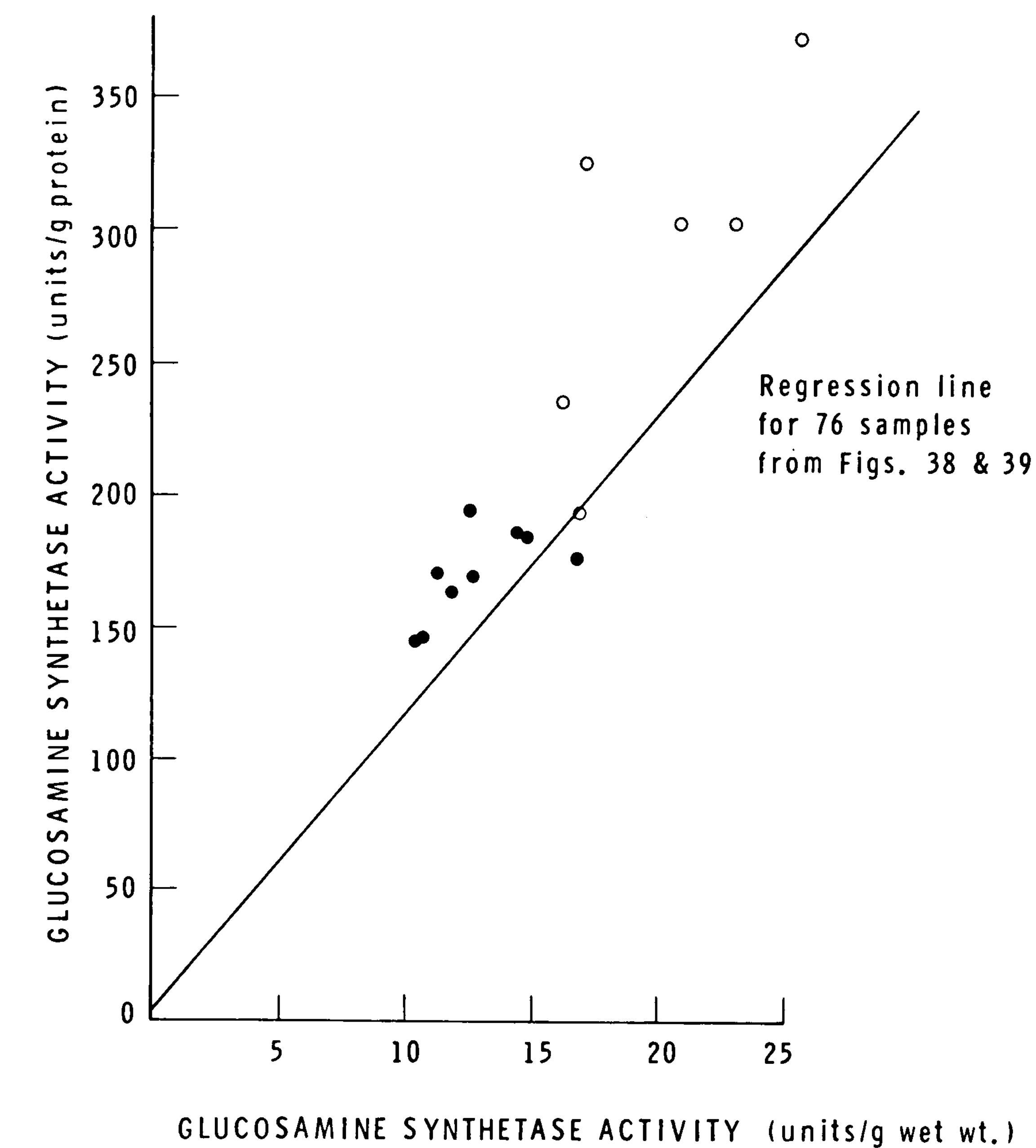
Thus the estimation of the protein concentration of the homogenates was rather inaccurate. The wet weight of the tissue sample, measured on a fine balance weighing up to 0.0001 g, was a more reliable quantity.

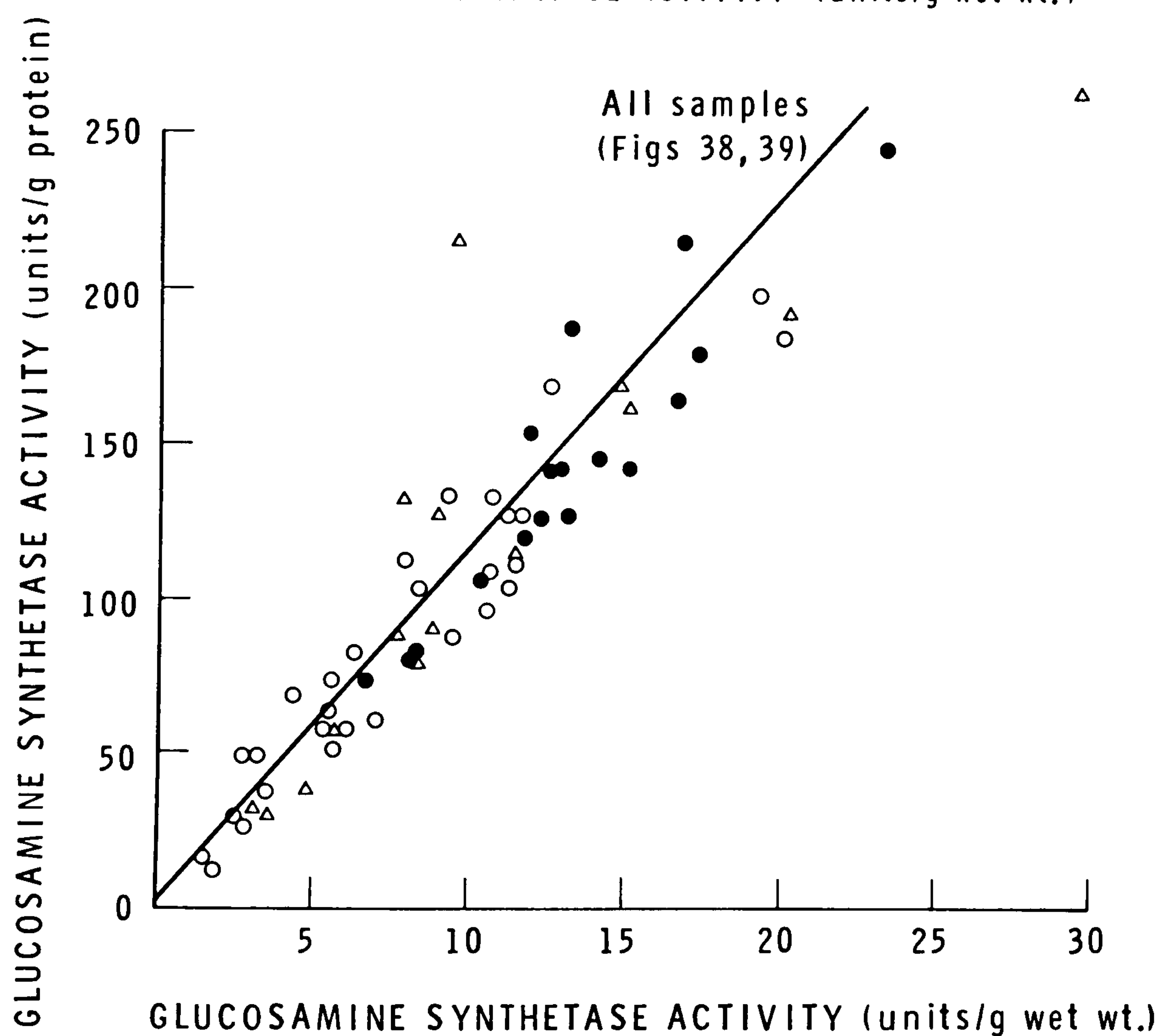
Tissue protein versus wet weight

Eight pieces of adjacent normal colonic mucosa from one patient and 6 pieces of adjacent mucosa from a second patient were assayed for glucosamine synthetase using the 10 mg method and the protein concentrations of the homogenates were also estimated. The glucosamine synthesized was assayed with the same batch of standards and dilute DAB for all the specimens from a single patient and the protein concentrations were estimated at the same time and from the same standard curve for all the homogenates from one patient, although the estimations from the two patients were made with different batches of dilute DAB and different batches of protein standards.

The glucosamine synthetase activity in μmoles glucosamine synthesized/h/g wet wt was plotted against the activity in μmoles glucosamine synthesized/h/g protein (Fig. 38) and there was a close correlation between the two types of units for expressing the enzyme activity. This correlation was also confirmed in pathological tissue from patients with ulcerative colitis and Crohn's disease (Fig. 39).

It was concluded that tissue wet weight was a satisfactory denominator for glucosamine synthetase assays.





FIGS. 38, 39. Glucosamine synthetase activity: units/g wet wt versus units/g protein.

FIG. 38 (above): 8 adjacent pieces of normal mucosa from one patient (closed circles) and 6 adjacent pieces from another patient (open circles).

FIG. 39 (below): 18 samples of normal mucosa from 15 patients (closed circles); 28 samples of mucosa from 10 patients with ulcerative colitis (open circles); 16 samples from 8 patients with Crohn's disease of the colon (open triangles).

THE METHOD ADOPTED FOR THE CLINICAL STUDIES

The 4 mg assay and the 10 mg assay

The method was used at two degrees of miniaturization. For tissue obtained at surgical operation, larger volumes were used, in the "10 mg assay", which required at least 10.5 mg tissue. For biopsy specimens, smaller volumes were used, in the "4 mg assay", which required only 4.5 mg tissue.

In the description of the method which follows, the volumes quoted with a single asterisk (*) are those used in the 4 mg assay. The volumes used in the 10 mg assay are given in brackets, with two asterisks (**).

From time to time, biopsies were taken which weighed less than 4.5 mg. In these cases, it was not possible to obtain further tissue as the instrument for taking the biopsy had been removed before the deficiency was discovered. In some of the clinical studies, such biopsies were considered to be important to the study. In such cases, if the biopsy weighed less than 4.5 mg but more than 2.5 mg, the 4 mg assay was used without duplication of the incubations, thus reducing the minimum tissue requirement to just over 2 mg. This was termed the "2 mg assay" and its errors in relation to the 4 mg assay are discussed later in this chapter.

Stock solutions

Double-strength homogenizing buffer was made by dissolving 21.8472 g $Na_2HPO_4.12H_2O$ (61 mmoles), 6.0866 g $NaH_2PO_4.2H_2O$ (39 mmoles), 14.910 g KCl (200 mmoles) and 0.7444 g EDTA (disodium, $2H_2O$, 2 mmoles) in 1000 ml deionized water. This solution was diluted 1:1 with deionized water to make the homogenizing buffer.

*8M (**9M) KOH

M KOH

Borate buffer was made up by dissolving 34.630 g boric acid (0.56 mole) and 15.711 g KOH (0.28 mole) in 500 ml water.

Acetic anhydride 1.5% in acetone was made by taking 1.5 ml acetic anhydride and making it up to 100 ml with acetone.

Stock dimethylaminobenzaldehyde solution (DAB) was made up monthly and kept in a refrigerator. 5 g DAB were dissolved in a mixture of 87.5 ml

glacial acetic acid and 12.5 ml concentrated hydrochloric acid.

Preparation of the tissue

Surgical specimens were collected from the operating theatre as soon as they were removed from the patient. They were placed in dry containers.

The tissue to be assayed was removed from the surgical specimen as soon as possible and placed in ice-cold homogenizing buffer. When it was the mucosa which was to be assayed, which was usually the case, it was stripped from the submucosa by blunt dissection. The tissue was rinsed in fresh homogenizing buffer and then plotted dry on filter paper. When the tissue was to be examined histologically, it was oriented flat on the filter paper and cut in half at right angles to the plane of the mucosa with a scalpel blade. The portion for histology was taken with the filter paper adherent and placed in formal saline.

Biopsy specimens were collected in bijou bottles containing ice-cold homogenizing buffer. The bottles were kept in ice until the time of homogenization.

The preparation of the homogenate

The tissue for the enzyme estimation was weighed in parafilm on a fine balance, to the nearest 0.0001 g.

The tissue was then placed in a Potter-Elvehjem homogenizer and homogenized in the homogenizing buffer, using 0.0099 ml buffer per 0.1 mg tissue, to the nearest 0.01 ml.

*0.2 ml (**0.5 ml) of the homogenate was pipetted into each of 2 small plastic test-tubes, using an Eppendorf pipette with an unused tip.

Preparation of the substrate-buffer mixture

The substrate-buffer mixture was made up on the morning of the incubation. A minimum of 2.0 ml substrate-buffer mixture was made.

For 2.0 ml substrate-buffer mixture, 0.0243 g (dessicated weight) fructose-6-phosphate disodium (80 μmoles) was dissolved in 1.0 ml double-strength homogenizing buffer. 0.0468 g L-glutamine (320 μmoles) was dissolved in 5.0 ml deionized water. 0.0080 g (8000 μg) gentamicin base, as gentamicin sulphate 0.0130-0.0160 g, was dissolved in 10.0 ml deionized water. 0.5 ml of the glutamine solution (32 μmoles) and 0.5 ml of the gentamicin solution

solution (400 μg) were added to the 1.0 ml fructose-6-phosphate in double buffer, making a solution containing fructose-6-phosphate 40 mM, glutamine 16 mM, gentamicin 200 μg/ml and the components of the homogenizing buffer in single strength.

Incubation of the homogenate

*0.2 ml (**0.5 ml) substrate-buffer mixture was added to each *0.2 ml (**0.5 ml) volume of homogenate. The *0.4 ml (**1.0 ml) volumes contained fructose-6-phosphate 20 mM, glutamine 8 mM, and gentamicin 100 μg/ml. As soon as the substrate-buffer mixture was added, the test-tubes were placed in a water-bath at 37°. The incubation was commenced within 2 hours of the removal of the tissue from the patient.

After exactly 3 hours, the incubation was terminated by the addition of *0.02 ml (**0.05 ml) 71% perchloric acid. The test-tubes were then placed in a deep freeze at -20°, unless they were to be assayed for glucosamine on the same day.

Standard solutions

Standard solutions of 0.05, 0.10, 0.20 and 0.30 mM glucosamine hydrochloride were made up freshly for each batch of test solutions. A stock solution of 2 mM glucosamine hydrochloride was made up weekly by dissolving 0.2156 g glucosamine hydrochloride in 500 ml deionized water. The 0.30 mM standard was made by pipetting 7.5 ml of the 2 mM solution into a 50 ml volumetric flask and making up the volume with water. The 0.05 mM standard was made by taking 2.0 ml of the 0.30 mM standard and adding 10.0 ml water; the 0.10 mM standard was made by taking 4.0 ml of the 0.30 mM standard and 8.0 ml water; and the 0.20 mM standard was made by taking 8.0 ml of the 0.30 mM standard and 4.0 ml water.

Assay of glucosamine in the incubates

The glucosamine assay was performed on batches of up to 19 incubated test-tubes. The two incubates from each homogenate were not assayed in the same batch. The test-tubes were not stored for longer than 30 days before they were assayed.

The incubates to be assayed in a batch were thawed, if they had been

frozen, and centrifuged at 2500 rpm for 15 minutes.

*0.3 ml (**0.8 ml) of each supernatant was pipetted into another test-tube, using a Finpipette. *0.5 ml (**1.0 ml) of each standard was pipetted into a test-tube and *0.025 ml (**0.05 ml) 71% perchloric acid was added to each standard.

*0.02 ml 8M KOH (**0.05 ml 9M KOH) was added to the pipetted supernatant of each incubate and *0.035 ml 8M KOH (**0.065 9M KOH) to each acid-treated standard.

M KOH was added dropwise to each supernatant and to each standard until the pH was between 6 and 11 according to Merck non-bleeding indicator strips. The volume of M KOH used was recorded to the nearest 0.005 ml.

The test-tubes containing the neutralized supernatants and standards were centrifuged at 2500 rpm for 5 minutes to precipitate the $KClO_4$.

*0.2 ml (**0.5 ml) of each solution was pipetted into a screw-cap heat-resistant test-tube. Into one screw-cap tube a similar volume of water was pipetted.

*0.2 ml (**0.5 ml) borate buffer was then pipetted into each screw-cap tube and then *0.05 ml (**0.1 ml) acetic anhydride in acetone was added to each tube.

The caps were screwed on and all the tubes were placed in a boiling water-bath for 3 minutes.

The tubes were then placed in an ice water-bath for at least 5 minutes.

Dilute DAB solution was made by diluting 1 volume of stock DAB with 9 volumes of glacial acetic acid, no more than 15 minutes before the dilute DAB was to be used.

*1.6 ml (**3.9 ml) dilute DAB was added to each screw-cap tube and the cap was replaced. Dilute DAB was added to 3 tubes at a time and the tubes were immediately placed in a 37° water-bath, at a time which was recorded to the nearest $\frac{1}{4}$ min. The DAB was added to one set of 3 tubes every 2 minutes, there being up to 24 tubes in a batch.

After 20 minutes in the 37° water-bath, the tubes were placed in an ice water-bath. As soon as all the tubes in a batch had been removed from the 37° water-bath, the optical density of the magenta colour formed was measured in a Unicam SP 500 spectrophotometer at 545 nm, using *the half-height

slit and the focussing lens (**the normal-height slit without the focussing lens). Three glass cuvettes of 1 cm light path were used which had previously been filled with water and their optical densities measured against the water-filled cuvette of lower O.D. which served as the blank for the glucosamine estimations.

The calculation of the glucosamine synthetase activity

Prepared forms were used for the calculation of the glucosamine synthetase activity (Fig. 40).

The value B (B_s) was calculated for the standard solution from A_s which was 100 times the concentration of glucosamine in mM in the original standards. *q′ ml (**q ml) was the volume of M KOH added to the standard in the neutralization procedure.

$$*B_s = \frac{A_s}{1 + 1.8\,q'} \qquad \left(**B_s = \frac{A_s}{1 + 0.90\,q} \right).$$

D_s, 1000 times the O.D. in the colour reaction, was plotted against B_s for the zero water standard and for the four glucosamine hydrochloride standards. The scale used on the graph paper was 20 units per cm on the ordinate for D and 2 units per cm on the abscissa for B. The straight line which appeared visually to come the closest to the five points was ruled on the graph paper (*Fig. 29, **Fig. 21).

From this graph, a value of B for each test solution was derived from D (1000 times the observed O.D.).

*p′ ml (**p ml) was the volume of M KOH used in the neutralization procedure for each test solution. A for each test solution was calculated from the formula

$$*A = B\,(1 + 3.1\,p') \qquad (**A = B\,(1 + 1.18\,p)).$$

The decay factor, f, was calculated from d, the number of days of storage of the incubate, by the formula

$$\log_{10} f = 0.00169\,d.$$

For each of the duplicated readings of each homogenate, fA was calculated. For the two readings f_1A_1 and f_2A_2, the glucosamine synthetase activity, x, in μmoles glucosamine synthesized/h/g wet wt, was calculated from the

Spec or Std	Time	Cell	Reading	Cell blank	D	Pip	q or r	1.18q 1 0.90r + 3.1q′ 1.8r′	B	A
0		a								0
5		b								5.0
10		c								10.0
20		a								20.0
30		b								30.0
		c								
		a								
		b								
		c								
		a								
		b								
		c								
		a								
		b								
		c								
		a								
		b								
		c								
		a								
		b								
		c								
		a								
		b								
		c								

FIG. 40. Pro forma used in the calculation of A in the determination of glucosamine synthetase activity. Batches of 5 standards and up to 19 test solutions.

formula

$$x = \frac{f_1A_1 + f_2A_2}{3}\ .$$

THE ACCURACY OF THE METHOD

The potential sources of error in the method adopted for the assay of glucosamine synthetase were errors in the technique, sampling errors, and errors in the use of wet weight rather than tissue protein as the denominator of enzyme activity.

Errors in the technique

The sources of error in the technique were the following:—

(a) Cleaning the tissue.
(b) Drying the tissue.
(c) Weighing the tissue.
(d) Pipetting.
(e) The composition of the homogenizing buffer.
(f) The composition of the substrate-buffer mixture.
(g) Homogenization.
(h) The temperature of the incubation.
(i) The duration of the incubation.
(j) The pH following the neutralization of the perchloric acid.
(k) The composition of the borate buffer and the acetic anhydride solutions.
(l) The composition of the DAB solution.
(m) The decay of DAB.
(n) The decay of the colour formed in the Morgan-Elson reaction.
(o) The spectrophotometer readings.
(p) The composition of the standard solutions.
(q) The linearity of the standard curve.
(r) The accuracy of the standard readings.
(s) The drawing of the standard curve.
(t) The reproducibility of the standard curve.
(u) The delay in commencing the incubation.
(v) The storage of the incubated solutions and the application of the decay factor.
(w) The calculation of the enzyme activity.
(x) The linearity of the assay in relation to glucosamine synthetase concentration.
(y) Glucosamine in the unincubated tissue.

These sources of error are discussed in detail in Appendix A.

Errors of sampling

There was a source of error in the stripping of the mucosa, which is discussed in Appendix A, and an inherent sampling error due to the fact that a small rectal biopsy weighing between 5 and 15 mg or a piece of stripped mucosa weighing between 10 and 40 mg is only a small sample of the mucosa from which it was taken.

The sampling error in the 10 mg assay was estimated by taking 10 pieces of stripped mucosa at 1 cm intervals from the normal mucosa of each of two specimens removed for carcinoma of the colon, the samples weighing between 15 and 30 mg, and assaying each piece of mucosa by the 10 mg method (Table 12). To minimize errors in the reproducibility of the method, the duplicated incubates from each pieces of mucosa were assayed in the same two batches with dilute DAB.

The sampling error in the 4 mg assay was estimated similarly, using pieces of mucosa weighing between 5 and 10 mg (Table 12).

For the 10 mg assay, the S.D. of the samples was 16% of their mean for the first colectomy specimen and 18% for the second specimen.

For the 4 mg assay, the S.D.'s were 21% and 17% respectively for two other colectomy specimens.

Thus the sampling error had an S.D. of approximately 15-20% by the 10 mg method and 15-25% by the 4 mg method. A sampling error was not estimated for pieces of mucosa weighing less than 4.5 mg as were used in the "2 mg assay".

Errors in the use of wet weight rather than tissue protein as the denominator of enzyme activity

This was assessed for surgically removed mucosa assayed by the 10 mg method. Biopsies did not provide sufficient material in all cases to make it worthwhile to express the enzyme activity in terms of tissue protein even for those biopsies in which there was sufficient material.

Of the multiple samples of two specimens of colonic mucosa assayed by the 10 mg method in the investigation of the sampling error, satisfactory measurements of the protein concentrations of the homogenates were obtained for 8 samples of one specimen and for 6 samples of the other specimen. For the 8 samples of the first specimen, the S.D. of the enzyme measurements

TABLE 12. The sampling error of the glucosamine synthetase assay. 8 or 10 adjacent pieces of mucosa assayed in the same batch with dilute DAB. Experiment performed twice with the 10 mg assay and twice with the 4 mg assay

		Glucosamine synthetase activity						
		Units/g wet wt				Units/g protein		
Assay	Colectomy specimen	(No. of readings)	Readings	Mean + S.D. [S.D.%]		(No. of readings)	Readings	Mean + S.D. [S.D.%]
10 mg method	(a)	(10)	16.7 10.4 14.7 11.8 11.3 12.5 14.4 12.5 15.2 17.0	13.7 ± 2.2 [16%]	13.0 ± 2.1 [16%] (16.7–12.5)	(8)	178 146 186 164 171 171 187 195	175 ± 14 [8%]
	(b)	(8)	16.8 16.1 20.7 22.9 25.4 17.0 15.1 21.0	19.4 ± 3.4 [18%]	19.8 ± 3.5 [18%] (16.8–17.0)	(6)	195 237 304 305 374 327	290 ± 59 [20%]
4 mg method	(c)	(10)	15.0 18.7 12.8 17.3 10.5 15.8 18.1 19.4 21.9 15.5	16.5 ± 3.3 [20%]				
	(d)	(10)	17.4 17.9 15.5 22.1 11.5 20.7 20.3 17.9 17.7 19.0	18.0 ± 3.0 [17%]				

per g wet wt was 16% compared to 8% for the measurements per g protein, but for the 6 samples of the second specimen the S.D. was 18% for the enzyme activity per g wet wt and 20% for the activity per g protein (Table 12).

Glucosamine synthetase activity per g wet wt was plotted against the enzyme activity per g protein for these 14 samples (Fig. 38), for 18 other samples of normal colonic mucosa and for 44 samples of mucosa taken from colectomy specimens of patients with ulcerative colitis and Crohn's disease of the colon (Fig. 39), and there was a high coefficient of correlation ($r = 0.9$).

Thus it appeared almost as accurate to express glucosamine synthetase activity in μmoles glucosamine synthesized/h/g wet wt as it was to express the activity in μmoles glucosamine synthesized/h/g protein.

The overall error in the method

The reproducibility of the method had two components, the reproducibility of the assay of a single tissue homogenate and the sampling error.

The error in the reproducibility of the assay of glucosamine synthetase activity in a single tissue homogenate was estimated by taking two homogenates of normal colonic mucosa, 10 mg/ml, and assaying 12 samples of each homogenate by the 10 mg method and 12 samples of each by the 4 mg method. A new pipette tip was used for each pair of samples. The two homogenates were incubated on different days but all samples of each homogenate were incubated at the same time, using the same substrate-buffer mixture and the same water-bath, so that errors in the composition of the substrate-buffer mixture and in the temperature of the water-bath were eliminated. The incubated samples were stored at -20° for up to 32 days. Each sample of each homogenate was assayed for glucosamine in a separate batch with fresh standards using a fresh 2 mM stock standard on each occasion (Fig. 30).

The decay factor was applied, although it must be stated that these were the readings from which the decay factor was calculated (but the factor did not exceed 1.12). Pairs of consecutively assayed samples were taken as duplicates of a single reading, so that 6 readings were obtained from each homogenate, both for the 10 mg assay and for the 4 mg assay (Table 13).

The mean of the readings in the 4 mg assay was 6-8% higher than the mean in the 10 mg assay, for both homogenates. This may have been due to errors in the standard curves.

TABLE 13. Reproducibility of the glucosamine synthetase assay. Glucosamine synthetase activity of 2 homogenates of normal colonic mucosa, each assayed 6 times by the 10 mg method and 6 times by the 4 mg method

	Glucosamine synthetase activity (units/g wet wt)			
	10 mg assay		4 mg assay	
Homogenate	Readings	Mean ± S.D. [S.D. %]	Readings	Mean ± S.D. [S.D. %]
(a)	7.5		8.8	
	10.8		9.7	
	8.3	9.0 + 1.4 [15%]	9.9	9.8 + 0.8 [8%]
	10.2		10.4	
	9.3		8.9	
	8.1		10.8	
(b)	9.9		8.9	
	8.1		9.5	
	10.8	9.0 + 1.2 [14%]	10.3	9.6 + 1.2 [12%]
	8.1		10.7	
	8.0		7.8	
	9.1		10.5	

The standard deviation of the error in these reproducibility experiments was 10% for the 4 mg assay (8% and 12% respectively for the two homogenates studied) and 15% for the 10 mg assay (15% and 14% respectively for two homogenates). It was likely that this difference arose by chance since the 10 mg assay was inherently at least as accurate as the 4 mg assay.

Thus any additional error due to miniaturization from the 10 mg assay to the 4 mg assay was not detectable by these studies.

Error due to the omission of a duplicate in the 4 mg method—"the 2 mg method"

The error of reproducibility for the 2 mg assay (which was the 4 mg assay with the omission of the duplicate reading) was estimated by taking the 12 samples from each homogenate assayed by the 4 mg method as separate readings by the 2 mg method rather than as 6 pairs of readings by the 4 mg method. The S.D.'s from the two homogenates were 10% and 18% respectively by the 2 mg method, compared to 8% and 12% for the same homogenates by the 4 mg method.

Thus the use of one rather than two 0.2 ml samples of an homogenate gave rise to an additional error of only 3-4%.

SECTION III

CLINICAL STUDIES

CHAPTER 9

NORMAL COLON AND THE IRRITABLE COLON SYNDROME

NORMAL MUCOSA FROM PATIENTS WITH LOCALIZED COLONIC DISEASE

A normal range for glucosamine synthetase activity in human colonic mucosa was determined by measuring the enzyme levels in the histologically normal mucosa from the cut ends of colectomy specimens of patients undergoing colonic resection for carcinoma of the large bowel or for localized diverticular disease.

PATIENTS STUDIED AND METHODS

Surgical colectomy specimens were obtained from patients undergoing operation for large bowel cancer and for localized diverticular disease. The specimens were kindly supplied by the surgeons of the Radcliffe Infirmary.

Patients were excluded if they had had inflammatory bowel disease at any time in the past, if they had suffered from endocrine disorders (such as diabetes mellitus, thyrotoxicosis, myxoedema, Addison's disease or Cushing's disease) if they were taking hormonal preparations (such as corticosteroids, orally or topically), or if they had chronic liver disease (in view of the association of inflammatory bowel disease with chronic liver disease). Patients with peptic ulceration were not excluded.

As soon as the segment of bowel had been removed, it was placed in a dry container and the laboratory was informed. It was collected immediately and then opened and examined. The mucosa at one of the cut ends was considered to be normal if the bowel at that point had a normal appearance and was free from tumour and inflammatory tissue both macroscopically and histologically,

and if it was more than 10 cm clear of the carcinoma of diverticular mass. A portion of mucosa at the cut end was removed from the submucosa by blunt dissection. The remainder of the surgical specimen was placed in formol saline and taken to the Pathology Department for routine diagnostic histology. The pathologist was informed that portions had been removed from the specimen for research.

One such piece of apparently normal mucosa was obtained from each of 25 patients.

A piece of mucosa weighing between 15 and 40 mg was homogenized in the homogenizing buffer, 10 mg tissue per ml homogenate. The glucosamine synthetase activity of the homogenate was assayed by the 10 mg method. In 16 out of the 25 samples, the protein concentration of the homogenate was assayed to enable the glucosamine synthetase activity to be expressed both in units/g wet wt and in units/g protein. In the other 9 samples, glucosamine synthetase activity was measured only in units/g wet wt.

The clinical details of the patients and details of the operation and the region of the bowel from which the sample was taken were recorded on a pro forma (Fig. 41).

Normal submucosa

After the dissection and stripping of the mucosa at the normal cut ends of 3 colectomy specimens, a piece of submucosa was taken (2 pieces from one of the specimens) which was assayed for glucosamine synthetase.

RESULTS

The glucosamine synthetase levels in the normal colonic mucosa of the cut ends of the 25 colectomy specimens are shown in Table 14 and in Fig. 48. The mean glucosamine synthetase level was 13.66 units/g wet wt (±0.84, S.E.M.) and the standard deviation was 4.18, or 30.6%. For the 16 samples in which the enzyme activity was also measured per g protein, the mean glucosamine synthetase level was 147.8 units/g protein (±10.8, S.E.M.) with a standard deviation of 43.2, or 20.2%, compared to a standard deviation of 30.0% for the enzyme activity per g wet wt in these 16 samples (mean 13.48 units, S.D. 4.05) (Fig. 42).

GLUCOSAMINE SYNTHETASE ACTIVITY IN COLONIC MUCOSA

Lab No.

Hosp. No.

Surname: Date

First names:

Date of birth: Ward

Sex:

Consultant Surgeon

Diagnosis:

Operation:

Segment of colon:

Other diseases:

Drugs (3 days pre-op.):

Premed:

Anaesthetic:

Blood group:

Hb. WBC MCV ESR Date:

Urea Na K Albumin T.P. Date:

Time of removal of tissue:

Time in buffer:

Initial time of incubation:

Wet wt (1) Wet wt (2)

Protein (1) Protein (2)

Activity (1) Activity (2)

Histology:

FIG. 41. Pro forma used to record clinical details of patients whose colectomy specimens were assayed for glucosamine synthetase.

TABLE 14. Glucosamine synthetase activity in 25 samples of normal colonic mucosa (taken more than 10 cm away from the lesion) in patients undergoing colonic resection for carcinoma or for localized diverticular disease

Patient	Sex	Age	Diagnosis	Segment of colon	Glucosamine synthetase activity	
					Units/g wet wt	Units/g protein
S.Be.	M	69	Ca.	Rectum	13.1	127
M.By.	F	53	Ca.	Rectum	13.2	188
L.Cox	M	61	Ca.	Descending	10.3	
G.G.	F	72	Ca.	Caecum	11.8	119
R.Gos.	M	55	Ca.	Ascending	21.8	
A.Hi.	F	69	Ca.	Descending	9.0	
W.Hu.	M	70	Ca.	Rectum	10.1	133
T.K.	M	69	Div.	Sigmoid	16.8	216
R.Li.	M	74	Ca.	Rectum	14.1	146
G.L.	M	44	Ca.	Rectum	23.4	246
M.Mi.	F	44	Ca.	Sigmoid	13.4	
M.Na.	F	74	Ca.	Descending	15.2	141
H.N.	M	61	Ca.	Sigmoid	13.3	
R.Nu.	M	69	Ca.	Caecum	18.7	
M.P.	F	70	Ca.	Rectum	7.6	
R.Pe.	M	69	Div.	Rectum	17.4	179
A.Pec.	M	70	Ca.	Sigmoid	7.3	93
H.P.	M	73	Ca.	Rectum	8.2	80
M.Ra.	F	43	Ca.	Ascending	17.9	155
F.Ro.	M	61	Ca.	Transverse	11.9	154
E.Saw.	M	62	Div.	Sigmoid	12.6	141
K.T.	F	54	Ca.	Rectum	17.3	
A.Tov.	M	83	Ca.	Rectum	14.3	
Reg.Wa.	M	63	Ca.	Descending	12.9	141
Rex Wa.	M	54	Ca.	Rectum	9.9	105
(No. of patients)					(25)	(16)
(Males)					(17)	(12)
(Females)					(8)	(4)
[Mean age]					[63.4]	[63.8]
Mean					13.66	147.8
S.D.					4.18	43.2
S.E.M.					0.814	10.8

Ca. = carcinoma of colon or rectum.
Div. = localized diverticulitis

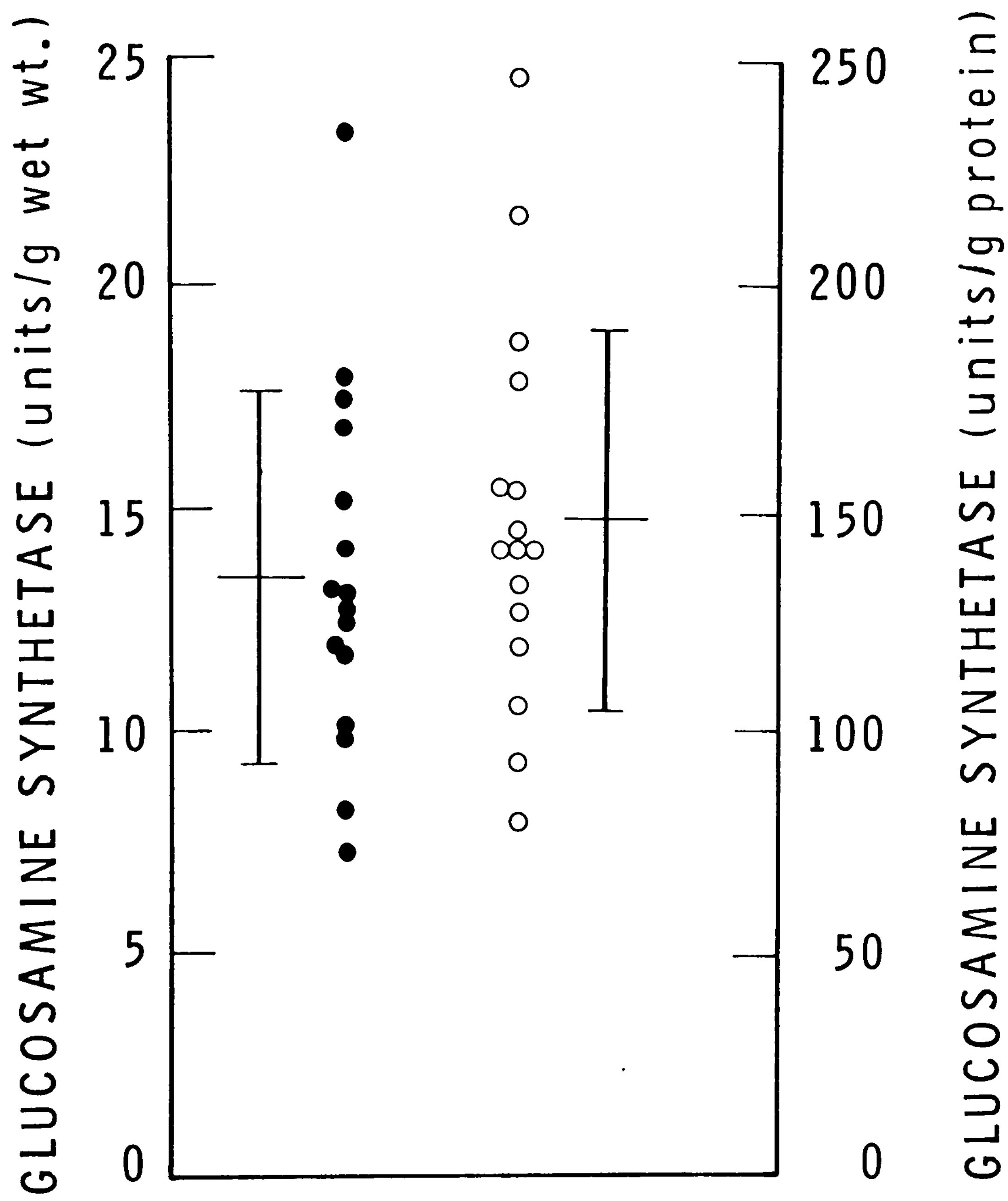


FIG. 42. Glucosamine synthetase levels in normal colonic mucosa obtained from the cut ends of 16 colectomy specimens, in units per g wet wt and in units per g protein.

Region of the bowel

The results are plotted in Fig. 43 in relation to the region of the large bowel. Also plotted are 8 readings of normal colonic mucosa proximal to the splenic flexure, taken from patients with carcinoma of the colon, assayed by the 10 mg method but with duplicates read in the same batch with dilute DAB, giving an estimated additional error of about 7% (see Chapter 8).

Normal submucosa

In the 4 samples of normal submucosa, the glucosamine synthetase levels were measured as 0.3, 0.6, 0.6, and 0.8 units/g wet wt. Readings of less than 1.0 unit can be considered as equivalent to no significant glucosamine synthetase activity detectable by this method.

DISCUSSION

The results from the mucosa are discussed later in this chapter in conjunction with those from the irritable colon syndrome.

Submucosa

There was no significant glucosamine synthetase activity in the submucosa. Kikuchi et al (1971) and Kaufman et al (1971) could find no detectable glucosamine synthetase in muscle (Table 2). The low levels recorded by these workers for glucosamine synthetase in rat intestine were measurements in full thickness bowel; this included the submucosa, which has been shown here to have no significant glucosamine synthetase activity.

A more accurate reading for glucosamine synthetase in the submucosa could have been obtained by assaying a more concentrated homogenate than one of 10 mg/ml, but this was not done and it was considered irrelevant to the assay of the enzyme in the mucosa.

THE IRRITABLE COLON SYNDROME

The irritable colon syndrome is a functional disorder in which the patient complains of abdominal pain, diarrhoea, or constipation, or more than one of these symptoms (Ritchie, 1973). The syndrome is almost certainly due to

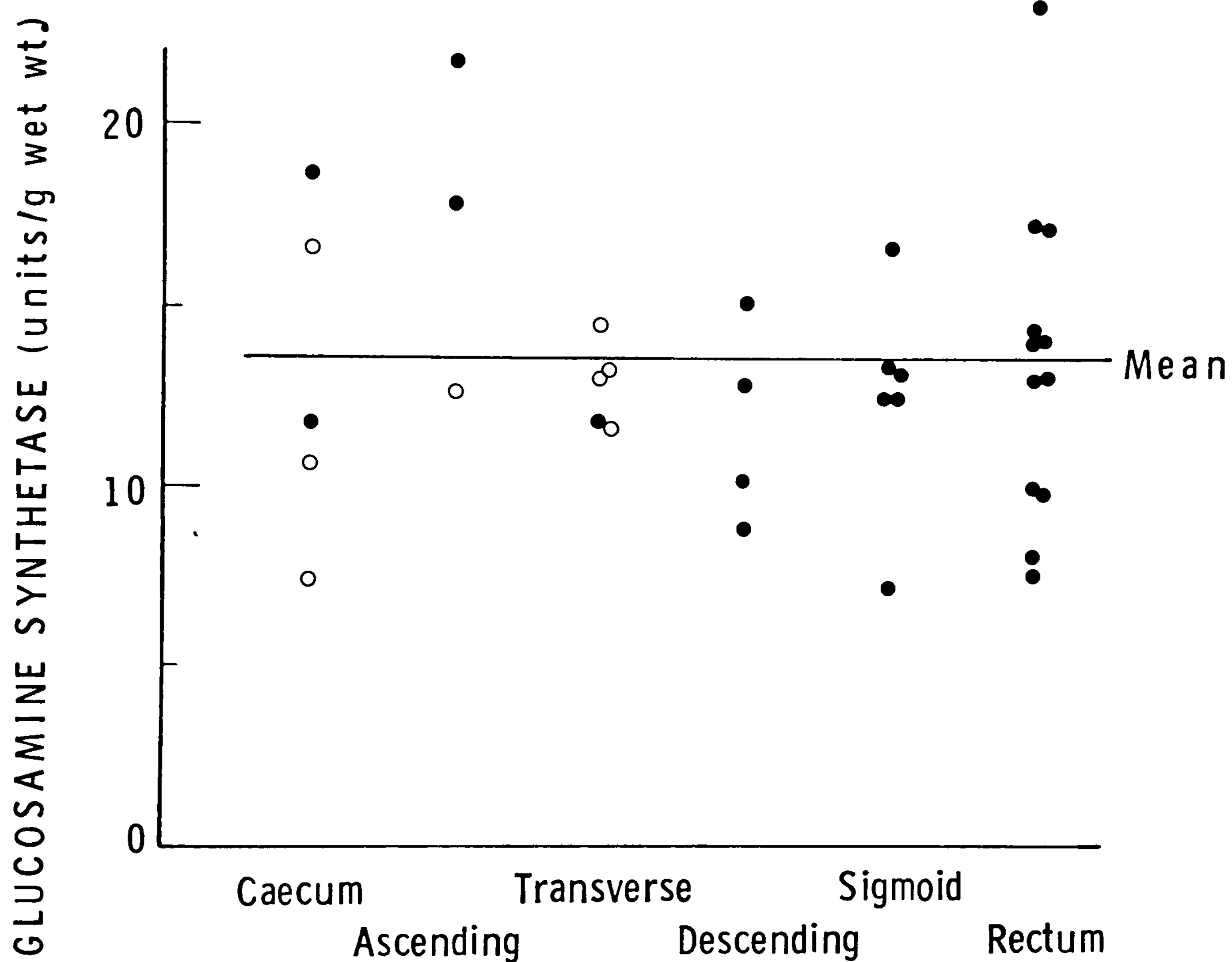


FIG. 43. Glucosamine synthetase levels in normal colonic mucosa, obtained from the cut ends of colectomy specimens for carcinoma of the colon and localized diverticulitis, in relation to the region of the large bowel from which the specimen was obtained. [Closed circles indicate duplicate readings in separate batches with dilute DAB (standard method). Open circles indicate duplicate readings in the same batch with dilute DAB (additional error of about 7%).]

abnormalities of colonic motility, although the symptoms are commonly aggravated by anxiety. Many patients complain of excessive amounts of mucus in their stools and this has resulted in the inaccurate term "mucous colitis" being applied to the condition.

The colonic mucosa is entirely normal in the irritable colon syndrome. There is no evidence of organic disease. The condition is diagnosed on the history and then by exclusion of organic disease. The sigmoidoscopic appearances and the histology of a rectal biopsy must be normal and the barium enema should show no abnormality apart from evidence of disturbed colonic motility, which may be observed in a proportion of the cases. The blood count and the ESR should be normal unless the patient is suffering from another disease which affects these.

Diverticulosis of the colon is an anatomical condition which affects a large proportion of the middle-aged and elderly population of the Western world. Diverticula can become infected and inflamed, giving rise to diverticulitis. Many patients with diverticulosis experience symptoms which are similar to those of the irritable colon syndrome although they are not suffering from diverticulitis (Painter, 1973). Such patients have a normal colonic mucosa apart from the presence of the diverticula, they have a normal blood count and ESR, and the sigmoidoscopic appearances and the histology of a rectal biopsy are normal, as with patients with the non-diverticular irritable colon syndrome.

Patients with the irritable colon syndrome (including those with diverticular disease presenting with symptoms of an irritable colon) are normally treated with anticholinergic drugs, tranquillizers and bulk laxatives. In the Gastroenterology Clinic at the Radcliffe Infirmary, the preparations most commonly used are Probanthine (propantheline bromide) as an anticholinergic, phenobarbitone as a tranquillizer, and Normacol Special (sterculia granules) as a bulk laxative.

<u>The irritable colon syndrome as a source of normal tissue</u>

Since the colonic mucosa is always histologically normal in the irritable colon syndrome, glucosamine synthetase levels were studied in rectal biopsies, taken at the time of diagnosis, in patients with this condition, as a source of normal tissue. The variability of the glucosamine synthetase level in rectal biopsies was studied by taking serial rectal biopsies in patients with the irritable colon syndrome.

The irritable colon syndrome in relation to glucosamine synthetase levels

In view of the role of glucosamine synthetase in mucus synthesis, the levels of glucosamine synthetase in the rectal biopsies of patients with the irritable colon syndrome were studied in relation to the symptom of excessive mucus production in the stools.

The effects on glucosamine synthetase levels of the standard drugs used in the irritable colon syndrome were also studied.

PATIENTS STUDIED

Eighteen patients with the irritable colon syndrome were studied. They were attending the Gastroenterology Clinic at the Radcliffe Infirmary for confirmation of this diagnosis. They had no history of organic gastrointestinal disease, no other organic illnesses, normal sigmoidoscopic appearances, normal barium enema apart from evidence of the irritable colon syndrome, and the rectal biopsies in which the glucosamine synthetase levels were measured were histologically normal. The patients had a normal haemoglobin, white cell count and ESR.

Two patients with diverticular disease and irritable colon symptoms were also studied. Neither patient gave a history suggestive of inflammatory diverticulitis, although one of them suffered from angina of effort.

All 20 patients were questioned closely about drugs they had received in the previous 2 weeks and hormonal preparations (including corticosteroids) that they had received in the previous 3 months. One patient was excluded from the study because he was taking prednisolone phosphate enemas, although he was diagnosed as suffering from the irritable colon syndrome and he was shown to have no organic disease. Two young women taking oral contraceptives were not excluded from the study.

All 20 patients were asked whether they had noticed mucus in their stools.

The clinical details of each patient were recorded on a pro forma (Fig. 44).

Two patients with the non-diverticular irritable colon syndrome who were on no drug therapy when a rectal biopsy was taken at the time of diagnosis agreed to try each of the three standard preparations used in the irritable colon syndrome for 2 weeks at a time and then to undergo a further sigmoidoscopy and biopsy. It was explained that this was in order to investigate the action of the drugs used in their condition.

GmS STUDY

Category　　No. in category

Name　　Hosp. No.　　Ward

Address

D.O.B.　　Sex

G.P.

History:

Ba. enema:

Treatment prior to study:

Biopsy details

Date	Biopsy No.	Sig.grade	Histology No.	Histology details	GmS (1)	GmS (2)	GmS (mean)

Clinical details

Date	Hb	WBC	ESR	SMA	Clinical progress	Treatment	Next appt.

FIG. 44. Pro forma used to record clinical details of patients whose rectal biopsies were assayed for glucosamine synthetase.

Four other patients, two with and two without diverticular disease, also agreed to undergo sigmoidoscopy and rectal biopsy 1 month and 2 months after the initial biopsy.

METHODS

The rectal biopsy, which was being obtained for the diagnosis of the irritable colon syndrome, was taken at 10 cm from the anal margin through a rigid sigmoidoscope using cupped biopsy forceps (Fig. 13) and transferred to a piece of filter paper using a fine paint brush which had been washed in normal saline to remove any antiseptic. The paint brush was used to lay the biopsy flat on the filter paper. A scalpel blade was used to cut the biopsy in half at right angles to the plane of the mucosa. One half was picked up with forceps and placed in a bijou bottle containing ice-cold homogenizing buffer for the glucosamine synthetase assay. The other half was placed in formol saline, with the filter paper adherent to ensure orientation, and sent to the Pathology Department for routine diagnostic histology.

Patients who were due to have a barium enema on the same day as their sigmoidoscopy and rectal biopsy, as was the practice at the Radcliffe Infirmary at the time of this study, had the rectal biopsy taken prior to the wash-out for the barium enema (so that the wash-out would not interfere with the mucosal enzyme) but, as a precaution against perforation, a plain abdominal X-ray was taken after the biopsy and before the wash-out.

Serial studies

The two patients in whom the effects of the drugs used in the irritable colon syndrome were studied were prescribed one preparation for 2 weeks then had a second sigmoidoscopy and rectal biopsy, then were prescribed a second preparation for 2 weeks then had a third rectal biopsy, and were then prescribed the third preparation for a further 2 weeks followed by a fourth biopsy. In one patient, the drugs prescribed were phenobarbitone, Normacol Special, and Probanthine, in that order; the other patient was prescribed Probanthine, phenobarbitone, and Normacol Special, in that order. Each patient was questioned about how diligently the prescribed drug was taken.

The other four patients in whom serial biopsies were taken were not treated according to any particular schedule. They were seen monthly in the out-patient

clinic for their irritable colon syndrome, and sigmoidoscopy with rectal biopsy was performed at the second and third visits.

Rectal biopsy does carry a very slight risk of bowel perforation, although this usually heals with conservative management. This risk was minimized by taking the biopsy always 10 cm from the anal margin, a position which is below the peritoneal reflection.

RESULTS

The results of the glucosamine synthetase readings in the 20 patients with the irritable colon syndrome (including the two with diverticular disease) are shown in Table 15 and in Fig. 48. For the 6 patients who had serial biopsies, only the first reading is shown, in order to obtain a range of readings comparable with the readings from other series of patients.

The mean glucosamine synthetase level was 13.91 units/g wet wt (± 0.85, S.E.M.) with a standard deviation of 3.78, or 27.2%.

The glucosamine synthetase levels were similar in the 5 patients who complained of excessive mucus production and the 15 patients who did not notice mucus in the stools (Table 16 and Fig. 45).

Serial biopsies

The serial readings in the two patients in whom the effects of drugs were studied are shown in Fig. 46. The serial readings of the other 4 patients who had serial biopsies are shown in Fig. 47.

Excessive mucus in the stools

There were no differences in the glucosamine synthetase activity of the rectal mucosa between the 5 patients with the irritable colon syndrome who complained of excessive mucus in the stools and the 15 patients who did not have this symptom (Table 16 and Fig. 45). In one of these patients (W.F.), whose glucosamine synthetase level was 17.6 units, the mucus in the stools was so abundant that he had been referred from Birmingham to Oxford as an in-patient on account of this symptom.

Thus the presence of excessive mucus in the stools of some patients with the irritable colon syndrome is not associated with abnormal glucosamine synthetase levels.

TABLE 15. Glucosamine synthetase activity in 20 rectal biopsies of patients with the irritable colon syndrome

Patient	Sex	Age	Diverticular disease	Mucus in the stools	Current drug therapy	Glucosamine synthetase units/g wet wt
V.Buc.	F	31	-	+	Amitryptiline	12.6
M.Cl.	F	24	-	+	-	15.4
E.Co.	F	65	-	-	-	16.6
W.F.	M	57	-	+	-	17.6
R.Ga.	F	46	-	+	-	15.5
W.Gr.	F	40	-	-	-	10.1
D.H.	F	61	+	-	Practalol	20.2
A.Hol.	F	18	-	-	-	10.3
P.Hu.	M	39	-	-	-	8.3
B.Ly.	M	35	-	-	Normacol Special	15.8
R.Mo.	M	50	-	-	Probanthine	17.6
G.O.	F	48	+	-	Valium, Dulcolax	9.9
P.Ph.	F	29	-	-	-	19.8
A.Pr.	F	31	-	-	-	14.7
I.P.-D.	F	27	-	-	-	12.3
P.Ro.	F	49	-	+	-	9.8
M.Si.	M	23	-	-	-	14.6
L.Ta.	F	21	-	-	Norinyl-1	11.1
G.Wh.	F	22	-	-	-	8.3
A.Wo.	F	35	-	-	Minilyn, Normacol Standard	17.8
Mean age		37.6				
(Number)						(20)
(Males)						(5)
(Females)						(15)
Mean						13.91
S.D.						3.78
S.E.M.						0.85

TABLE 16. Glucosamine synthetase levels in the rectal biopsies of 20 patients with the irritable colon syndrome in relation to the complaint of excessive mucus in the stools

		Excessive mucus	No excessive mucus
(Number of patients)		(5)	(15)
Glucosamine synthetase levels (units/g wet wt)	Mean	14.18	13.82
	S.D.	3.02	4.07
	S.E.M.	1.35	1.05

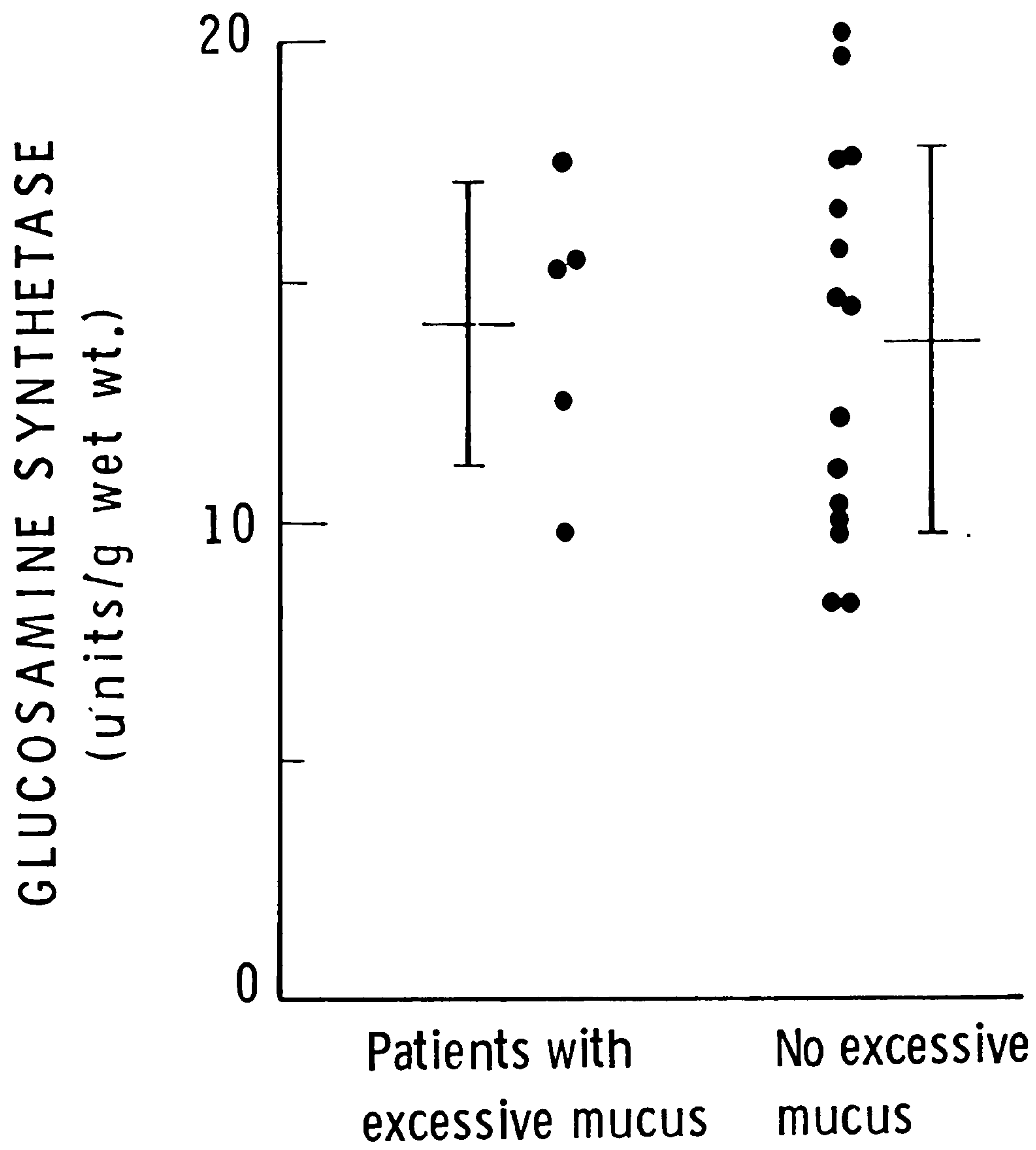
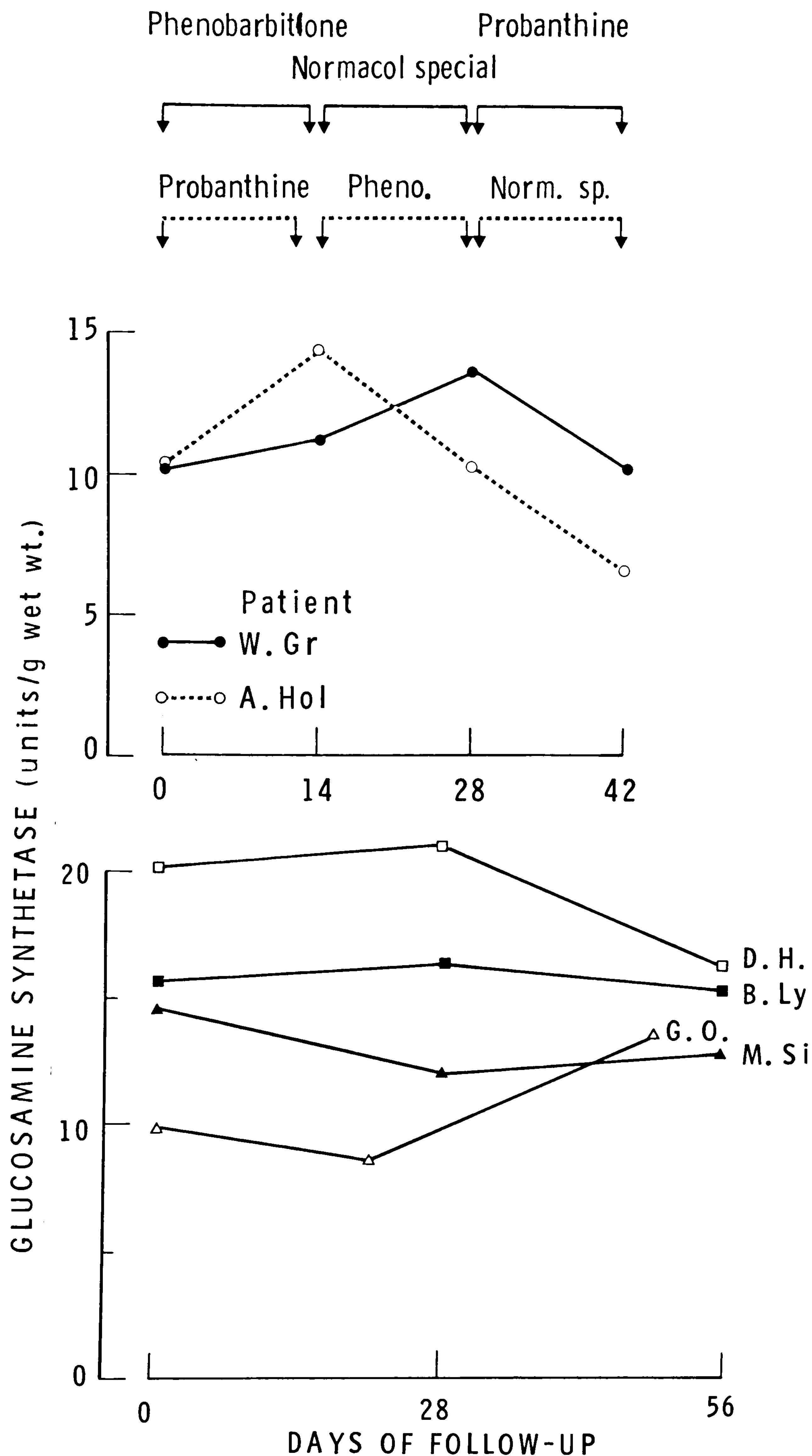


FIG. 45. Glucosamine synthetase levels in the rectal biopsies of patients with the irritable colon syndrome in relation to the complaint of excessive mucus in the stools. (See Table 20.) (Mean ± 1 S.D.)



FIGS. 46, 47. Glucosamine synthetase levels in serial rectal biopsies of patients with the irritable colon syndrome.

FIG. 46 (above): 2 patients receiving each of 3 drugs for a 2-week period.

FIG. 47 (below): 4 other patients.

In Chapter 5, it was suggested that the excessive mucus may be due to impaired bacterial decomposition of mucus in the presence of faecal hurry, but this mechanism was considered unlikely because some mucus-producers have abnormally slow bowel transit. Chaudhary and Truelove (1961) reported exaggerated responses of intraluminal pressure to standard doses of injected prostigmine in patients with the irritable colon syndrome, and animal experiments have shown increased mucus output by the intestine following stimulation by acetylcholine and by prostigmine (Werner, 1953). It is possible that excessive parasympathetic activity is responsible for the abnormal colonic motility found in this syndrome and, if so, this would explain the excessive production of mucus.

The drugs used in the irritable colon syndrome

In the two patients in whom the effects of phenobarbitone, Probanthine and Normacol Special were studied, no differences were observed in the glucosamine synthetase activity of the rectal mucosa (Fig. 46) beyond the expected sampling variation (Fig. 47). It was concluded that none of these drugs had any important effects on colonic mucosal glucosamine synthetase activity and no further studies were carried out. In any event, it was a troublesome study, both for the patient, who had to undergo repeated sigmoidoscopies and rectal biopsies, and for the doctor, who had to recruit the patients and to follow them up and perform the repeated biopsies. There is also a very slight risk of bowel perforation in performing a rectal biopsy, and it was considered that further studies of this aspect of glucosamine synthetase activity were not justified.

Other drugs

The two patients with the irritable colon syndrome taking oral contraceptives, in one case ethinyloestradiol with lynoestrol (Minilyn) and in the other case mestranol with norethisterone (Norinyl-1), had normal glucosamine synthetase levels (17.8 and 11.1 units respectively).

One patient with the irritable colon syndrome who was not included in the series because he was taking prednisolone retention enemas had a glucosamine synthetase level of 13.3 units, which was completely normal.

Apart from the drugs already mentioned, one patient in the irritable colon series was taking the antidepressant drug amitryptiline and one was taking diazepam (Valium) and bisacodyl (Dulcolax). These patients had normal glucos-

amine synthetase levels. One patient, D.H., was taking practalol 100 mg twice daily for angina pectoris and she had the highest glucosamine synthetase level in the series, 20.2 units. This is still within the normal range as determined in the colectomy specimens, and the mean of 3 readings in this patient was 19.2 units, giving a level of 1.35 standard deviations above the normal mean (P = 0.09). In view of the occurrence of sclerosing peritonitis with fibrosis of the peritoneum (Brown et al, 1974), oral ulceration, and fibrosis and scarring of the conjunctiva (Wright, 1975) in patients receiving practalol, this patient perhaps should not have been included in the series. This rather high level of glucosamine synthetase might be related to the mucosal abnormalities caused by this drug.

NORMAL COLONIC MUCOSA: COMBINED RESULTS OF COLECTOMY SPECIMENS AND RECTAL BIOPSIES

The glucosamine synthetase levels in the normal colonic mucosa from the cut ends of the 25 colectomy specimens and from the 20 rectal biopsies of patients with the irritable colon syndrome are compared in Fig. 48 and in Table 17. The mean levels and the standard deviations were similar for the two sources of normal mucosa despite a great difference in mean age of the two groups of patients.

TABLE 17. Glucosamine synthetase in normal colonic mucosa. Comparison of material obtained from the cut ends of surgical specimens for carcinoma of the colon and localized diverticulitis and material obtained from rectal biopsies of patients with the irritable colon syndrome. (Shown diagrammatically in Fig. 48.)

		Colectomy specimens	Rectal biopsies	Colectomies and biopsies together
Number of specimens		(25)	(20)	(45)
Mean age		63.4 years	37.6 years	51.9 years
Glucosamine	Mean	13.66	13.91	13.77
synthetase levels	S.D.	4.18	3.78	3.97
(units/g wet wt)	S.E.M.	0.84	0.85	0.59

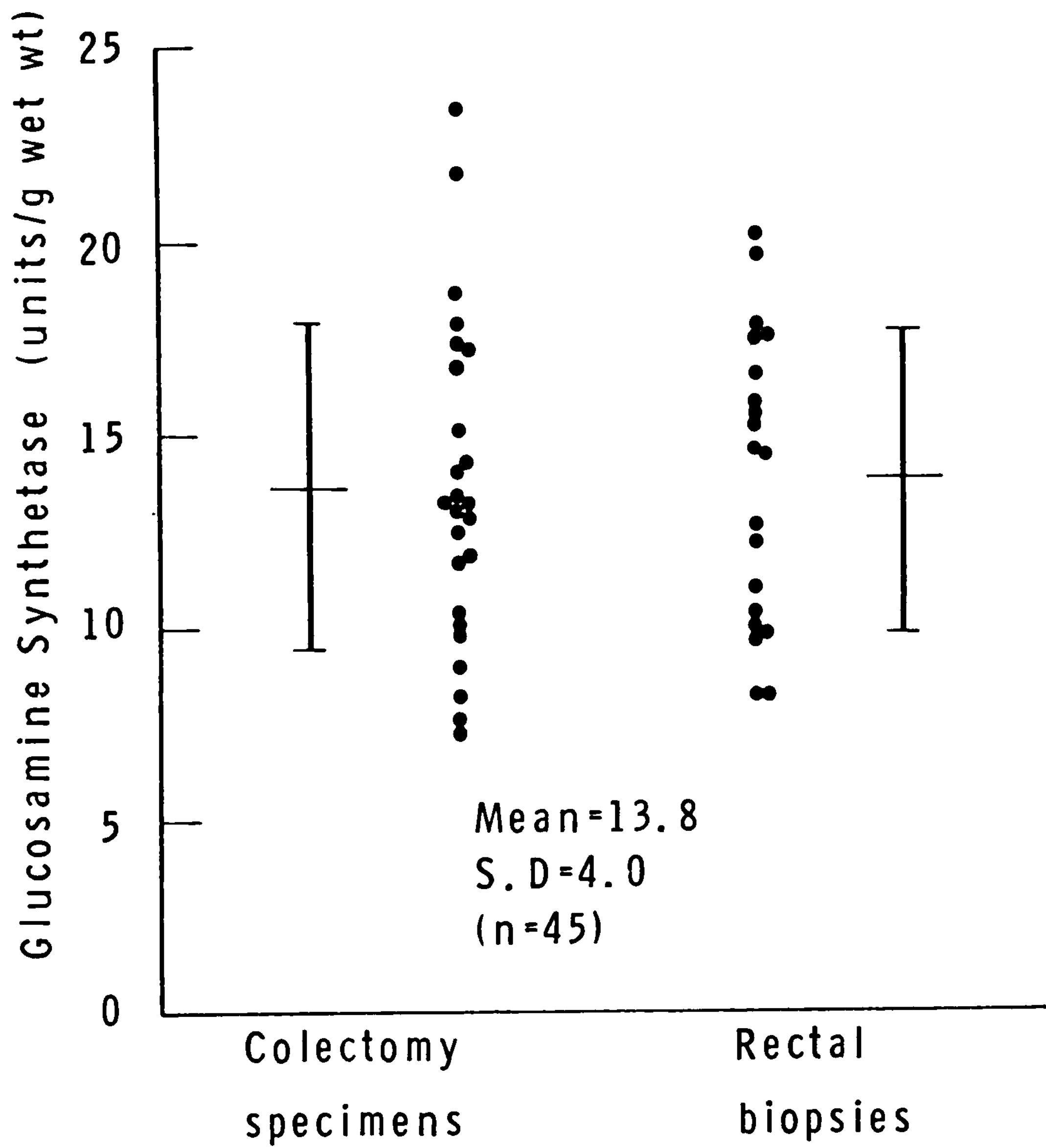


FIG. 48. Glucosamine synthetase levels (in units/g wet wt) in normal colonic mucosa, obtained from the normal cut ends of 25 colectomy specimens (22 specimens removed for carcinoma of the colon and 3 removed for localized diverticulitis), and from the rectal biopsies of 20 patients with the irritable colon syndrome. (See Table 17.)

Relationship to sex

The 45 normal samples of colonic mucosa were taken from 22 male patients and 23 female patients. The mean and standard deviation for each sex is shown in Table 18 and the sex of the patient in relation to the glucosamine synthetase level is indicated in Fig. 49.

TABLE 18. Glucosamine synthetase in normal colonic mucosa. Males and females compared.

		Males	Females
Number of patients		(22)	(23)
Mean age		59.6 years	44.6 years
Glucosamine	Mean	14.1	13.5
synthetase levels	S.D.	4.3	3.7
(units/g wet wt)	S.E.M.	0.91	0.77

Relationship to age

The glucosamine synthetase levels in the 45 samples of normal mucosa are plotted in relation to the age of the patient in Fig. 49. Only patients aged 18 years and over were included in the study.

DISCUSSION

The normal range

The range of glucosamine synthetase levels in the normal colonic mucosa from the cut ends of colectomy specimens was similar to that in rectal biopsies from patients with the irritable colon syndrome, despite a considerable age difference in the two series and despite the fact that the colonic mucosa from the colectomy specimens was obtained from all parts of the colon whereas the rectal biopsies were all taken 10 cm from the anal margin. Also, the 10 mg assay was used for the rectal biopsies.

For the 45 samples of normal colonic mucosa, all from different patients, the mean glucosamine synthetase level was 13.77 units (μmoles glucosamine synthesized/h/g wet wt). The standard error of this mean was 0.59. The standard deviation of the series was 3.97 units.

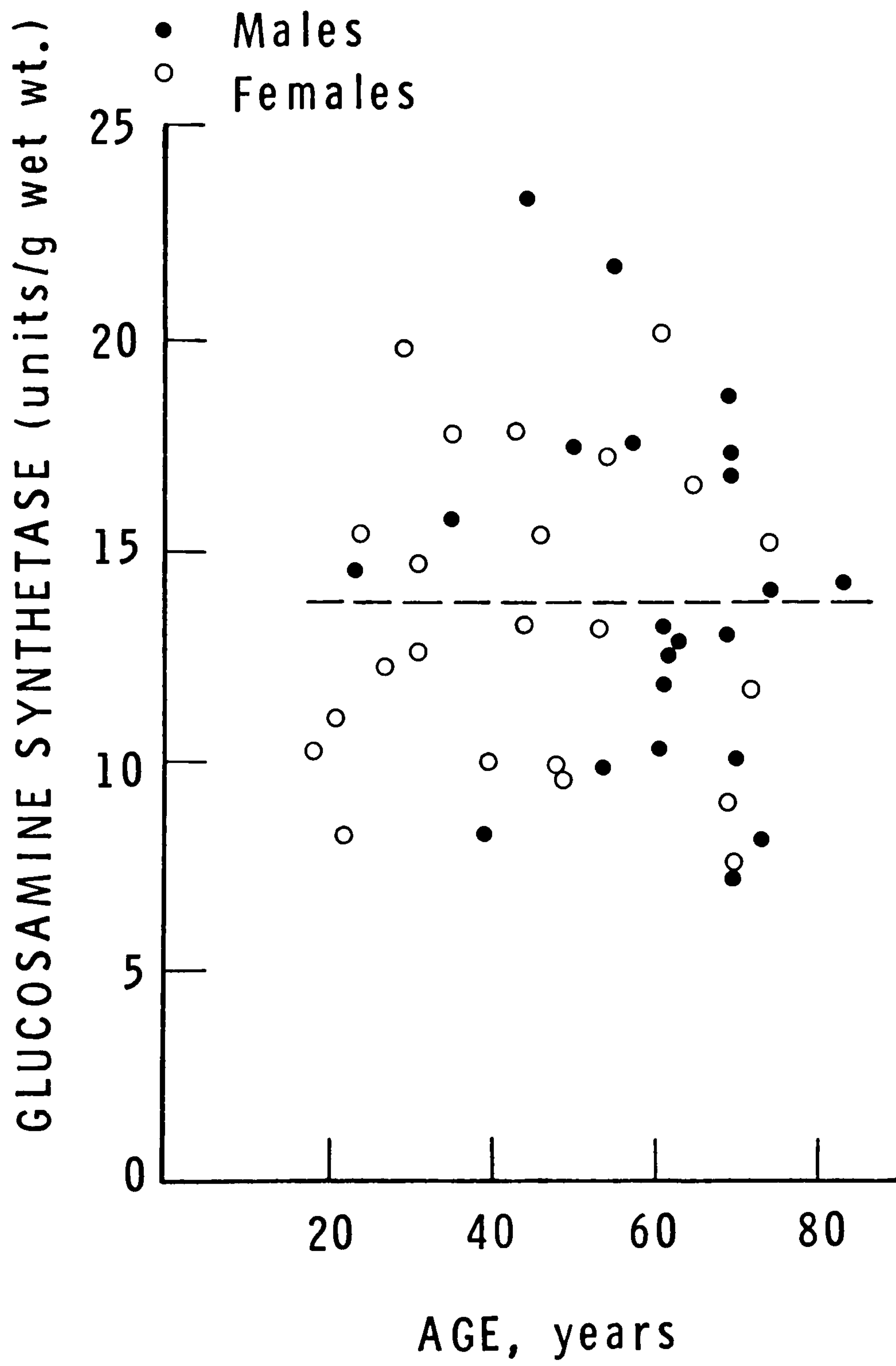


FIG. 49. Glucosamine synthetase levels in normal adult colonic mucosa, according to age and sex.

When plotted as a histogram, the distribution actually appeared to be slightly skew with a tail at the upper end (Fig. 50). On a logarithmic scale, the distribution tails more at the lower end (Fig. 51).

Enzyme activity per gram protein

The glucosamine synthetase activity was measured both in units/g wet wt and in units/g protein in 16 of the colectomy specimens (Fig. 42). The mean level was 147.8 units/g protein (± 10.8, S.E.M.) and the standard deviation was 43.2 units. For these 16 specimens, the mean enzyme level in units/g wet wt was 13.48 ± 4.05 (S.D.). Thus the S.D. was 29.2% when the enzyme level was expressed in units/g protein and 30.0% when expressed in units/g wet wt, confirming that readings per g protein gave little useful additional information. These samples are included in the plot of enzyme activity per g protein versus activity per g wet wt in Figs. 38 and 39.

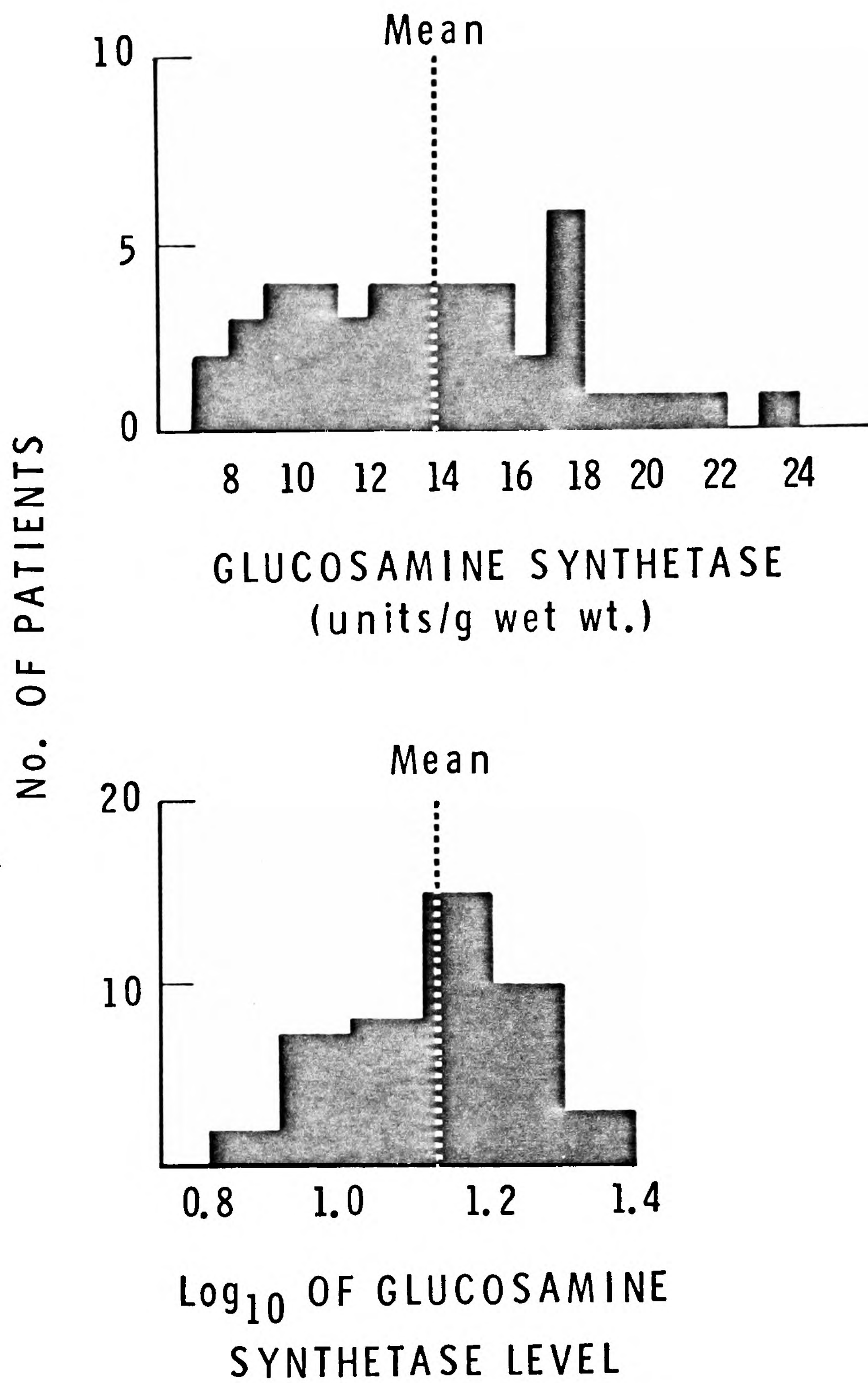
Relationship to the diagnosis

The mean glucosamine synthetase level in the normal mucosa of the 22 colectomy specimens for carcinoma of the colon was 13.39 (± 0.92, S.E.M) and the standard deviation was 4.32 units. Glucosamine synthetase in the normal mucosa immediately adjacent to the tumour is discussed in Chapter 12.

In the 18 patients with the irritable colon syndrome and without diverticulosis coli, the mean glucosamine synthetase level of the rectal mucosa was 13.78 (± 0.84, S.E.M.) with a standard deviation of 3.57.

The 3 patients with diverticulitis in the colectomy series and the 2 patients with diverticulosis in the biopsy series had a mean glucosamine synthetase level in the normal mucosa of 15.38 (± 1.88, S.E.M.); the standard deviation of this small series was 3.76 units.

Thus the normal colonic mucosa in patients with carcinoma of the colon has similar glucosamine synthetase activity to the rectal mucosa in patients with the irritable colon syndrome. These two disorders of the large bowel have no common features, and they tend to affect different age groups, so that it can be assumed that these levels of glucosamine synthetase represent the normal range. The 5 readings from patients with diverticular disease were also within this range.



FIGS. 50, 51. Histograms of glucosamine synthetase levels in the normal colonic mucosa of 45 patients.

FIG. 50 (above): Linear distribution

FIG. 51 (below): Logarithmic distribution.

The region of the bowel

No gross differences were observed in the glucosamine synthetase activity of colonic mucosa in different regions of the large intestine (Fig. 43). Only 5 readings were taken proximal to the splenic flexure in the definitive series of results but these were supplemented by 8 readings of normal colonic mucosa proximal to the splenic flexure in preliminary work in which the duplicates in the assay had been read in the same batch with dilute DAB, which increased the error of the method by an estimated 7%.

Sex

No detectable differences were found in glucosamine synthetase activity in the colonic mucosa between males and females (Table 18).

Age

All the patients studied were 18 years of age or more. Specimens were obtained from all decades of age and the glucosamine synthetase levels in adult colonic mucosa did not appear to have any relationship to age (Fig. 49).

Sampling variation

Serial studies of drug therapy in the irritable colon syndrome in 2 patients showed no differences due to the drugs beyond those shown by 4 other patients who had serial biopsies but were not in the formal drug study. When these 6 patients were taken together, the standard deviations of the 3 or 4 serial readings in each patient (mean, 3.3 readings) had a mean of 16.2%. This degree of variation is similar to that of the series of 10 samples of normal colonic mucosa taken from each of 4 colectomy specimens (Chapter 8), which was 17.6% (Table 12).

CONCLUSIONS

Glucosamine synthetase levels have been measured in the normal human colonic mucosa in the normal cut ends of colectomy specimens and in rectal biopsies of patients with the irritable colon syndrome. In both of these series there was a similar range, and a normal range for glucosamine synthetase has been established for the adult colonic mucosa of 13.77 ± 3.97 (S.D.) units/g wet wt. The distribution has a tail at the upper end. In all regions of the

large intestine, the levels were similar. There were no detectable differences with regard to age or sex.

There was no glucosamine synthetase activity detectable by this method in the submucosa of normal colon.

Phenobarbitone, Probanthine and oral contraceptive drugs containing oestrogen and progestogen did not appear to affect the activity of the enzyme. One patient taking practalol had a level which was high but within the normal range.

In the irritable colon syndrome, patients who complained of excessive mucus in the stools had normal glucosamine synthetase levels.

CHAPTER 10

ULCERATIVE COLITIS AND CROHN'S DISEASE

Goblet cell depletion in ulcerative colitis

This term is used to describe two features that are often found in the mucosa of patients with ulcerative colitis. One is a diminution in the number of crypts, which may in themselves contain a normal proportion of goblet cells (Fig. 52). This can occur in mild ulcerative colitis and in chronic cases in which there is no inflammation at all in the lamina propria. It is best described as atrophy of the crypts. The second feature is a diminution, which may be gross, in the proportion of goblet cells in the crypts, which instead contain mainly columnar cells which do not appear to contain mucus (Fig. 54). The two features often occur separately probably more often than they occur together (Fig. 55). In the presence of severe inflammation, the inflammatory cells can destroy the epithelium (Fig. 62) but this should not be considered as goblet cell depletion and it can occur in acute Crohn's colitis as well as in ulcerative colitis.

A diminution in the proportion of goblet cells amongst the epithelial cells is easiest to establish by using specific stains for mucus, such as Alcian blue (Figs. 53, 54). Filipe and Dawson (1970) found that there was depletion of mucus in the mucosa in ulcerative colitis in proportion to the degree of inflammation in the lamina propria. In normal colon, the lower two-thirds of the crypt contains sulphated mucus and the upper one-third and the superficial epithelium have a mixture of sulphated and acidic non-sulphated mucus (Fig. 86). Filipe and Dawson found that in ulcerative colitis sulphated mucus rather than non-sulphated mucus was depleted (Fig. 56).

When the goblet cells are diminished as a proportion of the epithelial cells, this may simply mean that there are more immature cells in the mucosa.

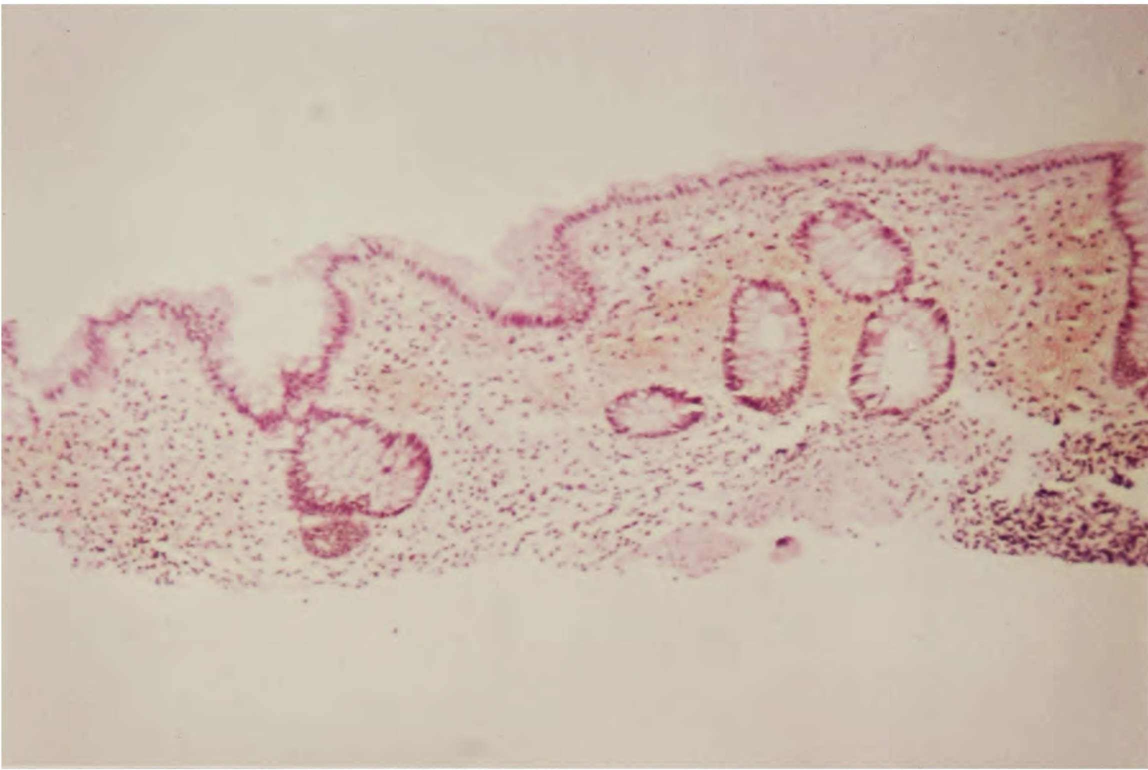


FIG. 52. Atrophy of the crypts in chronic ulcerative colitis.

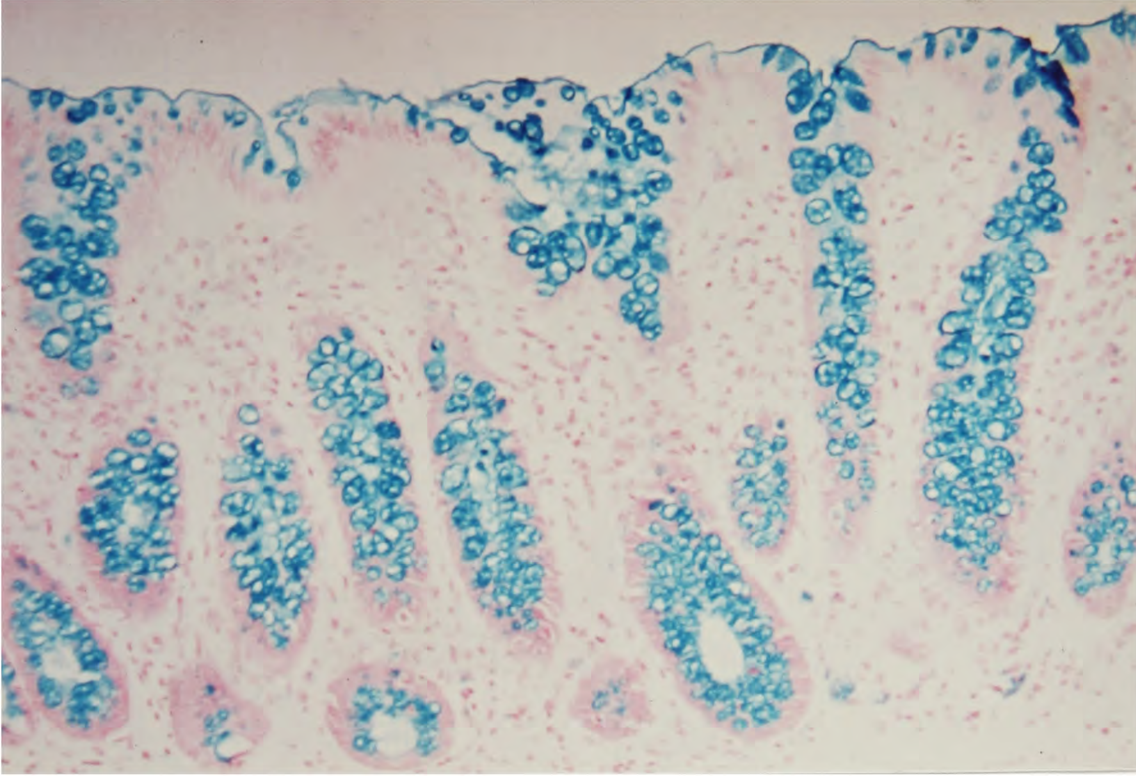


FIG. 53. Normal rectal mucosa, stained with Alcian blue at pH 2.5.

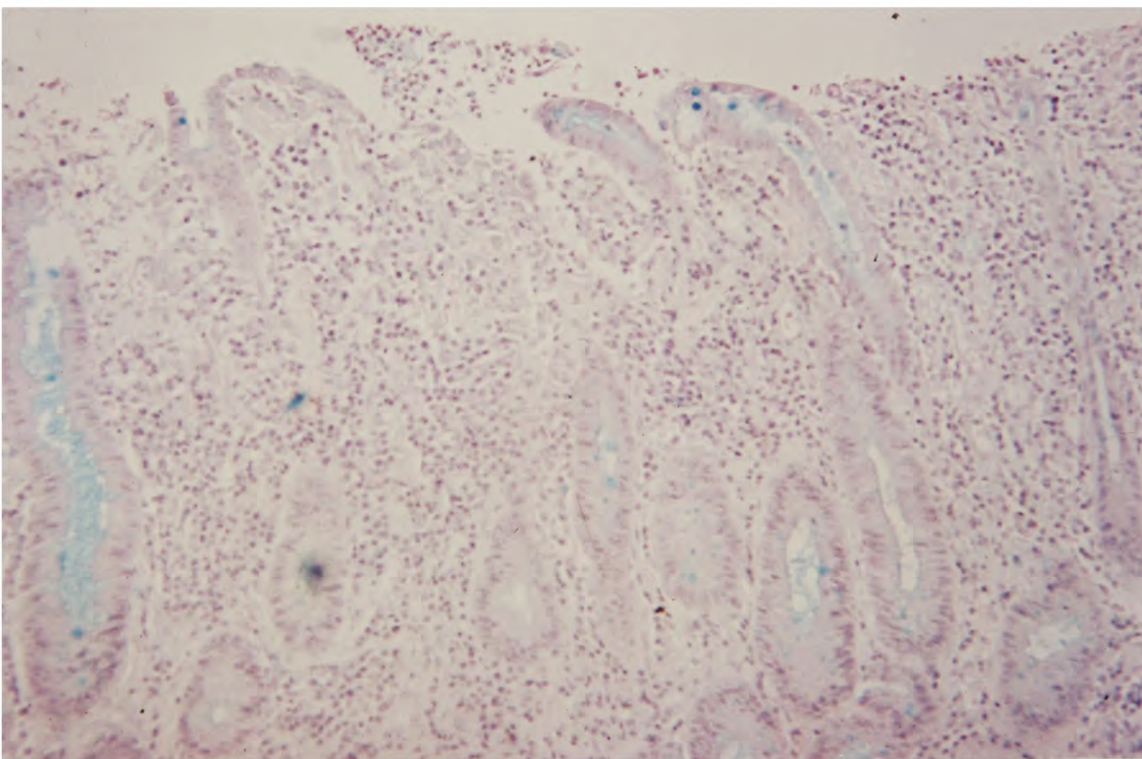


FIG. 54. Ulcerative colitis. Diminution in the proportion of goblet cells in the crypts. Stained with Alcian blue at pH 2.5 to show mucus in the goblet cells.

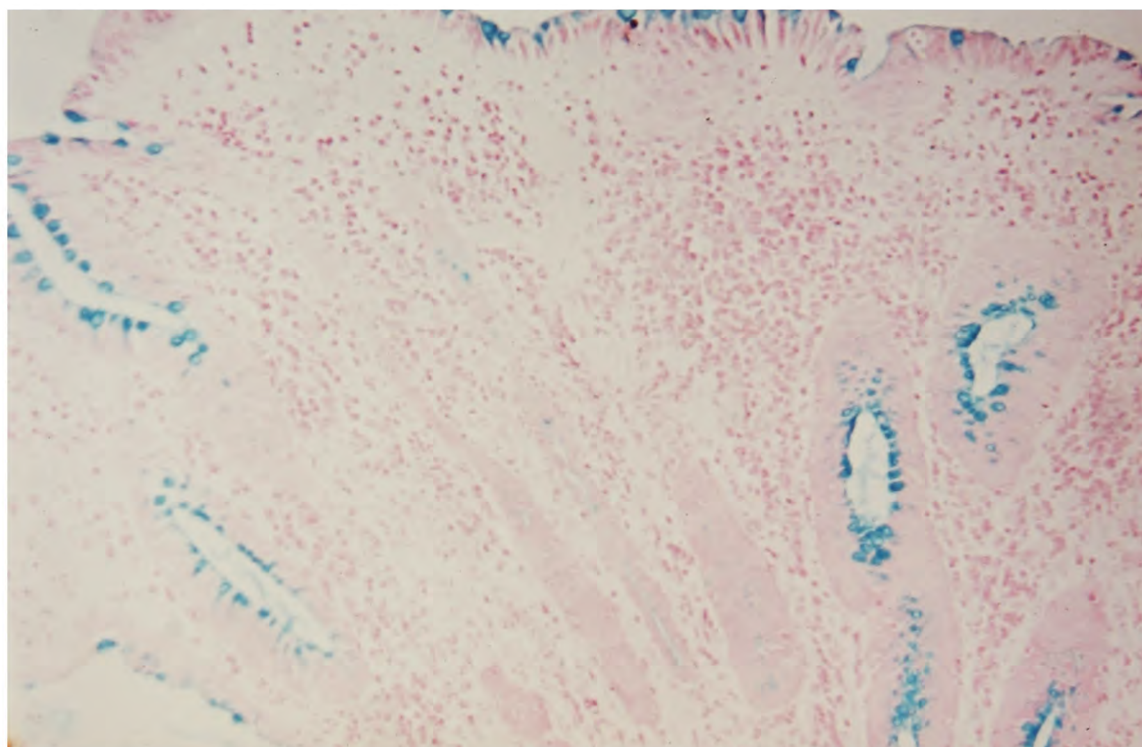


FIG. 55. The rectal mucosa of a patient with ulcerative colitis, showing both crypt and goblet cell depletion. Stained with Alcian blue at pH 2.5.

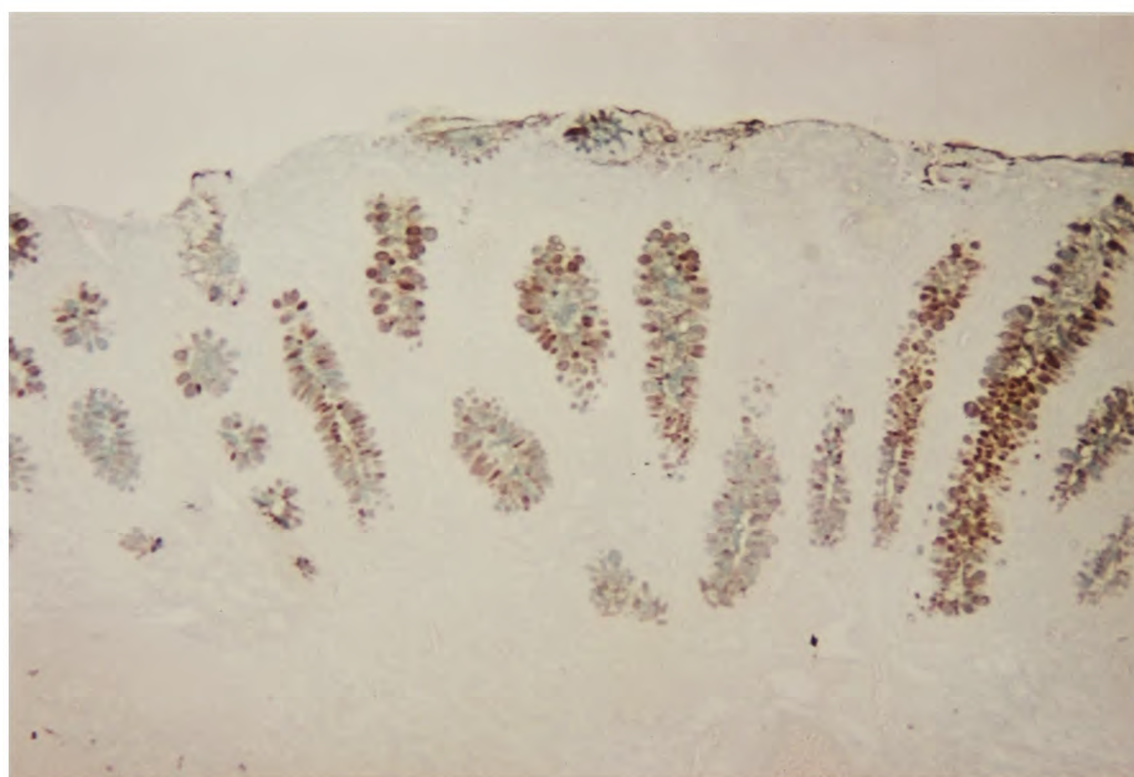


FIG. 56. Colonic mucosa in ulcerative colitis, stained with high iron-diamine (to show sulphated mucus) and Alcian blue (to show acidic non-sulphated mucus).

Weiser (1973b) showed that the mucus molecule acquires longer side-chains as its parent cell migrates towards the tip of the villus in the small intestine and it may be that the goblet cell represents a mature cell in which the mucus molecules have been completed. Merzel and Leblond (1969) demonstrated on electron microscopy of the mouse small intestine that as well as columnar cells and goblet cells there are also intermediate forms of intestinal epithelial cell which contain only one or a few mucous granules. These were termed "oligomucous cells" and "granulomucous cells" and they occurred mainly in the lower parts of the crypts. On autoradiography after the injection of ^{3}H-thymidine, the label was seen in the columnar cells and in the oligomucous cells at 1 hour after injection but it did not appear in the goblet cells until 12 hours. Also, Merzel and Leblond considered that there were not enough oligomucous cells to account for the immature forms of all the goblet cells. Their conclusion was that goblet cells are derived from columnar cells, with oligomucous cells as an intermediate stage.

Thus the goblet cell depletion in ulcerative colitis may simply be a reflection of a more rapid turnover of epithelial cells, resulting in the presence in the mucosa of more of the immature columnar cells and fewer of the mature goblet cells. The mean turnover time of the epithelial cells in ulcerative colitis has been calculated by Bleiberg et al (1970) to be 34 hours, compared to 90 hours for the epithelium of normal mucosa.

The suggestion in Chapter 3 that goblet cells containing sulphated mucus are less mature than cells containing non-sulphated mucus is not consistent with the finding of Filipe and Dawson that non-sulphated rather than sulphated mucus is seen in the remaining goblet cells in the mucosa in ulcerative colitis when it is depleted of goblet cells, if these are less mature goblet cells. The mucus in ulcerative colitis may, of course, be different from that in normal goblet cells.

The differential diagnosis of ulcerative colitis and Crohn's disease of the colon

Goblet cell depletion in the colonic mucosa is uncommon in Crohn's disease of the colon (Fig. 57) (Cook and Dixon, 1973). This difference has been used by pathologists in the differential diagnosis of ulcerative colitis and Crohn's disease (Morson and Dawson, 1972). In view of the clinical importance of making an accurate diagnosis (Truelove and Lee, 1973a,b) and in

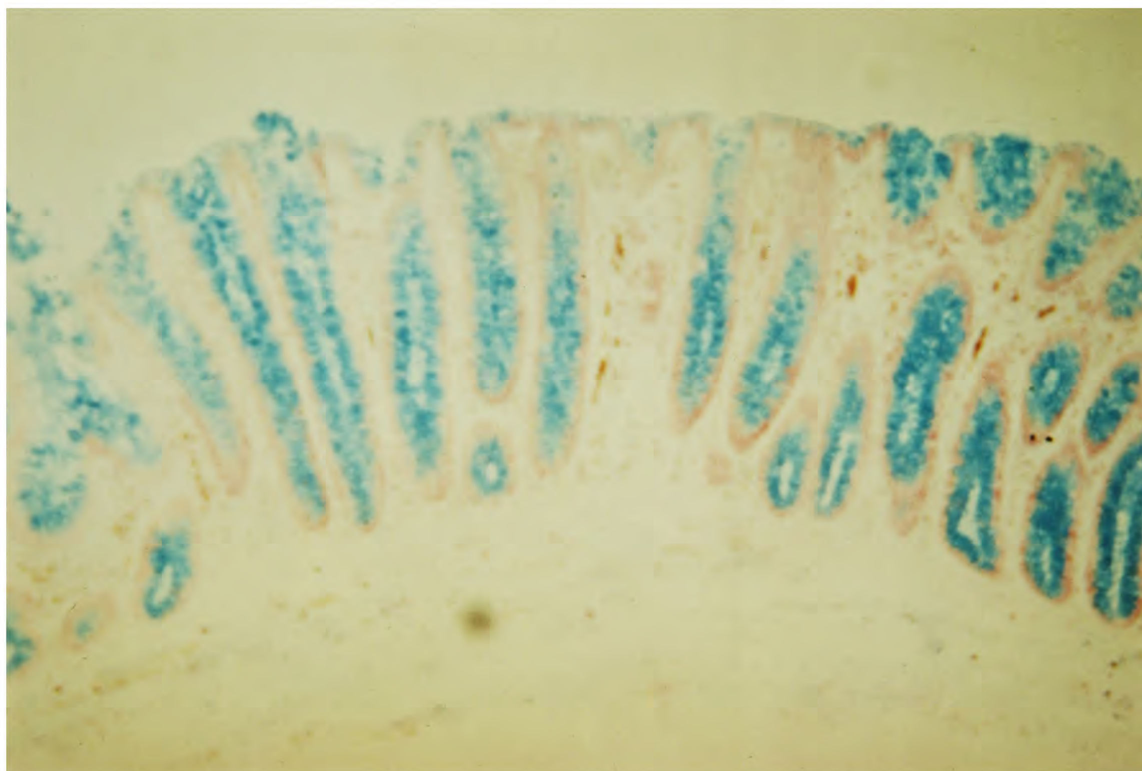


FIG. 57. Rectal mucosa in Crohn's disease, stained with Alcian blue at pH 2.5.

view of the difficulty which even the most experienced clinicians can find in differentiating ulcerative colitis and Crohn's disease confined to the colon (Lee and Truelove, 1975), it would be a great help in clinical practice if this difference in the appearance of the mucus-producing cells could be expressed biochemically and result in the ability to distinguish between these two diseases more reliably than by present histological and radiological criteria.

The biochemistry of the mucus in ulcerative colitis

This has received very little attention. Soergel and Ingelfinger (1964) found serum proteins, especially the α-glycoproteins, in the rectal mucus obtained by irrigation of the rectum with hypertonic phosphate solutions in patients with ulcerative colitis. There was 50% more nitrogen in the mucus in ulcerative colitis than in normal patients but the carbohydrate components and the tyrosine components were similar and no differences could be detected on electrophoresis of the proteins.

Teague et al (1973) obtained biopsies of specimens of colonic mucosa from the recto-sigmoid junction through a sigmoidoscope and at fibreoptic colonoscopy. The monosaccharide content of the glycoprotein in the biopsies was analysed by gas chromatography and the results were expressed in proportion to the galactose content. There was a significantly higher proportion of mannose in the mucosa of patients with ulcerative colitis when compared to normal subjects and when compared to patients with diarrhoea due to other causes, although patients with Crohn's disease were not studied specifically. This additional mannose may have come by exudation through the inflamed mucosa of serum glycoproteins, which typically contain mannose, or it may have been due to the local production of immunoglobulins, which also contain mannose. The immunocytes producing specific immunoglobulins can be demonstrated in histological sections using an immunoperoxidase method and the application of quantitative histology has shown that such immunocytes are greatly increased in the lamina propria in ulcerative colitis (Skinner, 1975).

Glucosamine synthetase has not previously been studied in the colonic mucosa in ulcerative colitis or in Crohn's disease.

PATIENTS STUDIED AND METHODS

Colectomy specimens

Twenty colectomy specimens were studied. Nineteen were panprocto-colectomy specimens for ulcerative colitis or Crohn's disease of the colon, and one was a subtotal colectomy specimen with the rectal stump being left *in situ* in a patient with Crohn's disease of the colon. Fourteen of the pan-proctocolectomy specimens were removed for severe ulcerative colitis, one was removed for Crohn's disease of the colon, and four were removed for persistent colonic disease in patients who had received a diversionary split ileostomy 1-4 years previously for Crohn's disease of the colon (Lee and Truelove, 1975). These patients with a split ileostomy had an isolated colon which was perfused daily with solutions of hydrocortisone hemisuccinate.

Personal and clinical details of the patient and details of the operation and the anaesthetic were recorded on a *pro forma* (Fig. 41). All medications taken by the patient in the 3 days pre-operatively were recorded, as well as any hormonal preparations such as corticosteroids which had been taken in the preceding 3 months.

Ulcerative colitis or Crohn's disease?

The differential diagnosis of ulcerative colitis and Crohn's disease of the colon can be difficult on clinical grounds, even when the histology of rectal biopsies is known and even when there has been a laparotomy, as in the patients with a split ileostomy. The diagnosis can only be made with maximum certainty when the whole colon is removed and examined macro-scopically and histologically by an expert pathologist. Even then, there are sometimes cases that cannot be definitely diagnosed as having one disease rather than the other (Filipe and Dawson, 1970). In this study, all 20 colect-omy specimens for ulcerative colitis and Crohn's disease were examined by one of two expert pathologists, Dr. R. Whitehead and Dr. J. M. Skinner, and their decision was taken as the final diagnosis. They considered that two of the 4 patients who had had a panproctocolectomy following a split ileostomy for Crohn's disease of the colon had, in fact, ulcerative colitis (Lee and Truelove, 1975).

Thus, on the final diagnosis, 16 out of the 20 colectomy specimens

were from patients with ulcerative colitis and 4 were from patients with Crohn's disease of the colon.

The sampling of the mucosa

From the 19 panproctocolectomy specimens, samples of mucosa were taken from three areas: (1) 10 cm from the anal margin, corresponding to the level of rectal biopsies; (2) from the most severely inflamed area as judged macroscopically; and (3) from the least inflamed area. From the subtotal colectomy specimen, the first sample was taken from the sigmoid colon.

The assay of the samples

From the 20 colectomy specimens, 59 samples of mucosa were obtained (the sample from the least inflamed area in one patient was lost due to laboratory error). All were assayed for glucosamine synthetase using the 10 mg method.

In 41 of the samples (29 from 11 patients with ulcerative colitis and 12 from 4 patients with Crohn's disease) the glucosamine synthetase activity was measured in units per g wet wt and in units per g protein. In the other 18 samples, the enzyme activity was measured only in units per g wet wt.

Submucosa

In one of the specimens, in the 10 cm sample (which was inflamed) and in the most inflamed sample, after dissection of the mucosa a portion of the submucosa was also assayed for glucosamine synthetase.

The apparently normal mucosa in Crohn's disease

It was not intended to study specifically the apparently normal mucosa in Crohn's disease. However, the enzyme was assayed in the macroscopically normal cut end at the colonic resection line in 2 right hemicolectomy specimens for Crohn's disease of the ileo-caecal region in patients who had never been diagnosed as having Crohn's disease distal to the caecum.

Histological correlations

The three samples of mucosa from each of 12 of the panproctocolectomy specimens (10 of ulcerative colitis and 2 of Crohn's disease) were orientated on filter paper and divided in two. One portion was placed in formol saline

and sectioned in the Pathology Department and the other portion was homogenized for the glucosamine synthetase assay. The 36 histological sections (from the 12 colectomy specimens) were stained with haematoxylin and eosin and with Alcian blue at pH 1.

The 36 histological sections so obtained were coded and examined without the glucosamine synthetase activity being known. They were graded according to the degree of inflammation and according to the appearances of the epithelial cells (see below).

Correlative histology was also obtained for the two samples of submucosa and for the mucosa that was stripped from the submucosa in these two samples.

Accurate correlative histology was not obtained for the remaining 21 samples of mucosa.

Rectal biopsies

Rectal biopsies from 105 patients who were considered by the clinician treating them to have ulcerative colitis or Crohn's disease were assayed for glucosamine synthetase by the 4 mg method. Eighty of the patients were thought to have ulcerative colitis, 24 were thought to have Crohn's disease, and in one patient the diagnosis was undecided between Crohn's disease and ulcerative colitis. They were patients attending the Ulcerative Colitis Clinic at the Radcliffe Infirmary (which patients with Crohn's disease and other forms of colitis also attend). All biopsies were taken 10 cm from the anal margin.

Serial biopsies

In 26 patients who were considered to have an acute attack of ulcerative colitis, as defined by moderate or severe inflammation on sigmoidoscopy and a clinical condition which required treatment with at least 20 mg prednisolone daily, serial rectal biopsies were taken, with half of each biopsy being sent for histological examination and half being assayed for glucosamine synthetase. The aim was to take biopsies at the time the attack was diagnosed and after 5 days, 10 days, 6 weeks, 3 months and 6 months. It was not always possible to obtain biopsies at these particular times and

patients were included in this series if two or more biopsies were taken, with the second biopsy no later than 14 days after the first biopsy. In 3 patients, serial biopsies were taken in two separate attacks of the disease.

In 20 of the attacks, the patients were treated with 5 days of intravenous prednisolone-21-phosphate, 40-60 mg daily, intravenous tetracycline (1 g daily), and twice daily rectal infusions of a solution of hydrocortisone hemisuccinate, and they received only intravenous nutrition, being allowed to take nothing orally apart from sips of water (Truelove and Jewell, 1974). Following this 5-day regime, the patients were usually treated orally, with prednisolone, sulphasalazine (Salazopyrin), and prednisolone retention enemas. Prednisolone was given 40 mg daily for 5 days, then 30 mg daily for 5 days, then 20 mg daily until 6 weeks after the start of the intravenous regime. If the patient had a normal-looking mucosa at 6 weeks, the prednisolone was tailed off over the next week; if the mucosa was still mildly inflamed or if the patient had been on steroids for a long period before the regime had started, the prednisolone dosage was reduced more slowly. Salazopyrin was prescribed at a dosage of 0.5 g four times daily and was continued indefinitely. Prednisolone retention enemas, or in some patients hydrocortisone enemas, were given twice daily for the first 10 days following the intravenous regime and then once daily until 6 weeks. If the mucosa appeared normal, they were tailed off in the week after the cessation of oral prednisolone.

Nine attacks which were less severe were treated orally, with prednisolone 20-40 mg daily, Salazopyrin, and prednisolone retention enemas twice daily. By the 10th day, all the patients were receiving prednisolone 20 mg daily, Salazopyrin, and one prednisolone enema daily, and this was continued until 6 weeks, after which the same protocol was followed as for the patients who had had the intravenous regime.

Serial biopsies were also taken from one patient with an acute attack of Crohn's disease of the colon which spared the rectum, who was treated with the intravenous steroid regime as for ulcerative colitis.

<u>Control samples</u>

With 3 biopsies from patients with ulcerative colitis, 0.2 ml of the homogenate was treated with perchloric acid without being incubated, in

order to assess the hexosamine content of the mucosa in ulcerative colitis in case these control samples gave results which significantly affected the glucosamine synthetase reading, which was not the case with control samples containing normal mucosa (Chapter 8).

Confirmation of the diagnosis

The hospital case-notes, reports of the histology of rectal biopsies and of resected segments of bowel, and reports of barium X-ray examinations were studied in detail. The information available included pathological reports on the panproctocolectomy specimens of 11 patients who were subsequently treated surgically.

On the basis of this more detailed examination of the diagnosis, 73 patients were considered to have ulcerative colitis, 22 to have Crohn's disease, 5 to have either ulcerative colitis or Crohn's disease but with no certainty as to which, and in 5 there was insufficient evidence to be certain that the patient had inflammatory bowel disease, one of these patients almost certainly having only the irritable colon syndrome.

Sigmoidoscopic appearances

These were classified by the author personally in all cases. For the patients with ulcerative colitis, the sigmoidoscopic appearances were graded as normal, mild inflammation, moderate inflammation, and severe inflammation, according to the classification of Truelove (Truelove and Reynell, 1972). These were taken as grades 0, 1, 2, and 3, respectively. In many cases, the appearances were considered to be intermediate between these grades and were designated, for example, 1-2 for mild-to-moderate inflammation.

For patients with Crohn's disease, a similar classification was used, from 0 to 3 according to whether the rectum appeared normal, mildly inflamed, moderately inflamed, or severely inflamed.

Histological grading

Of each rectal biopsy assayed for glucosamine synthetase, half was used for the enzyme assay and half was sent for routine histology. Histological reports for clinical use were written by various pathologists at the Radcliffe Infirmary, although mainly by Dr. R. Whitehead and Dr. J. M. Skinner,

who graded the degree of inflammation as mild, moderate, severe, or no inflammation; intermediate gradings such as mild-to-moderate were also used. Where such grading was not stated in the report, the details of the report were studied and the degree of inflammation was graded according to the detailed description. The pathologist was always informed of the diagnosis but he was kept unaware of the clinical and sigmoidoscopic severity of the disease.

Besides the pathologist's grading, each biopsy was also graded by the author, who was unaware of the patient's name, the pathologist's grading or the glucosamine synthetase level. Two variables were graded separately by the author — the epithelial cell pattern and the inflammatory infiltrate.

The epithelial cell pattern was graded from 0 to 4, plus an additional grade H for hypertrophic goblet cells, as follows:—

Grade H. Crypts containing goblet cells clearly hypertrophied (Fig. 58).

Grade 0. Grossly normal crypt pattern with normal goblet cell complement (Fig. 59).

Grade 1. Distorted crypt pattern, normal goblet cell complement (Fig. 60).

Grade 2. Surface epithelium damaged or absent, but overall a normal density of epithelial cells. The epithelial cells could be mainly goblet cells (Fig. 61) or mainly columnar cells (Fig. 54).

Grade 3. Epithelial cell density clearly diminished (Fig. 62).

Grade 4. No epithelial cells (Fig. 63,a) or only very occasional epithelial cells (Fig. 63,b).

There were no intermediate gradings.

This was a classification of epithelial cell density rather than of goblet cell density. It was noted which sections had a clearly diminished number of goblet cells in proportion to the total epithelial cell density.

The numbering of this classification is the reverse of that of Yardley et al (1972) who graded the goblet cells in the rat small intestine, in relation to the effect of cholera toxin, from 0 to 4 where 0 indicated absent goblet cells.

The inflammatory infiltrate was graded by the author from 0 to 3 according to whether there was no inflammation (grade 0), mild inflammation (grade 1), moderate inflammation (grade 2), or severe inflammation (grade 3).

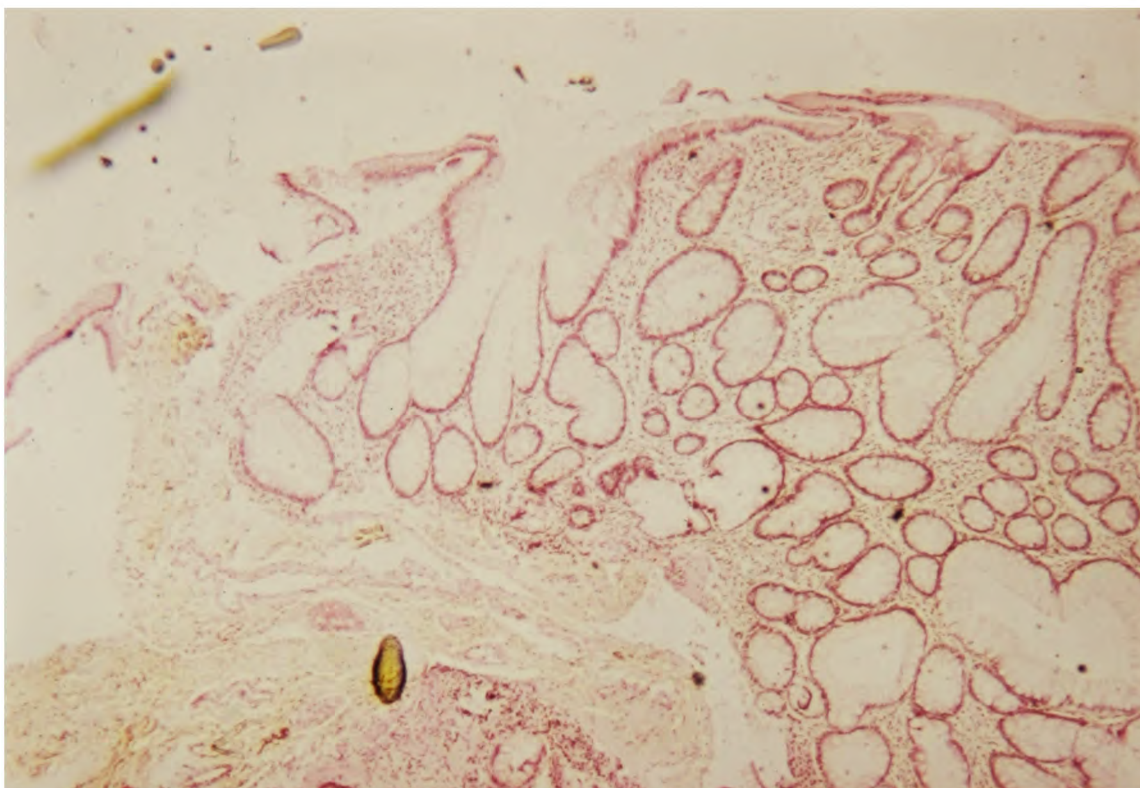


FIG. 58. Grade H epithelial cell density. Hypertrophied crypts. (H. and E.)

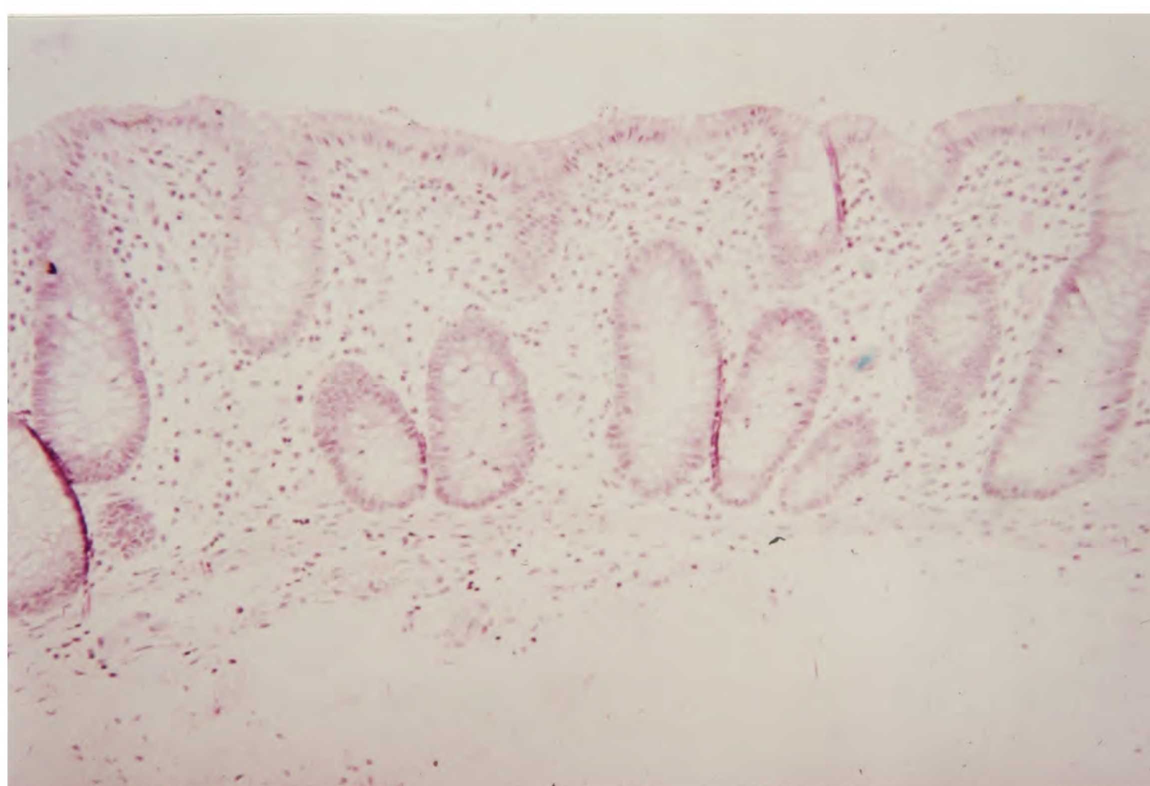


FIG. 59. Grade 0 epithelial cell density. Normal crypts. (H. and E.)

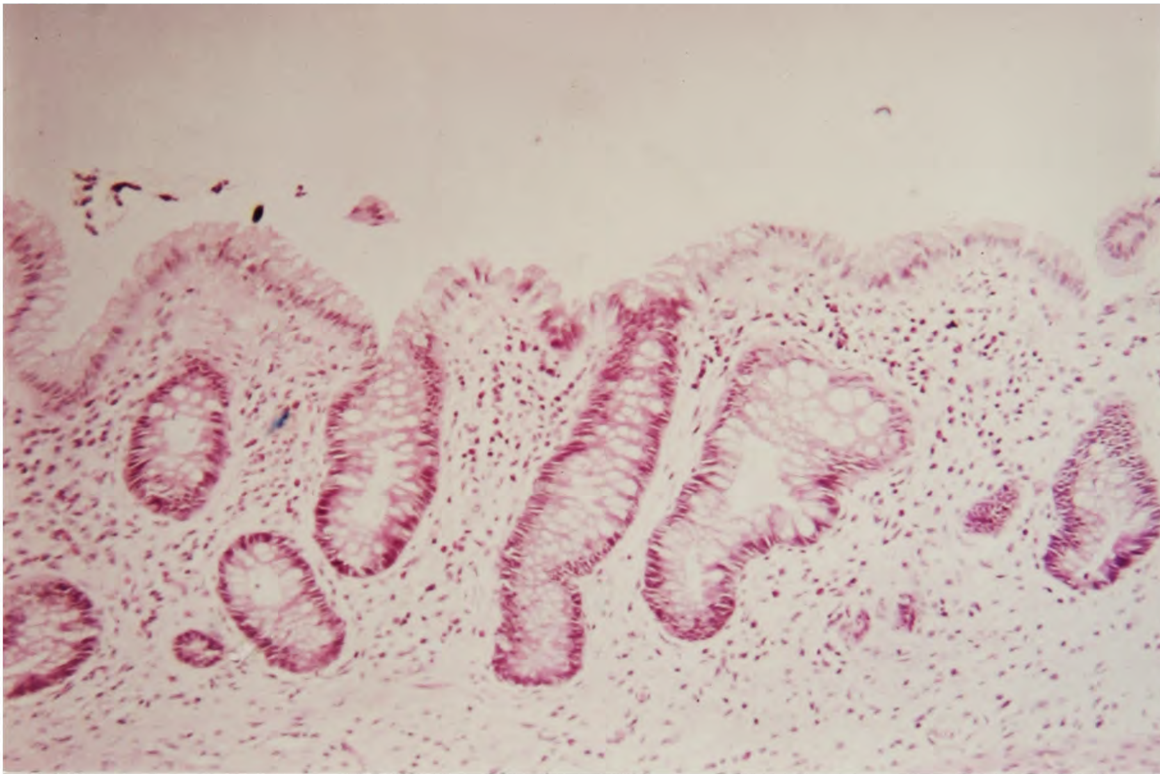


FIG. 60. Grade 1 epithelial cell density. Distorted crypts but normal goblet cell complement. (H and E.)

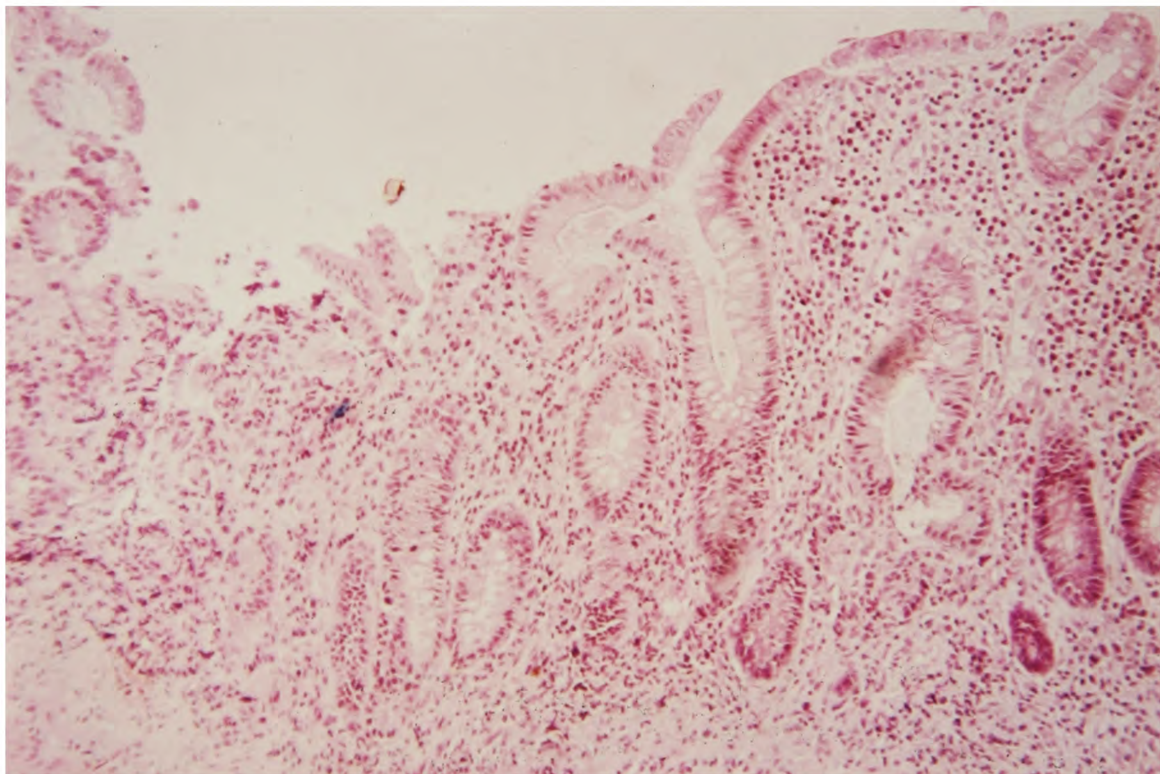


FIG. 61. Grade 2 epithelial cell density. Absent or damaged surface layer but overall density grossly normal. (H and E.)

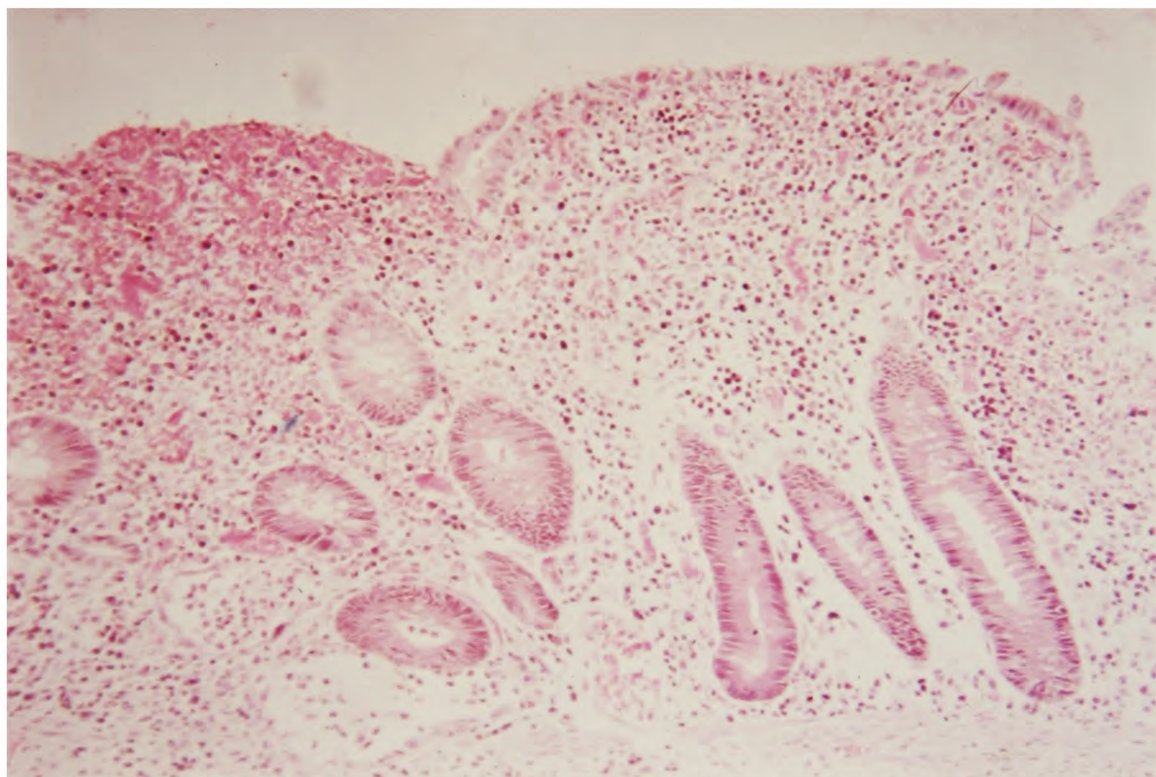
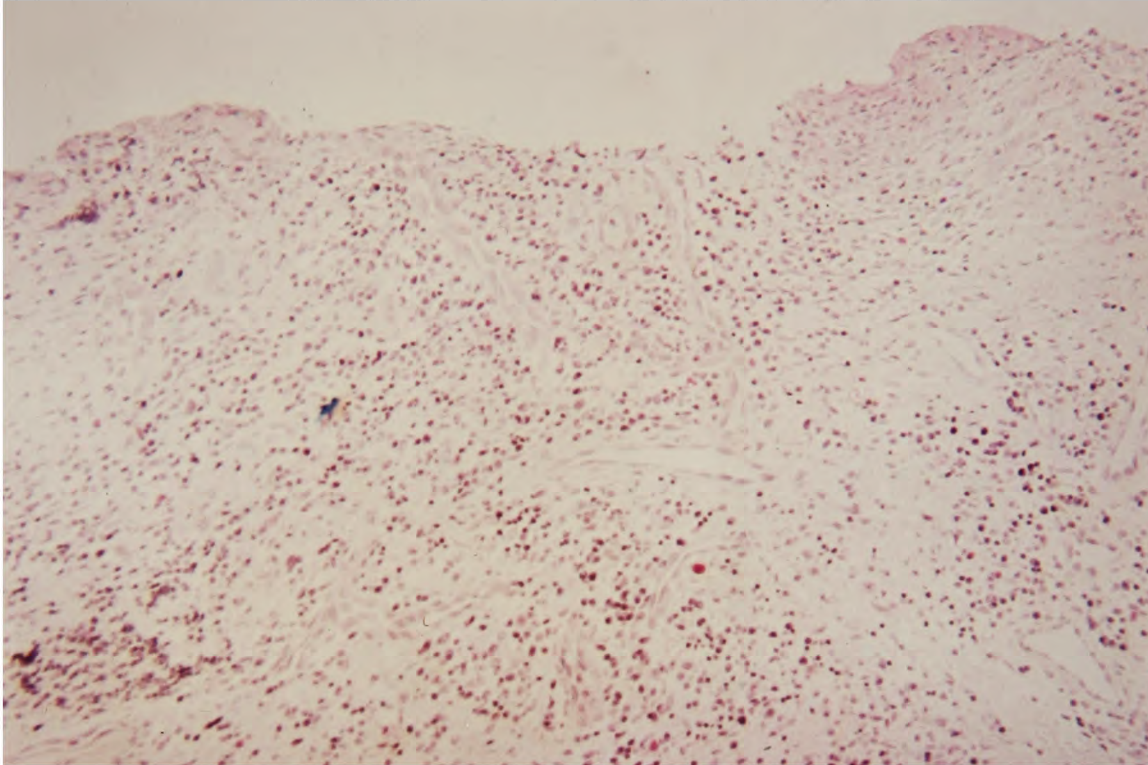
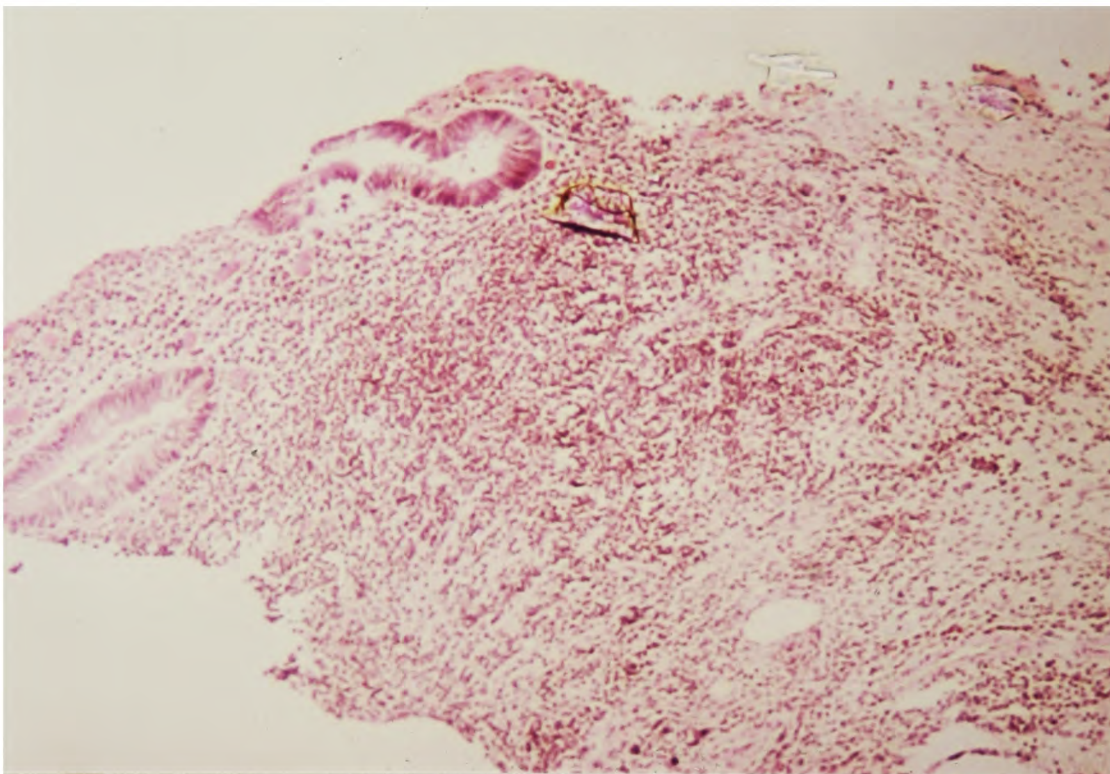


FIG. 62. Grade 3 epithelial cell density. Epithelial cells clearly diminished. (H and E.)



(a)



(b)

FIG. 63. Grade 4 epithelial cell density. (a) No epithelial cells to be seen or (b) only very occasional epithelial cells. (H and E.)

Many biopsies which were placed by the author in grade 0 for a normal epithelial cell pattern and grade 0 for absence of inflammation were considered by the pathologist to indicate chronic ulcerative colitis but showing no significant inflammation.

Clinical details of the patient

These were recorded on a *pro forma* (Fig. 44).

RESULTS

Colectomy specimens

The glucosamine synthetase activity in the 59 samples of mucosa, in units/g wet wt, is shown in Fig. 64 and in Table 19 compared to the normal series.

The mean glucosamine synthetase activity in 47 samples of colonic mucosa from 16 panproctocolectomy specimens for ulcerative colitis was 8.82 units/g wet wt (±0.87, S.E.M.) with a standard deviation of 5.24 units. This was 35% lower than the mean of the normal series (13.66 for the colectomies), a highly significant difference ($P < 0.001$).

In 12 samples of colonic mucosa from 4 colectomy specimens for Crohn's disease of the colon (3 panproctocolectomies and 1 subtotal colectomy), the mean glucosamine synthetase level was 8.36 units/g wet wt (±1.52, S.E.M.) with a standard deviation of 3.99 units. This was similar to the level in ulcerative colitis but was 40% lower than the mean of the normal series, a highly significant difference ($P < 0.001$).

Enzyme activity in units per g protein

The glucosamine synthetase activity in units per g protein in the 41 samples in which this was measured is shown and compared with the normal series in Fig. 65 and in Table 19.

The mean glucosamine synthetase activity in 29 samples of colonic mucosa from panproctocolectomy specimens for ulcerative colitis was 87.2 units/g protein (±9.0, S.E.M.) with a standard deviation of 48.3, compared to a mean of 147.8 for the normal series, a highly significant difference ($P < 0.001$). In the 12 samples of mucosa from colectomy specimens for

TABLE 19. Glucosamine synthetase activity in the mucosa of panproctocolectomy specimens for ulcerative colitis and Crohn's disease

		Ulcerative colitis	Crohn's disease of the colon	Normal colectomy series
Number of samples		47	12	25
Number of patients		16	4	25
Males		6	2	17
Females		10	2	8
Mean age of patients		41.9	27.2	63.4
Range		16-70	17-34	44-83
Glucosamine	Mean	8.82	8.36	13.66
synthetase	S.D.	5.94	1.52	0.84
units/g wet wt	S.E.M.	0.87	1.52	0.84
Difference from normal		$P < 0.001$	$P < 0.001$	
Number of samples		29	12	16
Number of patients		11	4	16
Mean age		40.5	27.2	63.8
Glucosamine	Mean	87.2	92.3	147.8
synthetase	S.D.	48.3	48.7	43.2
units/g protein	S.E.M.	9.0	14.1	10.8
Difference from normal		$P < 0.001$	$P < 0.005$	

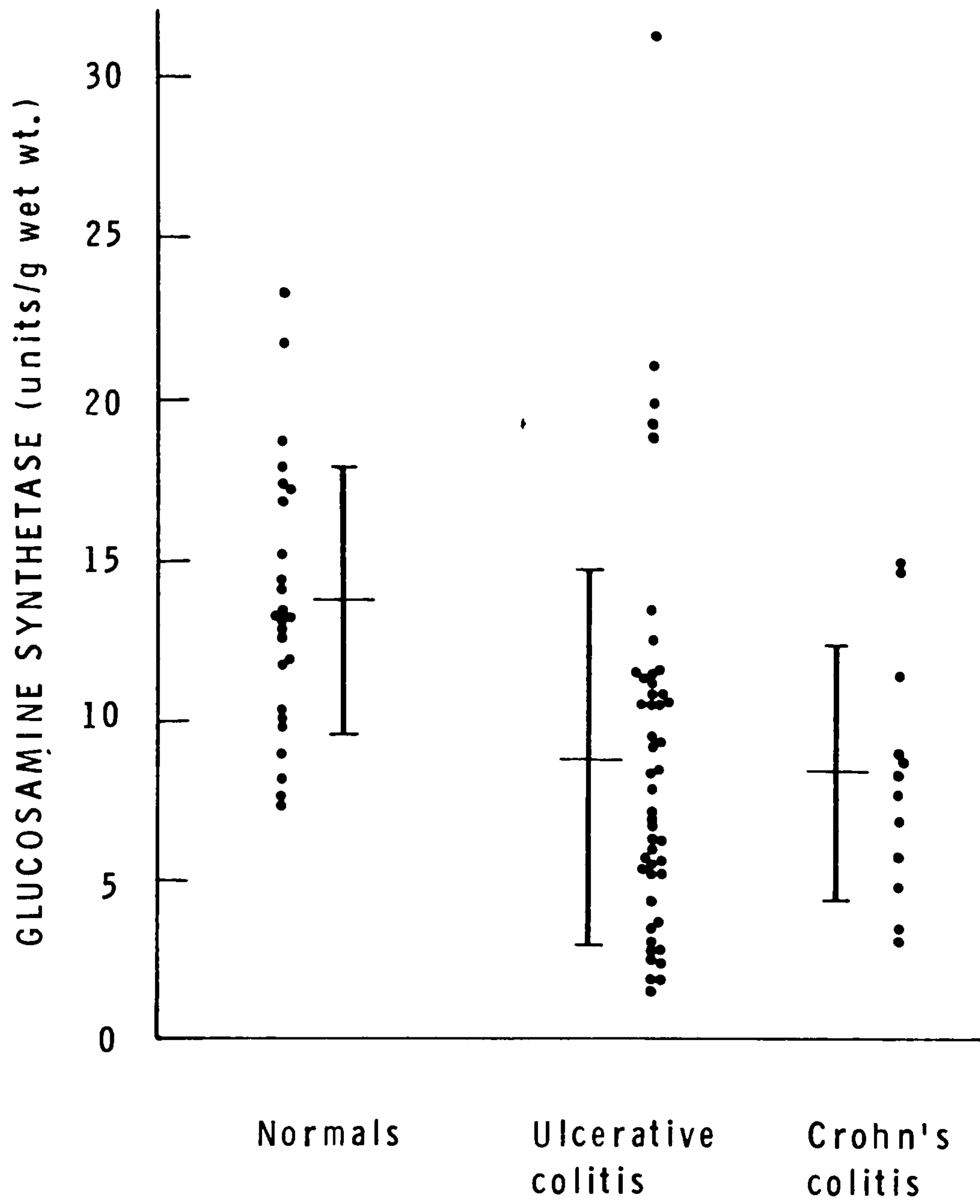


FIG. 64. Glucosamine synthetase activity in units/g wet wt in 20 panproctocolectomy specimens for ulcerative colitis and Crohn's disease, compared with the normal series of 25 colectomy specimens for carcinoma of the colon and localized diverticulitis (normal cut ends).

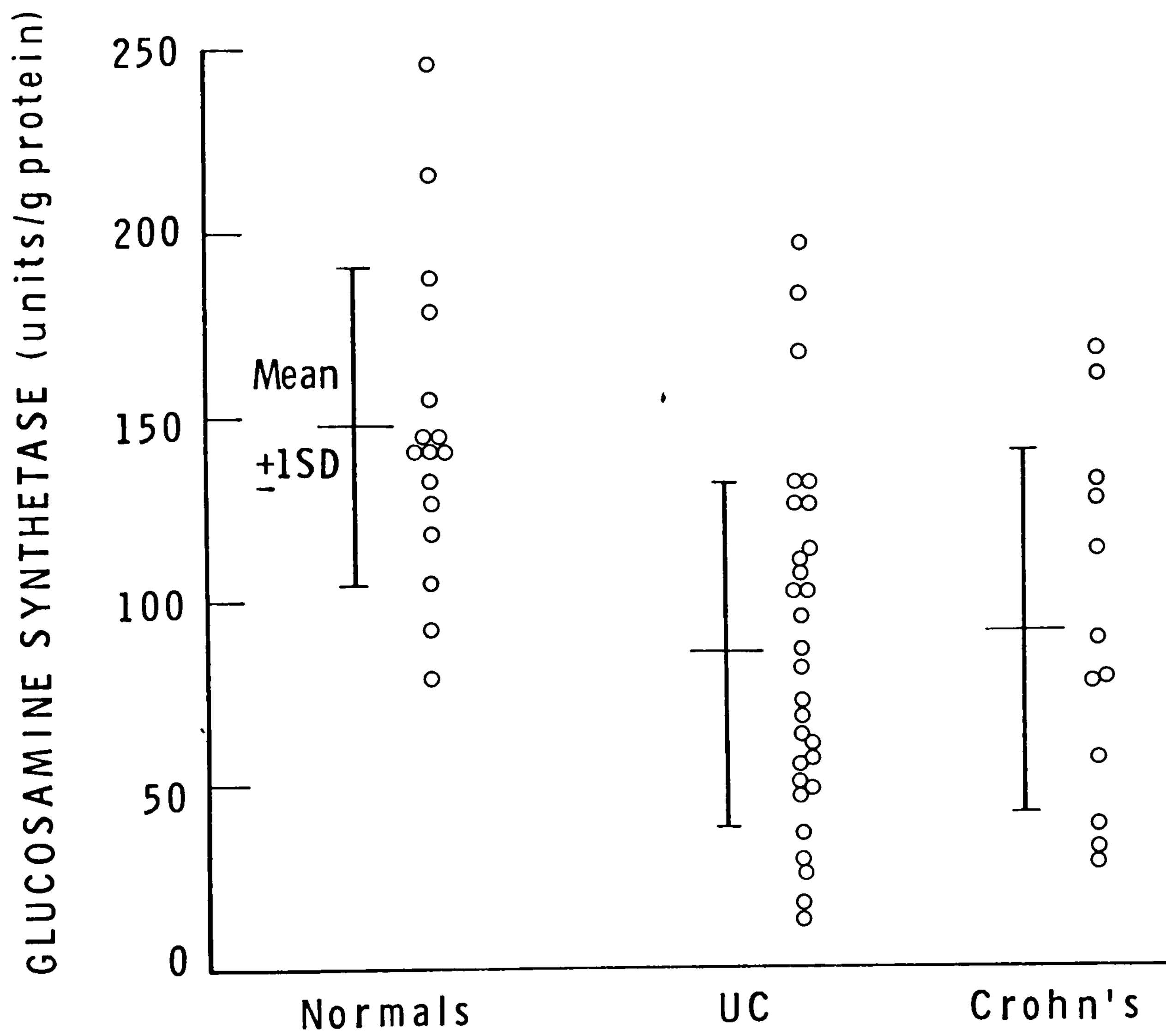


FIG. 65. Glucosamine synthetase activity in units/g protein in 41 samples of colonic mucosa from 15 panproctocolectomy specimens for ulcerative colitis and Crohn's disease, compared with 16 samples of normal mucosa.

Crohn's disease, the mean enzyme level was 92.3 units/g protein (±14.1, S.E.M.) with a standard deviation of 48.7, again a highly significant difference from the normal series ($P < 0.005$).

These 41 samples are included in the 44 samples of mucosa from ulcerative colitis and Crohn's disease in the plot of glucosamine synthetase activity per g protein against activity per g wet wt in Fig. 39. There was good agreement between the two ways of expressing the enzyme activity (5 ; 0.911), as good as for 32 samples of normal mucosa (for which $r = 0.911$).

Histological correlations

For the 36 specimens for which correlative histology was available, the glucosamine synthetase levels in units/g wet wt were plotted in relation to the grade of epithelial cell density (Fig. 66) and in relation to the grade of inflammation (Fig. 67).

The single sample with hypertrophic crypts (Fig. 58) had a very high glucosamine synthetase level of 31.6 units/g wet wt. In grades 0-2, the glucosamine synthetase levels were similar and had a mean value close to that of the normal series, although the degree of variation was greater. Taking grades 0-2 as a single group with a normal overall epithelial density, there was a highly significant correlation between the glucosamine synthetase levels and the epithelial cell density as assessed by subjective grading. This correlation was even more striking when the three readings from each panproctocolectomy specimens were compared in relation to the epithelial cell density (Fig. 68); this emphasized the similarity in glucosamine synthetase levels between grades 0, 1 and 2.

The one sample of mucosa with grade 2 epithelial cell density but with a grossly diminished goblet cell density (Fig. 54) had a glucosamine synthetase level of 12.6 units, which was similar to the other samples in grade 2 and close to the normal mean.

Fig. 69 shows the glucosamine synthetase levels in relation to the inflammatory infiltrate for (a) epithelial cell grades 0-2 and for (b) epithelial cell grade 3. All samples in epithelial cell grade 4 had severe inflammation.

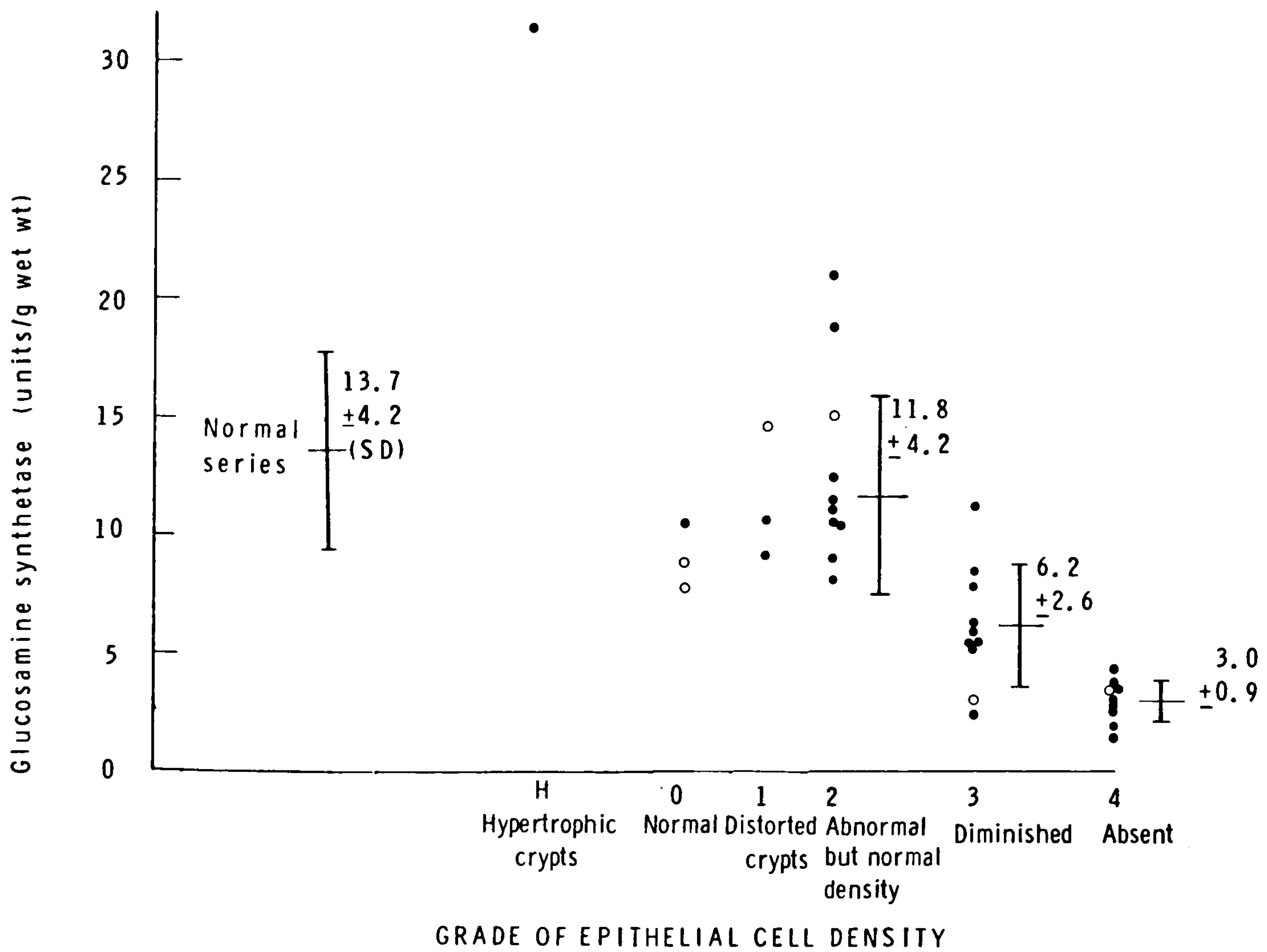


FIG. 66. Glucosamine synthetase activity in 36 samples of colonic mucosa taken from 12 panproctocolectomy specimens (10 for ulcerative colitis and 2 for Crohn's disease) plotted in relation to the graded epithelial cell density. (Closed circles: ulcerative colitis. Open circles: Crohn's colitis.)

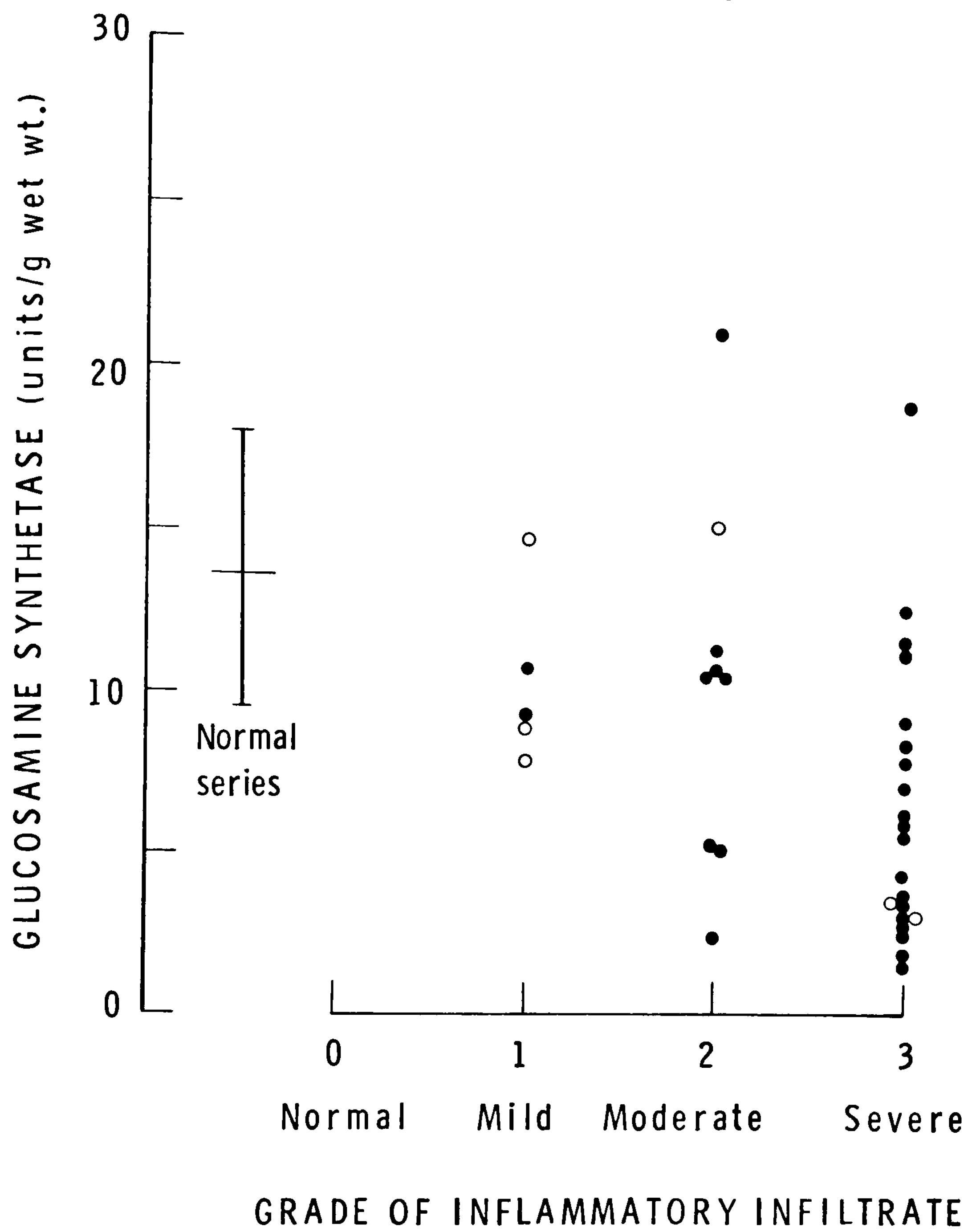


FIG. 67. Glucosamine synthetase activity in panproctocolectomy specimens for ulcerative colitis and Crohn's disease in relation to the grading of the inflammatory infiltrate. (Same samples as Fig. 66.)

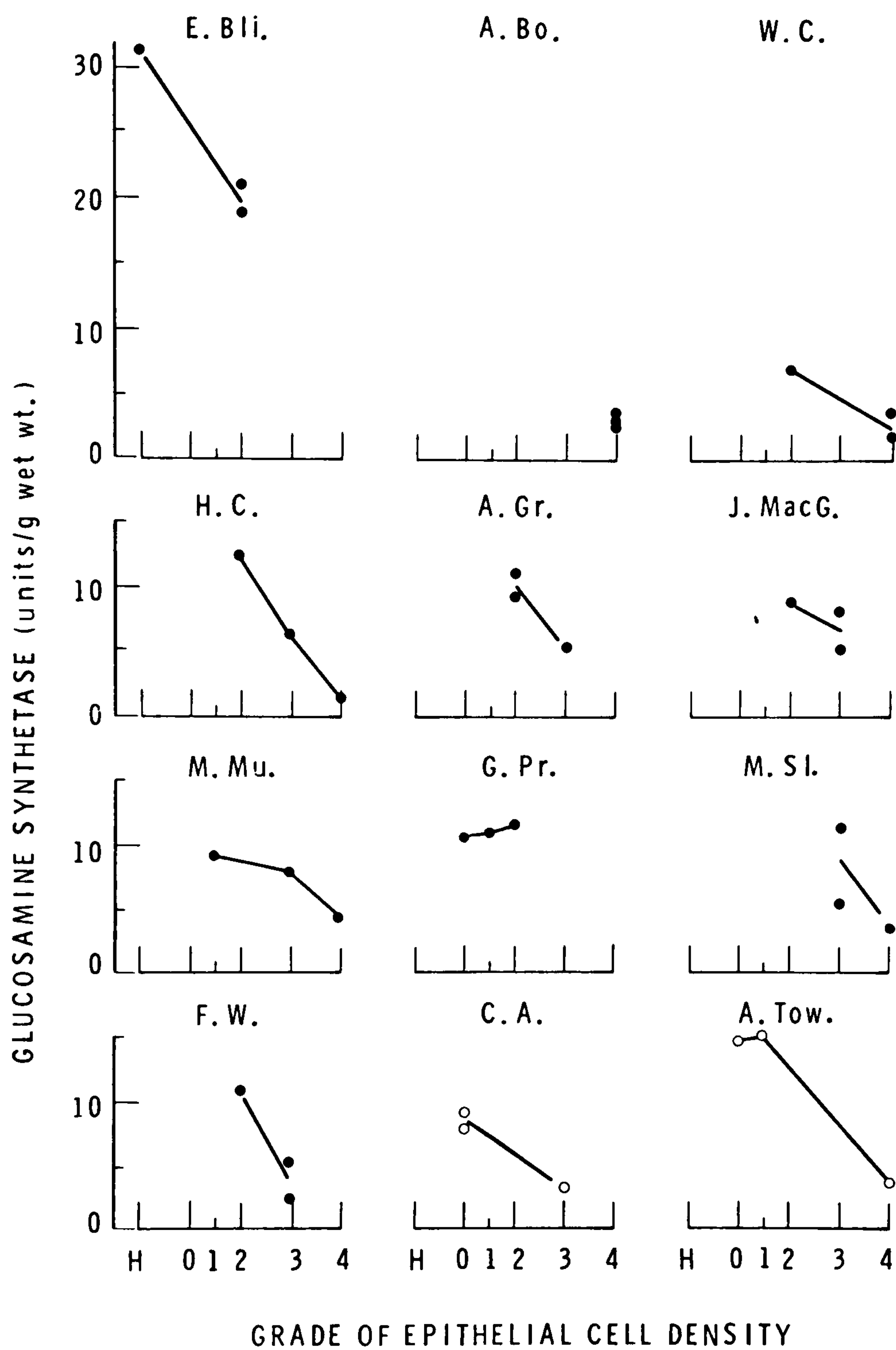


FIG. 68. Glucosamine synthetase activity plotted against epithelial cell density in 12 colectomy specimens for inflammatory bowel disease (10 of ulcerative colitis and 2 of Crohn's colitis). 3 samples of mucosa were taken from different regions of each specimen. (Closed circles: ulcerative colitis. Open circles: Crohn's colitis.)

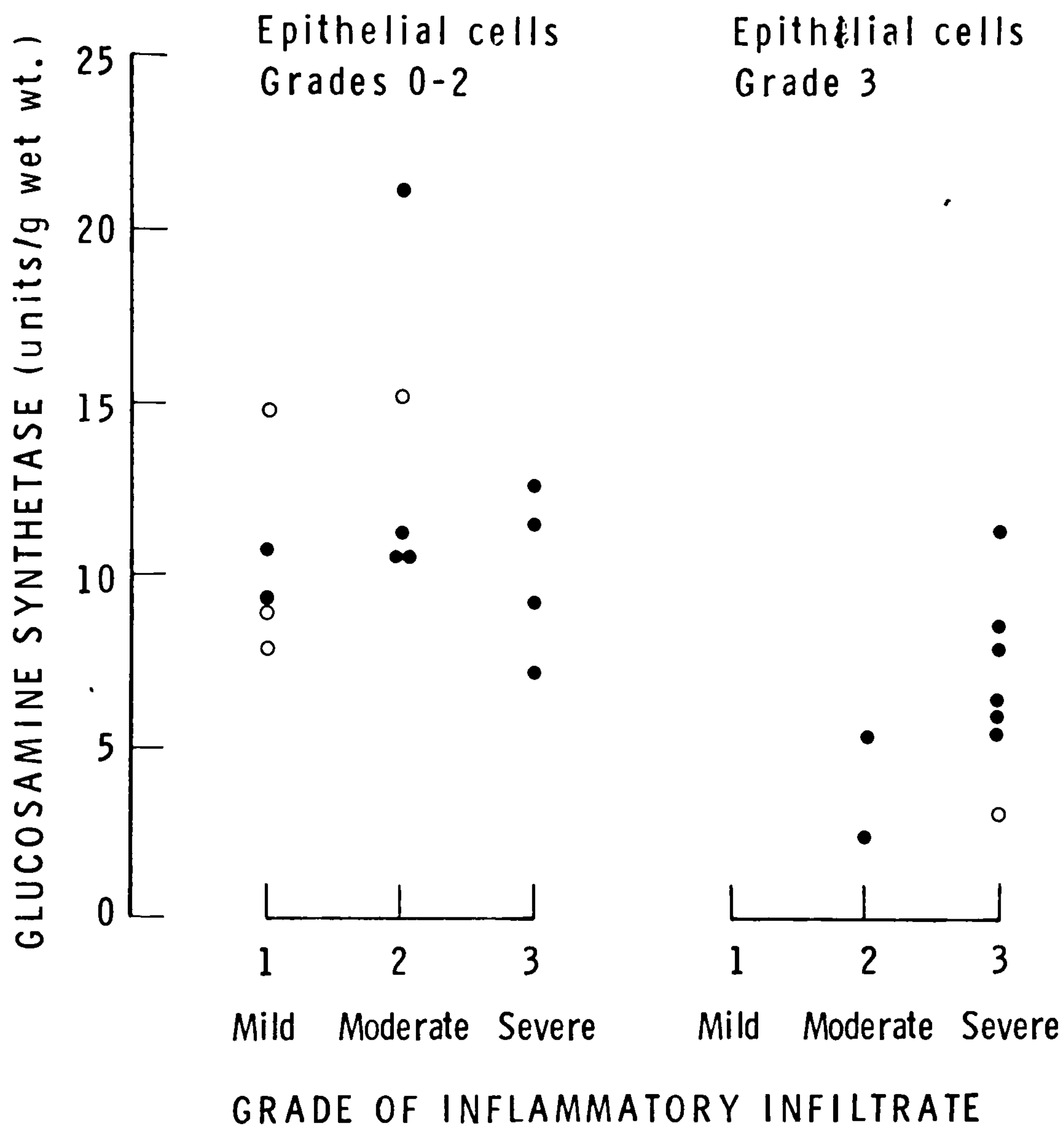


FIG. 69. Glucosamine synthetase in colectomy specimens for inflammatory bowel disease. Samples with epithelial cell density grades 0-2 and grade 3 in relation to the inflammatory infiltrate. (Closed circles: ulcerative colitis. Open circles: Crohn's colitis.)

Submucosa

In the one patient with ulcerative colitis in whom it was measured, the glucosamine synthetase levels in 2 samples of submucosa were 0.7 and 1.6 units/g wet wt. The sample with 1.6 units had a moderate inflammatory cell infiltrate (Fig. 70) and the sample with 0.7 units had a milder infiltrate.

Rectal biopsies — Ulcerative colitis

Table 20 shows the mean glucosamine synthetase levels in the rectal biopsies of 73 patients with ulcerative colitis, also shown according to whether the patient was taking steroids, orally or by enema or suppository. For the 26 patients who had serial biopsies, the reading of the first biopsy

TABLE 20. Mean glucosamine synthetase levels in the rectal biopsies of 73 patients with ulcerative colitis, according to whether or not the patient was taking steroids (orally or by enema or suppository)

		Ulcerative colitis			
		All patients	Patients taking steroids	Patients not taking steroids	Normal series (colectomies and biopsies)
Number of patients (1 biopsy each)		73	37	36	45
Glucosamine synthetase (units/g wet wt)	Mean	14.38	15.24	13.50	13.77
	S.D.	5.31	4.84	5.68	3.97
	S.E.M.	0.62	0.80	0.95	0.59
Mean dosage of steroids			18 patients taking average of 19 mg prednisolone daily orally. 18 taking enemas alone, 1 taking suppositories	0	0

was taken. The mean glucosamine synthetase level in ulcerative colitis (14.38 units/g wet wt ± 0.62 S.E.M.) was similar to that of the normal series (13.77 ± 0.59) but the standard deviation was greater. The 37 patients taking steroids had a higher mean (15.24 units) than the 36 patients not taking steroids (13.50 units) but this difference was not statistically significant

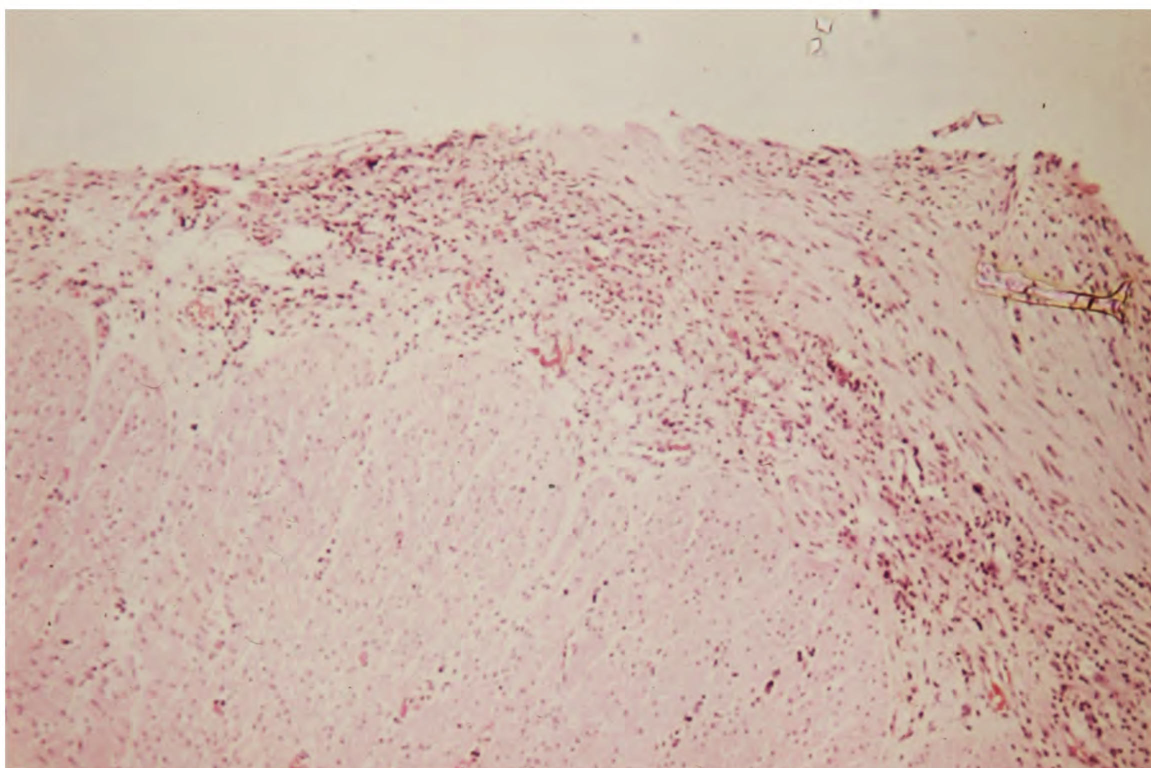


FIG. 70. Piece of submucosa from a panproctocolectomy specimen with ulcerative colitis in which the glucosamine synthetase level was 1.6 units/g wet wt.

($P > 0.20$) and the two groups of patients were not from a matched series. Within the steroid-treated group, there was no correlation with prednisolone dosage.

In 2 out of these 73 biopsies, the histological section was mislaid. The glucosamine synthetase levels in the other 71 biopsies are shown in Fig. 71 in relation to the grading of epithelial cell density, in Fig. 72 in relation to the grading of the inflammatory infiltrate by the author, and in Fig. 73 in relation to the pathologist's grading of the degree of inflammation. In Figs. 71-73, patients receiving oral steroids or steroid enemas are distinguished from patients not taking steroids. For the patients studied by serial biopsy, those biopsies with epithelial cell gradings H, 3 and 4 have been included in Fig. 71.

Control samples

The 3 unincubated control samples, taken from homogenates of two biopsies with moderate inflammation and one with severe inflammation, had glucosamine concentrations of 3.6, 5.4 and 3.6 μmoles/wet wt respectively. In the 3-hour assay, these gave false readings for glucosamine synthetase of 1.2, 1.8 and 1.2 μmoles glucosamine synthetized/h/g wet wt, respectively. The actual readings of glucosamine synthetase in these 3 biopsies were 21.2, 22.8 and 16.7 units respectively.

Epithelial cell density

Fig. 71 shows the means and standard deviations for each grading of epithelial cell density, taking only the 71 initial readings. These are given numerically in Table 21, which also includes the results from the 20 follow-up biopsies with epithelial cell gradings H, 3 and 4. As with the panproctocolectomy series (Figs. 66, 68), grades 0, 1 and 2 had similar glucosamine synthetase levels. Taking grades 0 - 2 together, there were 52 biopsies, comprising 30 from patients taking steroids and 22 from patients not taking steroids. There was a slightly higher mean in the patients who were taking steroids but this was not statistically significant ($0.20 > P > 0.10$). There was also a slightly higher mean for patients taking steroids in grade 3 but only when the 16 follow-up biopsies in this grade were included. As with the colectomy series, glucosamine synthetase levels correlated with the overall epithelial cell density.

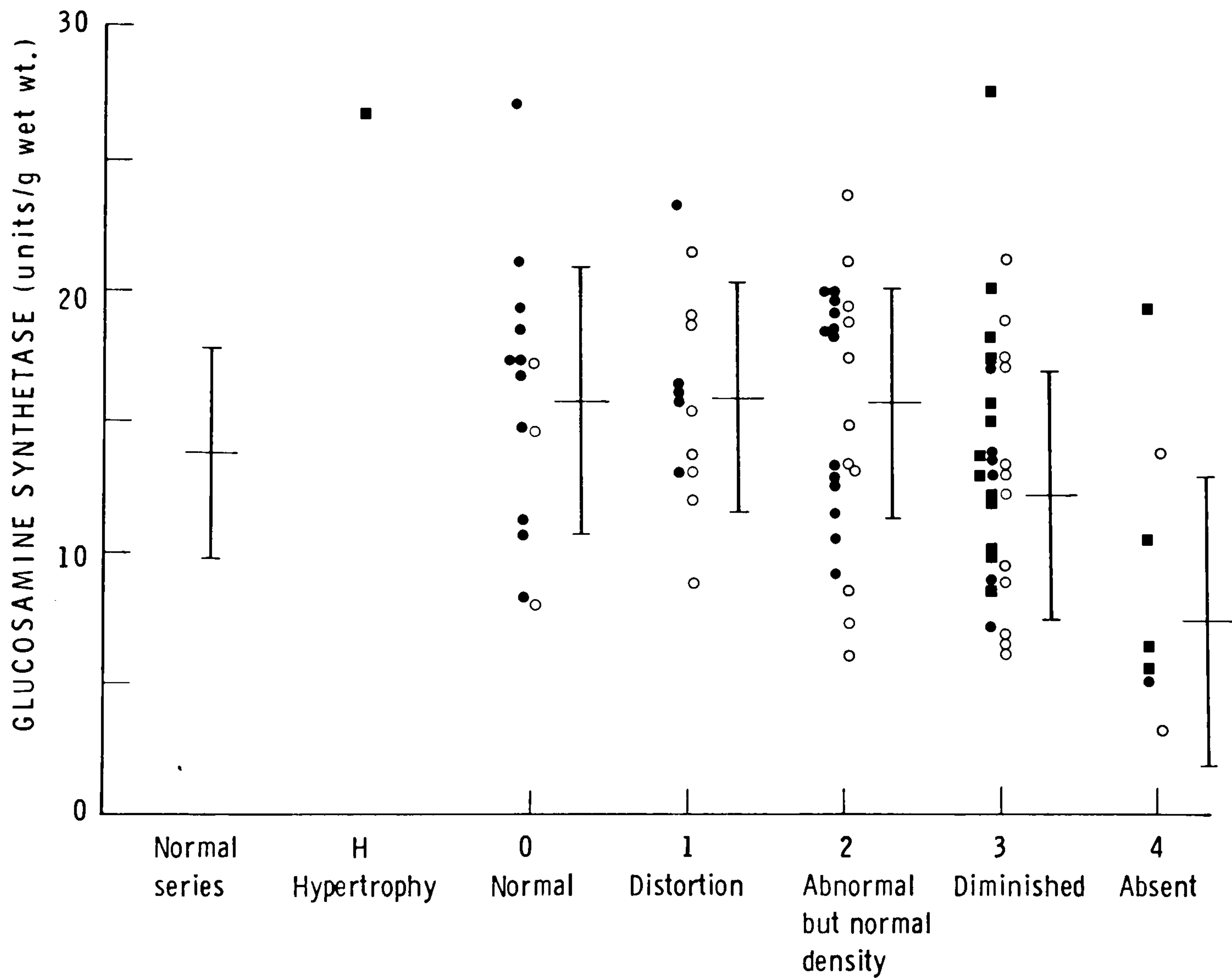
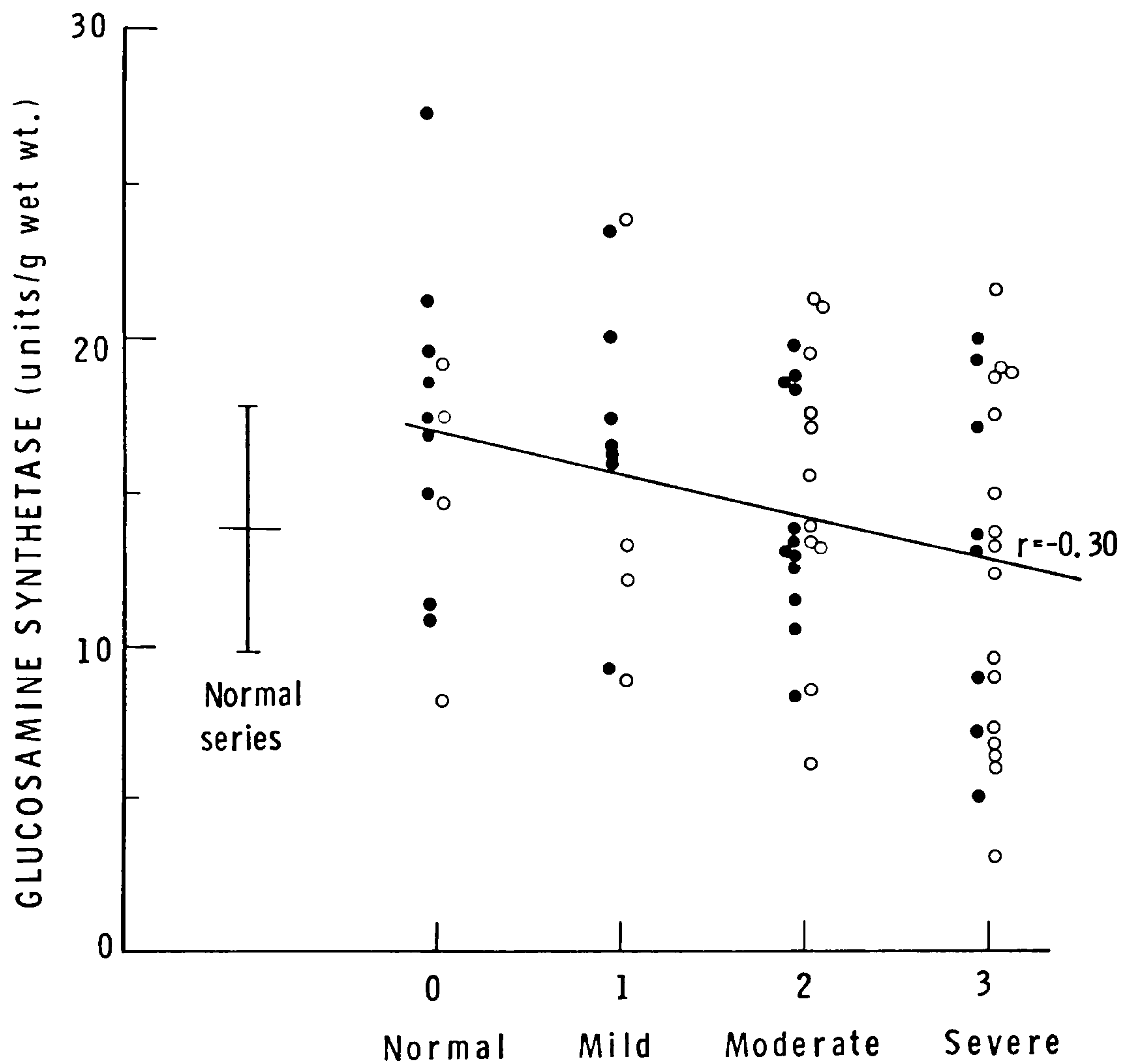
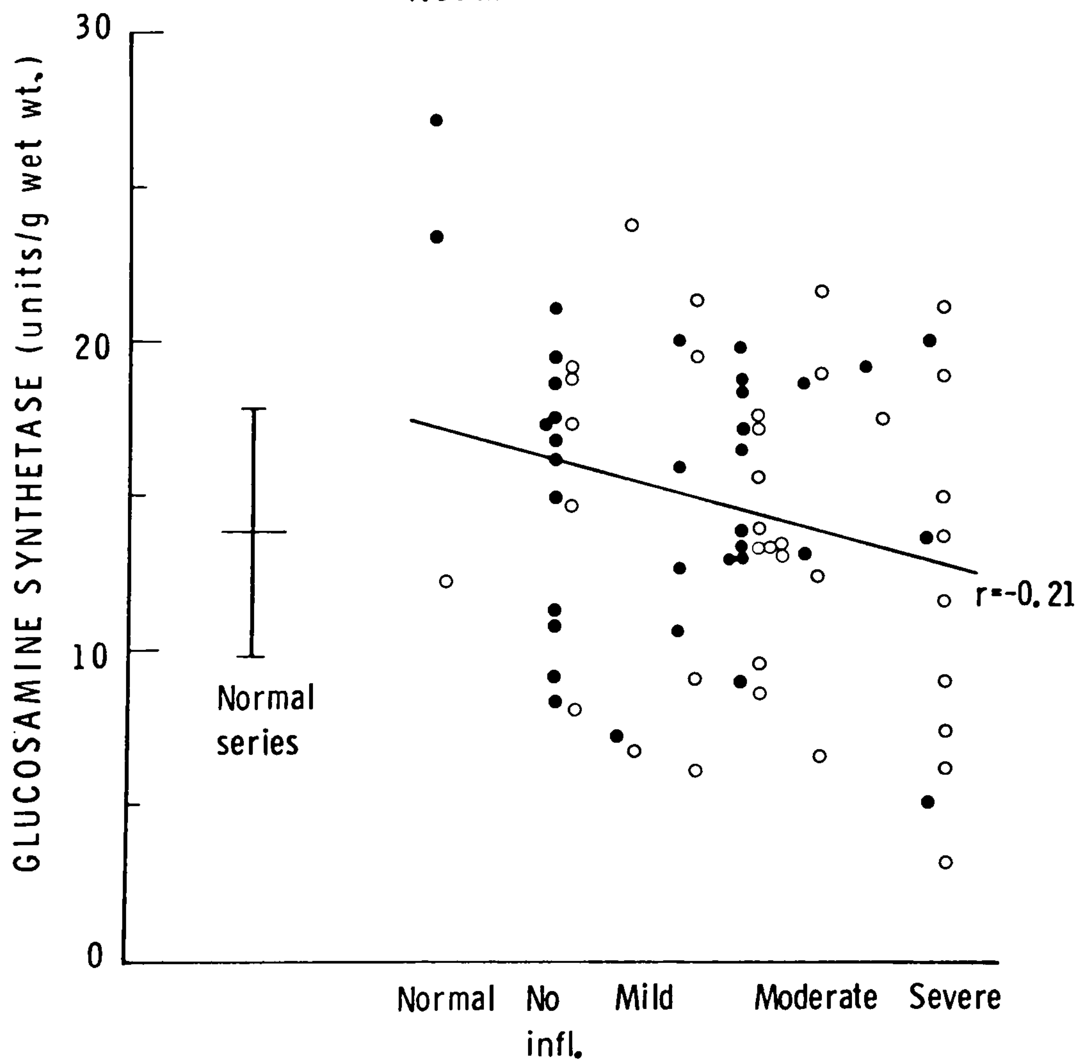


FIG. 71. Glucosamine synthetase in the rectal biopsies of 71 patients with ulcerative colitis (the initial biopsies in the case of patients who had serial biopsies), according to the grading of the epithelial cell density (closed and open circles). Also, glucosamine synthetase in 20 follow-up biopsies from 13 of these patients, being those biopsies in which the epithelial cell density was graded H, 3 and 4 (closed and open squares). Patients taking systemic steroids or steroid enemas shown as closed circles or squares. Patients not taking steroids shown as open circles or squares. Mean and standard deviation shown for each grade (initial biopsies only).





FIGS. 72, 73. Glucosamine synthetase in the rectal biopsies of 71 patients with ulcerative colitis, according to the severity of the inflammation in the biopsy.

FIG. 72 (above): Graded by the author.

FIG. 73 (below): Graded by the pathologist.

(Closed circles indicate patients taking systemic steroids or steroid enemas. Open circles indicate patients not taking steroids.)

TABLE 21. Mean glucosamine synthetase levels in 91 rectal biopsies from 72 patients with ulcerative colitis, according to the grading of epithelial cell density. The series consists of the initial biopsies from 71 patients, and 20 follow-up biopsies (from 13 patients) in which the epithelial cell density was graded H, 3 or 4

Epithelial cell pattern		Glucosamine synthetase (units/g wet wt)													
		Initial biopsies							Initial biopsies and follow-up biopsies						
					Patients taking steroids		Patients not taking steroids					Patients taking steroids		Patients not taking steroids	
Grade	Description	Mean ± S.E.M.	S.D.	(No. of patients)	Mean	(No. of patients)	Mean	(No. of patients)	Mean ± S.E.M.	S.D.	(No. of biopsies)	Mean	(No. of biopsies)	Mean	(No. of biopsies)
H	Hypertrophied crypts	—	—	(—)					26.8		(1)	26.8	(1)		
0	Normal	16.03 ± 1.37	5.13	(14)	16.74	(11)	13.43	(3)							
1	Distorted crypts	16.10 ± 1.22	4.40	(13)	17.08	(5)	15.48	(8)							
2	Damaged epithelium, grossly normal overall density	15.86 ± 0.88	4.37	(25)	16.66	(14)	15.02	(11)							
3	Diminished	12.25 ± 1.12	4.78	(16)	11.38	(5)	12.65	(11)	13.36 ± 0.88	4.92	(31)	13.78	(19)	12.69	(12)
4	Absent or very scanty	7.37 ± 3.24	5.62	(3)	5.1	(1)	8.50	(2)	9.16 ± 2.19	5.79	(7)	9.84	(5)	8.50	(2)
0-2		16.00 ± 0.62	4.47	(52)	16.76 ± 0.69 S.D. 3.77	(30)	14.97 ± 1.10 S.D. 5.18	(22)							
Normal series (colectomies + biopsies)		13.77 ± 0.59	3.97	(45)											

The goblet cell density

All the rectal biopsies in epithelial cell grade 2, including the follow-up biopsies, were examined to select those in which the goblet cell density was grossly diminished. Twelve such biopsies were found and in these the glucosamine synthetase levels had a mean of 14.87 units/g wet wt (Fig. 74), which is similar to the normal range of levels in grades 0 and 1 in ulcerative colitis.

The inflammatory infiltrate

The degree of inflammation represents more of a continuous grading than the grading of the epithelial cell density and therefore regression lines have been plotted in Figs. 72 and 73. There was a fall in glucosamine synthetase activity with increasing inflammation, but the correlation coefficient was low, being -0.30 with the author's grading and -0.21 with the pathologist's grading.

The negative correlation with the inflammatory cell infiltrate refutes the possibility that the glucosamine synthetase levels reflect the intensity of the inflammation. The fall in glucosamine synthetase levels with increasing inflammation almost certainly represents the coincident destruction of the epithelial cells. Fig. 75 shows the correlation between the degree of inflammation and the epithelial cell density.

Glucosamine synthetase in leucocytes, erythrocytes and plasma

In order to investigate the possibility that the inflammatory infiltrate in the inflamed mucosa might possess glucosamine synthetase activity, an experiment was performed with the blood of a patient with a leukaemoid reaction to a subphrenic abscess. This patient's white cell count was 25 000 per μl.

The blood was centrifuged to separate the red cells, the buffy coat and the plasma. 0.075 ml of each was homogenized in 1.425 ml homogenizing buffer, making homogenates containing 50 mg/ml of red cells, white cells and plasma. Glucosamine synthetase was assayed in each of these homogenates by the 10 mg method but no activity could be detected in any of them.

Serial biopsies in ulcerative colitis

A consistent pattern appeared in the glucosamine synthetase levels in serial rectal biopsies of the patients who were followed through an acute attack of

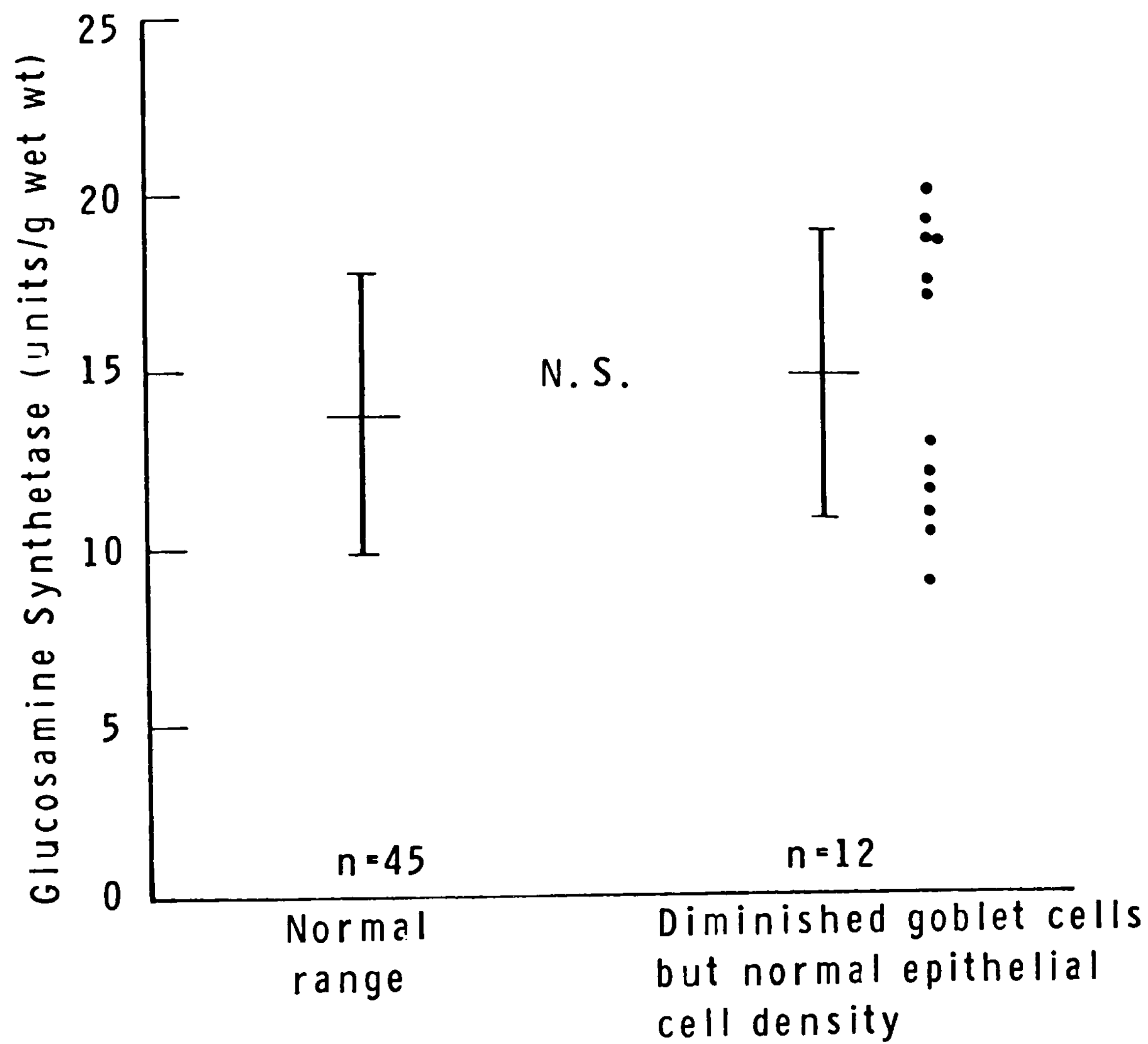


FIG. 74. Glucosamine synthetase levels in 12 rectal biopsies from patients with ulcerative colitis in which there was a normal overall epithelial cell density but in which the goblet cell density was much diminished.

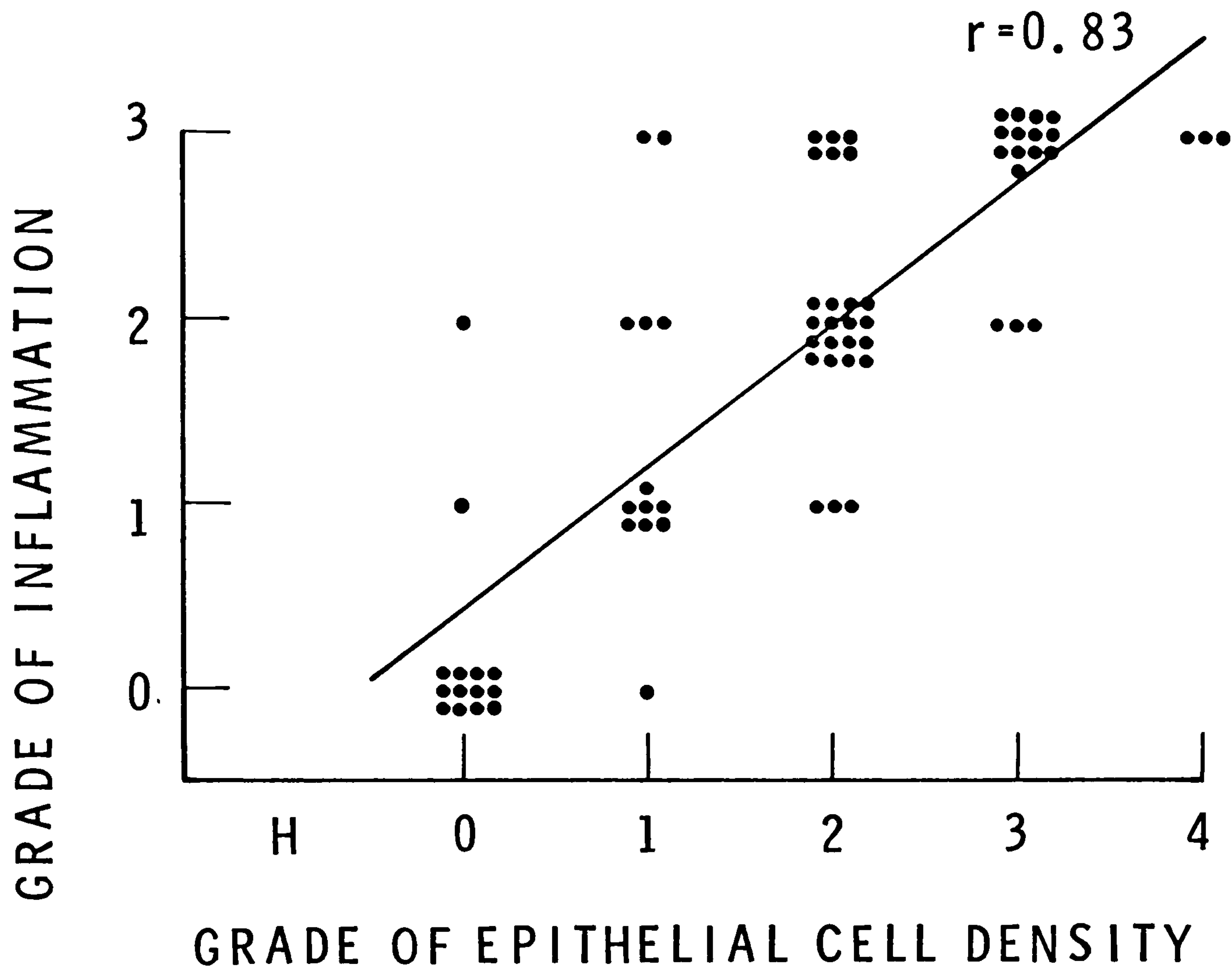
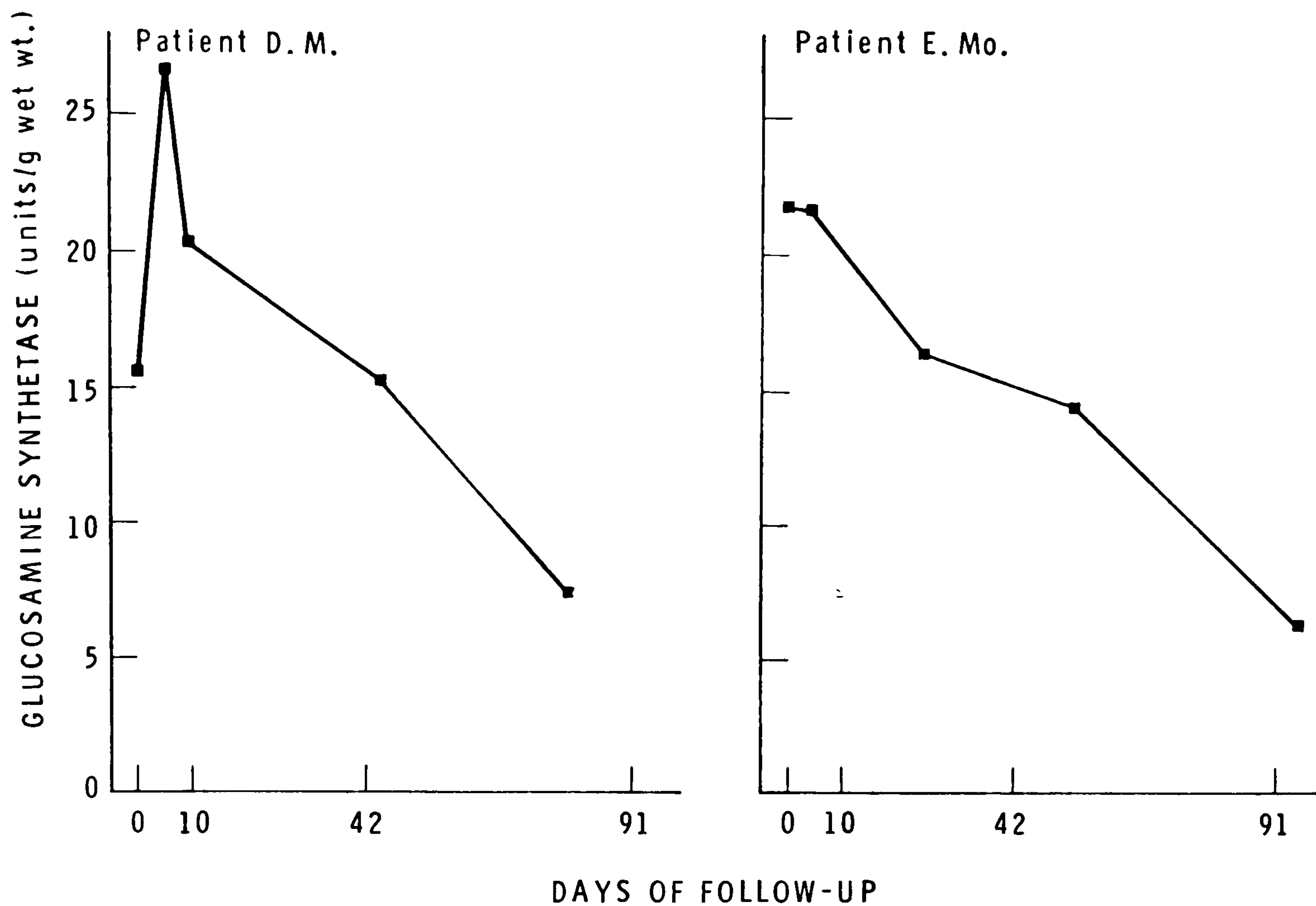


FIG. 75. The correlation between the epithelial cell density and the inflammatory infiltrate (on the author's grading) in the rectal biopsies of 71 patients with ulcerative colitis.

ulcerative colitis. Usually the enzyme levels rose to a peak at about the 5th - 10th day and then fell again gradually to a normal level (Fig. 76, a). Sometimes the enzyme levels were at a peak at the first reading and then fell later (Fig. 76, b).

Twenty-six patients were studied in the course of acute attacks of ulcerative colitis (23 during a single attack and 3 in the course of two attacks). In 20 of the attacks, the patients were treated with the 5-day intensive intravenous regime; the other 9 attacks, which were less severe, were treated with oral prednisolone. The outcome of the attacks is shown in Table 22. In 24 of the attacks (in 21 patients), the patient recovered as judged by the clinical state and by a return of the sigmoidoscopic appearances to normal or to no more than mild inflammation. One of these patients had a relapse 2 weeks after he had appeared to recover and he came to panproctocolectomy.

Table 23 shows the peak glucosamine synthetase level in these 21 patients in their 24 acute attacks of ulcerative colitis. In 19 of these attacks, further observations were made after the peak and these are also shown in Table 23. Of these 24 attacks, 15 were treated with the intensive intravenous regime and in these the peak level was 23.94 units/g wet wt, taken as the highest reading of a mean of 4.6 readings. This peak must be compared with the estimated highest reading of 4.6 readings in normal mucosa. (Actually, the variation in inflamed mucosa may be greater than in normal mucosa but an estimate of the variation in inflamed mucosa was not obtained.) The sampling variation was found to be 16.2% in 6 patients with the irritable colon syndrome (Figs. 46, 47) and 17.6% in the normal mucosa of 4 colectomy specimens for carcinoma (Table 12). Taking this variation as 17%, the highest of 4.6 readings from normal mucosa with a glucosamine synthetase level equal to the mean of the normal series should be the point on the curve of the normal distribution with a standard deviation of 17% which corresponds to an area of $\frac{4.6}{5.6}$ (or 0.821) under the curve up to that point, which is the mean plus 0.9192 standard deviations, or, for the normal series, $13.77 + 0.9192 \times 0.17 \times 13.77 = 15.92$ units, and the S.D. of this estimated mean will be $3.97 + 0.9192 \times 0.17 \times 3.97 = 4.59$ units. (This is probably an overestimate of the standard deviation since the highest readings in the normal series are more likely to be higher than the true mean for the mucosa from which they were taken than readings close to the mean of that series.)



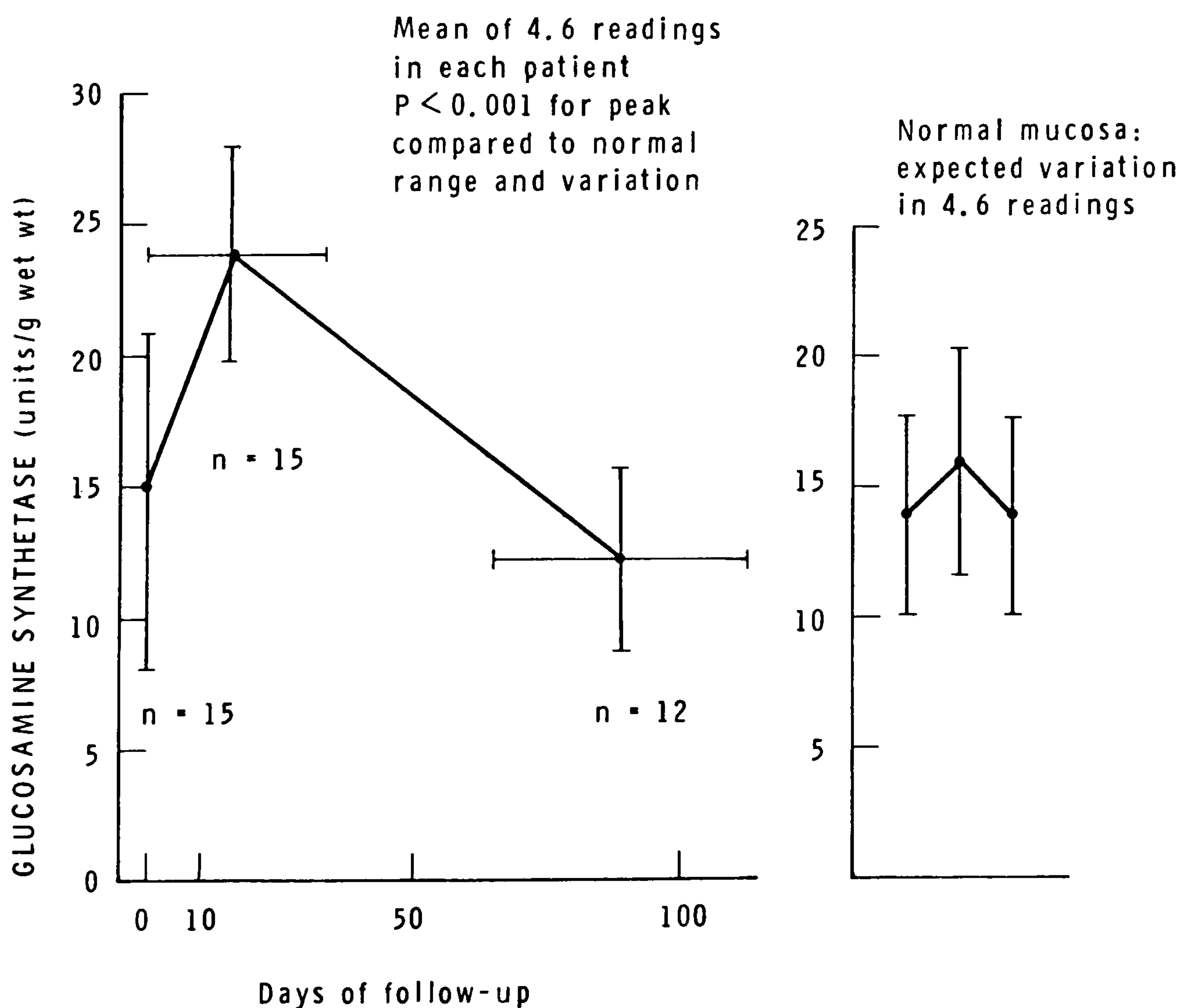


FIG. 76 (above): Glucosamine synthetase levels in serial rectal biopsies of two patients in an acute attack of ulcerative colitis.

FIG. 77 (below): Glucosamine synthetase levels in serial rectal biopsies of 15 patients in an acute attack of ulcerative colitis treated by the 5-day intensive steroid regime who recovered from the attack.

TABLE 22. Outcome of the acute attack of ulcerative colitis in 29 attacks (in 26 patients) in which glucosamine synthetase levels were measured in serial rectal biopsies

	Treated with intensive intravenous steroid regime (20 attacks)	Treated with oral steroids (9 attacks)
Clinical and sigmoidoscopic recovery	14	9
Recovery but subsequent relapse	1	0
Clinical recovery but mucosa remained inflamed sigmoidoscopically	2	0
No recovery, required panproctocolectomy	3	0

TABLE 23. Serial glucosamine synthetase levels (units/g wet wt) in the rectal biopsies in 21 patients who were treated for an acute attack of ulcerative colitis and subsequently had evidence of recovery (2 attacks in each of 3 patients)

		Treated with intravenous steroids	Treated with oral steroids only	All patients
(Number of attacks)		(15)	(9)	(24)
[Mean number of readings]		[4.6]	[4.1]	[4.4]
Initial reading	Mean	15.00	11.55	13.70
	S.D.	5.93	5.45	5.61
Peak reading	Mean	23.94	18.00	21.71
	S.D.	4.12	4.12	4.99
	S.E.M.	1.06	1.37	1.02
	Day (mean)	15.9 days	15.7 days	15.8 days
	Range	0-49 days	0-56 days	0-56 days
Lowest reading after peak	(Number of attacks)	(12)	(7)	(19)
	Mean	10.80	10.60	10.73
	S.D.	2.97	3.71	3.16
	Day (mean)	81.9 days	47.0 days	69.0 days
	Range	18-183 days	10-98 days	10-183 days
	Mean peak in these patients	23.54	19.02	21.86
	[Mean no. of readings after peak]	[2.8]	[2.4]	[2.6]

The peaks of 23.94 units for the patients treated by the intravenous regime and 18.00 for the patients treated only with oral steroids can also be compared with the lowest reading after the peak when such a reading was available (Fig. 77). In 12 patients treated intravenously, the mean of this reading was 10.80 units, and in 7 patients treated only orally this mean was 10.60 units. The difference between the peak and the subsequent lowest reading was highly significant even taking into account the fact that this was the lowest of 2.6 readings ($P < 0.001$).

The difference between the peak for this series of 24 attacks (21.71 units) and the estimated maximum peak for a normal series based on 4.4 readings is highly significant ($P < 0.001$). Comparing this peak with a similarly estimated peak for the 52 patients with ulcerative colitis in Table 21 with epithelial cell densities in grades 0-2, the difference is significant also ($P < 0.001$), although these 52 readings included some which were actually peaks in the series of serial biopsies. In 21 out of the 24 attacks, the peak was 19.0 units or more. In the other 3 attacks, none of which were treated with the intravenous regime, the peaks were 17.6, 13.6 and 9.3 units.

Serial glucosamine synthetase levels in individual patients

The 23 attacks of ulcerative colitis from which the patient recovered included 2 attacks in one patient (J. I.) who in fact appeared to have three peaks when studied serially over the course of 8 months. Fig. 78 shows the serial levels in this patient and also the grade of inflammation in the biopsy from which it can be seen that on biopsy she appeared to have a relapse before the second peak, which was not apparent clinically and was therefore not considered to be an attack of ulcerative colitis.

Fig. 79 shows the serial enzyme levels in the patient (L. Cof.) whose peak was only 9.3 units. This patient's initial biopsy had no epithelial cells in it (grade 4 epithelial cell density) and a glucosamine synthetase level of only 3.2 units, the lowest of all rectal biopsies studied in this thesis. The rise to 9.3 units cannot really be considered as a peak as it represents simply the recovery of the structure of the mucosa.

Patients who did not recover from their acute attack of ulcerative colitis

Fig. 80 shows the glucosamine synthetase levels in the serial rectal biopsies of the 5 patients who did not show sigmoidoscopic evidence of recovery from their acute attack of ulcerative colitis.

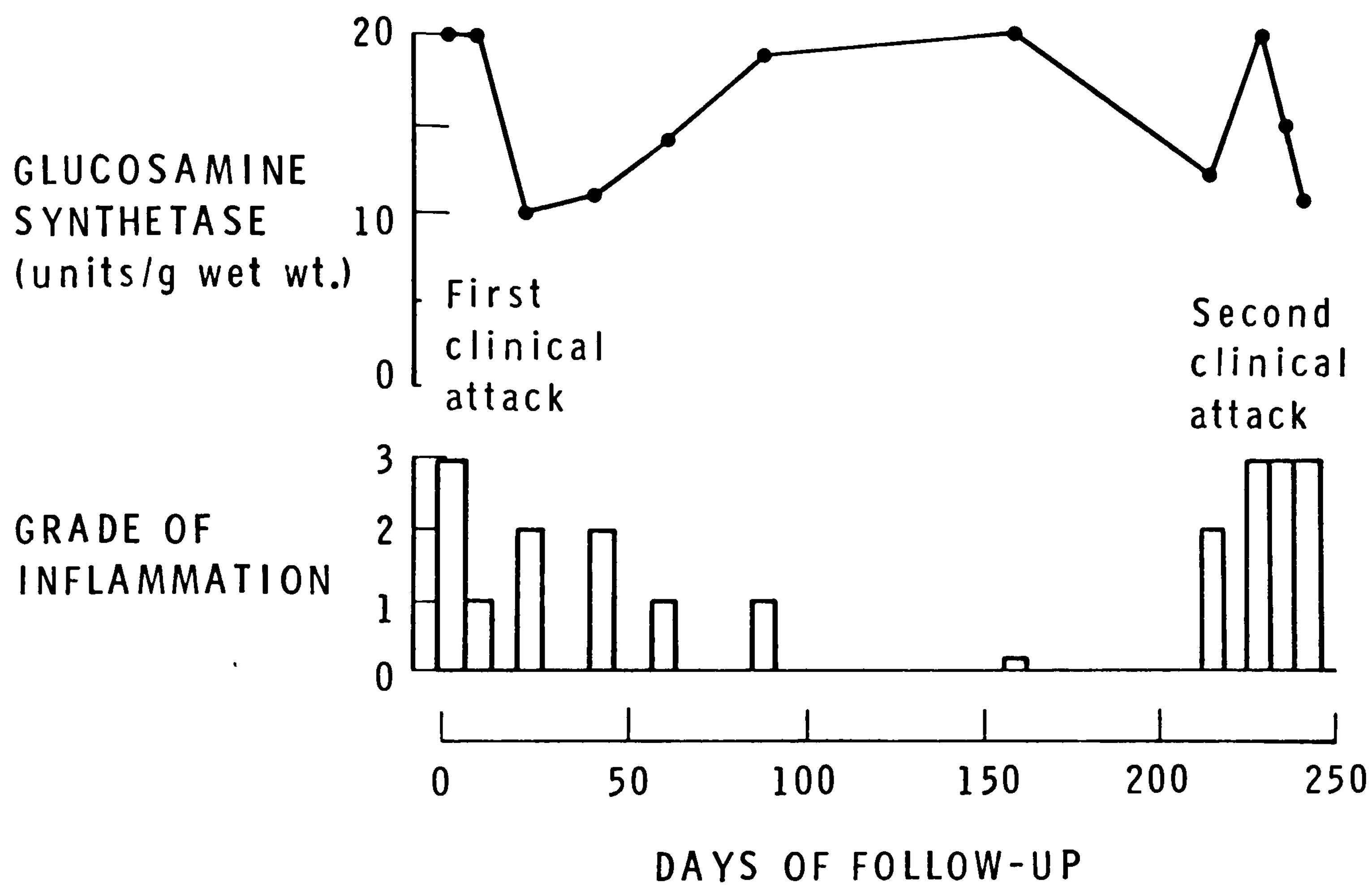


FIG. 78. Glucosamine synthetase levels in serial rectal biopsies in one patient (J.I.) who had two clinical attacks of ulcerative colitis over an 8-month follow-up period. (Grade of histological inflammation according to the author's assessment.)

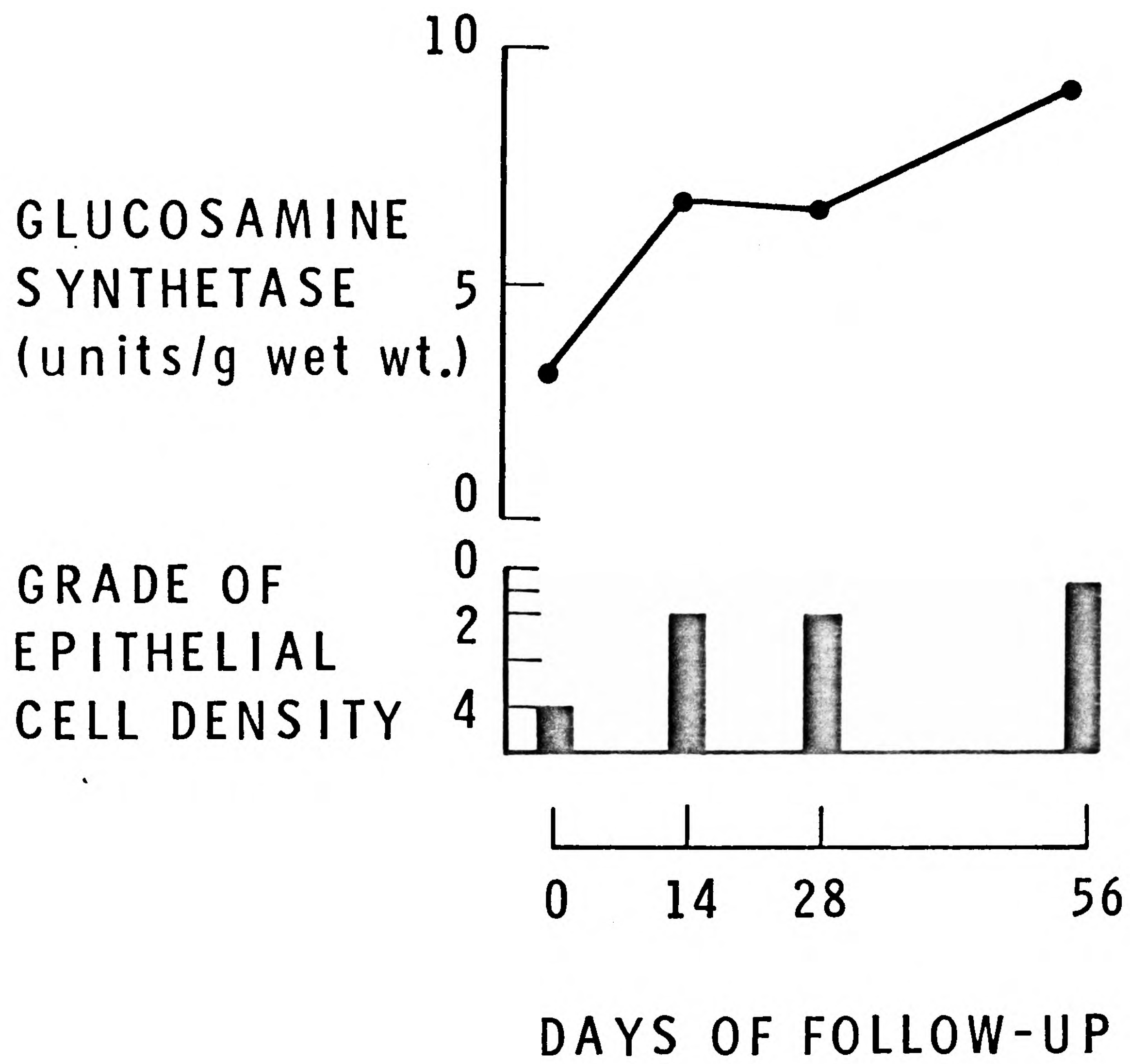


FIG. 79. Glucosamine synthetase levels in serial rectal biopsies in one patient (L. Cof.) treated for and recovering from an attack of ulcerative colitis.

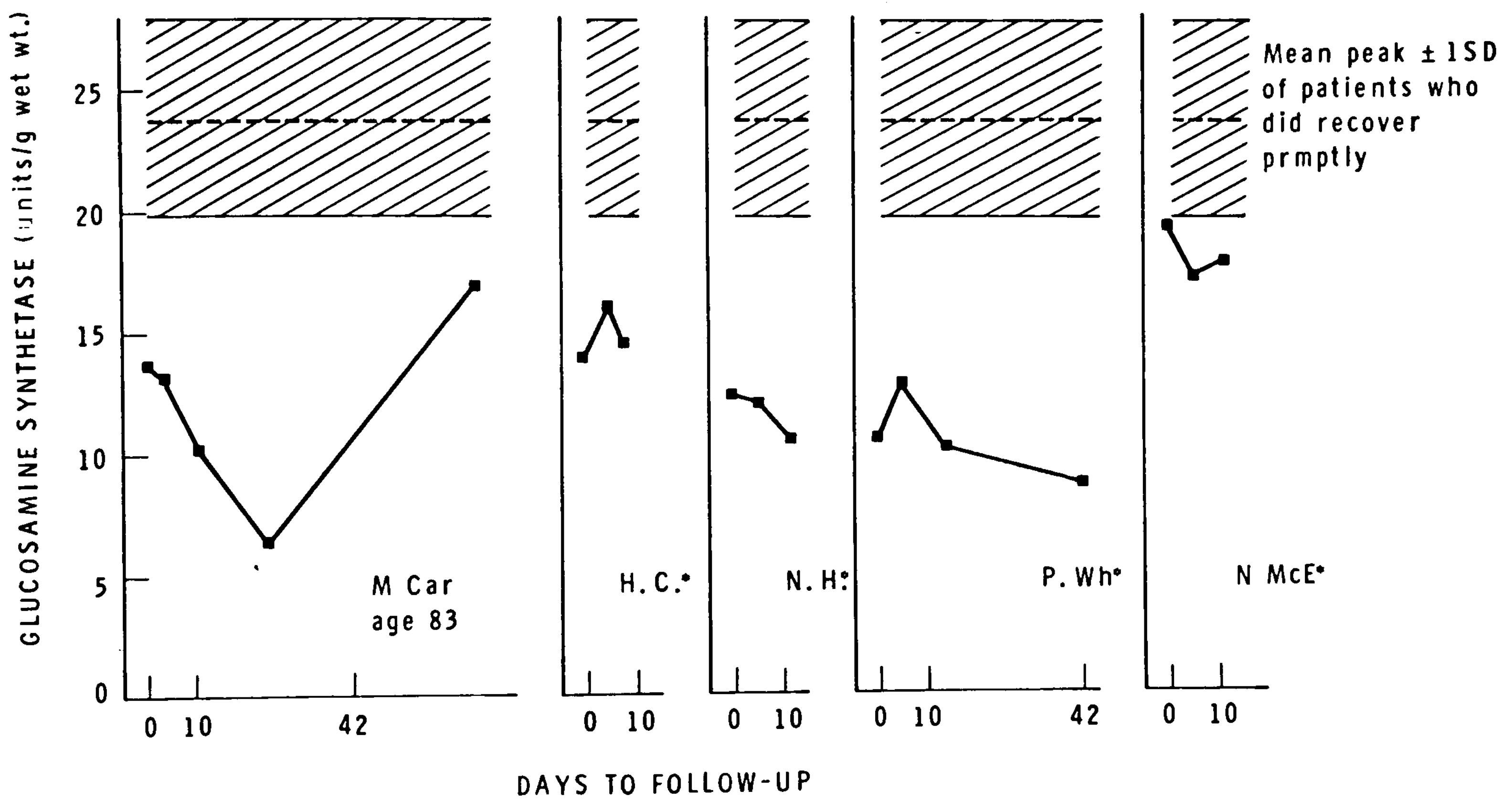


FIG. 80. Glucosamine synthetase levelsin serial rectal biopsies in 5 patients in an acute attack of ulcerative colitis who did not show sigmoidoscopic evidence of recovery from their attack. (Asterisk = panproctocolectomy.)

The patient M. Car. was an old lady of 83 years who improved clinically sufficiently to leave hospital on the 15th day but sigmoidoscopically the inflammation remained moderate until the 67th day, when it became mild. A peak can be seen at the 67th day.

The two patients H. C. and N. H. had severe attacks of ulcerative colitis, requiring emergency panproctocolectomy on the 9th and the 15th day respectively.

The patient P. Wh. was a resilient young man who seemed to make a good recovery clinically but his mucosa remained moderately inflamed on sigmoidoscopy and panproctocolectomy was subsequently performed. This showed severe and extensive ulcerative colitis.

The patient N. McE. showed some initial improvement but he failed to recover from the attack of ulcerative colitis and he was treated by panproctocolectomy 5 weeks after admission.

Relationship of the peak effect to steroid therapy

Four patients in the group who showed recovery had their peak on the initial reading, or this reading was within 1.0 unit of the peak, but they had not been taking steroids, orally or by enema. The mean peak for the 4 patients was 19.87 units (range 17.6 - 21.7) and the mean of the lowest reading after the peak was 10.40 units. The serial readings of one of these patients, E. Mo., are shown in Fig. 76(b). In two other patients not taking steroids prior to the initial reading, this level was 19.0 units in one patient and 18.9 units in the other patient, rising to peaks of 27.7 and 27.8 units respectively but falling to 10.8 and 15.7 units after the peak.

All patients were treated with prednisolone following the initial reading. Amongst the patients who showed recovery, in the 15 attacks treated intravenously the patients received in the first 5 days a mean of 48 mg prednisolone-21-phosphate daily, equivalent to 38 mg prednisolone daily. In the 9 attacks which were treated only orally, the mean initial prednisolone dosage was 30 mg daily.

Relationship of the peak effect to epithelial cell density

In the 24 patients who showed recovery, the epithelial cell grading of the initial biopsy was 0 - 2 in 18 instances, in 4 cases it was grade 3, and in 2 cases it was grade 4. The 4 initial biopsies in grade 3 had glucosamine synthetase levels of 6.2, 9.0, 17.6 and 19.0 units, and the last two of these

were in fact the peak readings. The two initial biopsies which were in grade 4 had very low glucosamine synthetase levels, of 3.2 units (patient L. Cof., Fig. 79) and 5.1 units respectively.

Of the 24 biopsies at peak glucosamine synthetase levels, 20 were in epithelial cell grades 0-2, three were in grade 3 (two of these were initial peaks already mentioned and the third had a level of 27.8 units), and one was in epithelial cell grade 4 and had a glucosamine synthetase level of 19.5 units.

Rectal biopsies — Crohn's disease

Twenty-two rectal biopsies from patients with Crohn's disease were assayed for glucosamine synthetase. The pathologist, Dr. R. Whitehead or Dr. J. M. Skinner, considered that the biopsies were abnormal in 9 patients and normal in 13 patients.

Crohn's disease not involving the rectum

In the 13 patients reported as having normal biopsies, sigmoidoscopy showed no evidence of inflammation in the rectum. The author considered that all these 13 biopsies had a normal goblet cell and crypt pattern (grade 0), that 11 out of the 13 had no inflammatory infiltrate but that 2 out of the 13 had a mild inflammatory infiltrate (grade 1) (Table 24). These 13 patients were considered to have Crohn's disease which did not involve the rectum. Examination of their case-notes showed that the rectum had never been involved. The clinical details of these 13 patients are shown in Table 24 together with their glucosamine synthetase levels. The mean glucosamine synthetase level in these 13 biopsies was 20.55 units/g wet wt, compared to a mean of 13.77 units for the 45 normal samples of colonic mucosa, a highly significant increase ($P < 0.001$). In addition, the glucosamine synthetase levels in the apparently normal cut ends of 2 right hemicolectomy specimens for ileo-caecal Crohn's disease were 20.4 and 29.6 units respectively; the author graded the histological sections in both of these patients as showing a normal epithelial cell pattern (grade 0) and a mild inflammatory infiltrate (grade 1).

Crohn's disease involving the rectum

The details of the 9 patients with Crohn's disease involving the rectum and the glucosamine synthetase levels in their biopsies are shown in Table 25. The mean level for these biopsies was 14.03 units/g wet wt. They were a heterogeneous group histologically although all were in epithelial cell grades 0-2.

TABLE 24. Glucosamine synthetase in the rectal mucosa of 13 patients with Crohn's disease not involving the rectum

Patient	Histology	Daily predni-solone dosage	Crohn's disease active	ESR	Previous resections	Furthest distal extent of Crohn's disease	Glucosamine synthetase (units/g wet wt)
F.Bu.	Normal	—	–	6	—	Caecum	18.2
C.B.	Normal	—	–		2 segments of colon (1 yr)	Descending colon	21.8
P.Ca.	Normal	20 mg	+	5	Ileo-caecal (1 yr)	Caecum	23.7
D.E.	Normal goblet cells; mild inflammation	10 mg	+	2	Ileo-caecal (7 yr)	Caecum	30.8
R.Gr.	Normal goblet cells; mild inflammation	40 mg + enemas	+	31	—	Sigmoid	23.9
F.Hor.	Normal	20 mg	+		—	Sigmoid	12.1
N.L.	Normal	—	–	2	Ileo-caecal (4 yr)	Caecum	23.7
A.Mit.	Normal	10 mg	+	5	—	Ileum	15.6
C.Mo.	Normal	15 mg	+	17	Caeco-proctostomy (11 yr)	Sigmoid	17.0
R.We.	Normal	—	+	65	Ileo-caecal (8 yr)	Caecum	14.6
M.Wilt.	Normal	—	–	5	Ileo-caecal (6 yr)	Caecum	20.8
G.Wo.	Normal	—	–		Ileo-caecal (3 yr)	Descending colon	26.3
C.Wo.	Normal	—	–	11	2 segments of small intestine (12, 4 yr)	Ileum	18.7
						(Number of patients)	(13)
						Mean	20.55
						S.D.	5.20
						S.E.M.	1.44

The mean was similar to that of the normal series and to that of similar biopsies in ulcerative colitis (Table 21). The standard deviation was greater than for the normal series.

Serial biopsies in one patient with Crohn's disease of the colon

One patient, R. Gr., (Table 24), had an acute attack of Crohn's disease of the colon with sparing of the rectum and was treated with the 5-day intravenous steroid regime (60 mg prednisolone-21-phosphate daily) as for ulcerative colitis. Serial biopsies in this patient were assayed for glucosamine synthetase and the results are shown in Fig. 81. The enzyme levels rose to 31.2 units as the patient's clinical state improved.

Rectal biopsies — Indeterminate group

Table 26 shows the glucosamine synthetase levels in the rectal biopsies of 5 patients in whom a firm diagnosis of ulcerative colitis or Crohn's disease could not be upheld although there was evidence that the patient had one or the other. In 3 patients, the glucosamine synthetase levels exceeded 20.0 units when the epithelial cell pattern was normal (grade 0) or only slightly abnormal (grade 1) and there was no inflammatory infiltrate.

DISCUSSION

Control samples

Although normal mucosa had glucosamine concentrations which gave apparent glucosamine synthetase levels by this assay of less than 1.0 unit, the three samples of inflamed mucosa assayed had glucosamine concentrations equivalent to glucosamine synthetase levels of 1.2-1.8 units. There must be a possibility that up to 2.0 units of glucosamine synthetase activity are accounted for by tissue hexosamine levels.

Glucosamine synthetase in relation to histological parameters

Epithelial cells

Glucosamine synthetase levels bore a close correlation with the overall epithelial cell density in the panproctocolectomy series (Fig. 66), which was also apparent in the biopsy series (Fig. 71). The colectomy series provided

TABLE 25. Glucosamine synthetase levels in the rectal mucosa of 9 patients with Crohn's disease involving the rectum

Patient	Histology (author's grading) Epithelial cells	Histology (author's grading) Inflammatory infiltrate	Daily prednisolone dosage	ESR	Glucosamine synthetase (units/g wet wt)
C.A.	1	0	20 mg	65	12.9
L.A.	0	1	—	20	17.9
I.Bi.	2	1	10 mg	50	19.4
K.D.	0	2	15 mg	4	15.2
M.El.	1	1	—	15	19.0
R.Fi.	1	1	Enemas	5	9.5
I.Si.	2	3	Enemas	46	7.9
E.V.	0	1	20 mg	2	20.1
M.Wils.	1	1	—	8	4.4
			(Number of patients)		(9)
			Mean		14.03
			S.D.		5.29
			S.E.M.		1.76

TABLE 26. Glucosamine synthetase levels in the rectal biopsies of 5 patients with either ulcerative colitis (U.C.) or Crohn's disease but no certainty as to which

Patient	Histology (author's grading) Epithelial cells	Histology (author's grading) Inflammatory infiltrate	Daily prednisolone dosage	Diagnosis favoured by the clinician	Glucosamine synthetase (units/g wet wt)
R.Ah.	0	0	20 mg	Crohn's	14.8
I.Ham.	1	1	—	U.C.	14.0
S.Hi.	3	3	—	Crohn's	24.5
42 days later	2	1	Enemas	Crohn's	22.8
98 days	0	0	—	Crohn's	22.7
C.S.	0	0	Enemas	U.C.	20.7
R.S.	1	0	—	Crohn's	22.4

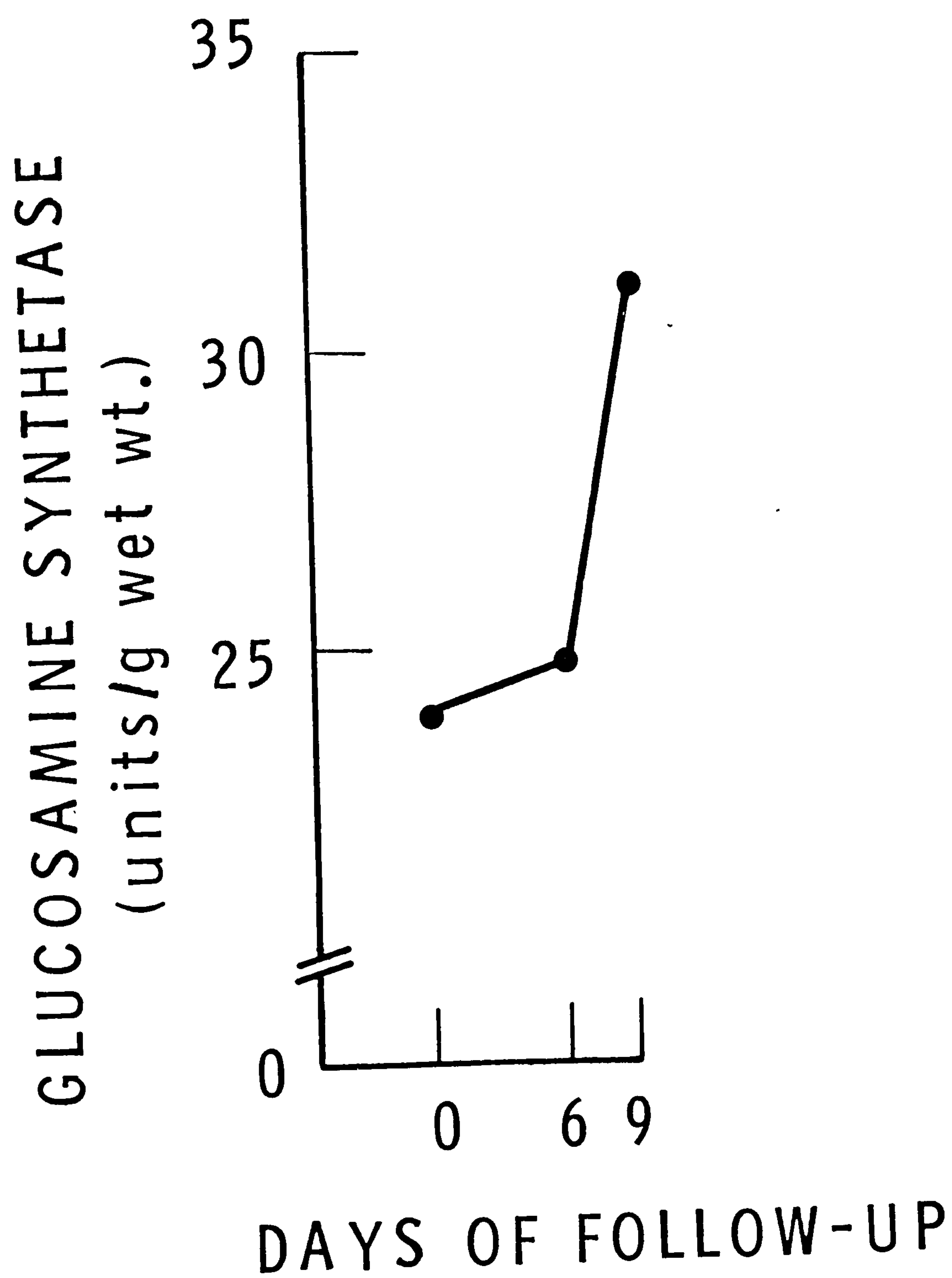


FIG. 81. Glucosamine synthetase levels in serial rectal biopsies of a patient (R.Gr.) with acute Crohn's disease of the colon sparing the rectum who was treated with the 5-day intensive intravenous steroid regime.

a more homogeneous group of samples than the biopsy group. In the colectomy series, all the patients suffered from disease of such severity that removal of the colon was required, whereas in the biopsy series some patients were already showing evidence of clinical recovery when first seen and this may have raised their initial glucosamine synthetase levels. Differences between patients, including differences in steroid and other therapy and in the phase of recovery from an acute attack of colitis, do not apply when 3 samples taken from different regions of the same colectomy specimen are compared (Fig. 68).

The results of two patients (G. Pr. and A. Tow.), shown in Fig. 68, confirm the impression derived from Figs. 66 and 71 that the glucosamine synthetase levels are similar in epithelial cell grades 0, 1 and 2; in other words, they are related to the overall epithelial cell density irrespective of mucosal destruction.

The definitions of grades 3 and 4 of epithelial cell density (grossly diminished, and absent, respectively) are clear-cut and the corresponding results, shown in Figs. 66 and 68, are so definite that quantitative histology is not required to show the correlation between glucosamine synthetase levels and epithelial cell density.

Inflammatory cells

The 9 samples in the colectomy series in grade 4 would be expected to have no glucosamine synthetase activity. In fact, they had levels of 3.0 ± 0.9 (S.D.) units. The inflammatory granulation tissue may have significant glucosamine synthetase activity. Schönhöfer and Anspach (1967) found elevated levels of glucosamine synthetase in the inflammatory tissue of the wall of granulomata induced in rats by carrageenin. One of the pieces of submucosa from a panproctocolectomy specimen with ulcerative colitis had a glucosamine synthetase level of 1.6 units and a significant inflammatory infiltrate (Fig. 70), although such a level might be accounted for by the hexosamine concentration of the tissue, perhaps from the immunoglobulins of lymphocytes and plasma cells.

The correlation of glucosamine synthetase levels with the degree of histological inflammation was negative (Figs. 67, 72 and 73), although with a low correlation coefficient, but this was probably because increasing inflammation is associated with concurrent destruction of the epithelial cells (Fig. 75). In 8 of the biopsies, the epithelial cell density was normal (grades 0-2) but there was a heavy inflammatory infiltrate (grade 3). These 8 biopsies

had a mean glucosamine synthetase level of 17.55 units, which was a little higher than the mean of 16.46 units of the 24 biopsies with a normal epithelial cell density (grades 0-2) and an inflammatory cell grading of 0 or 1. Thus the contribution of any inflammatory cell infiltrate to the glucosamine synthetase levels in the biopsies was not really detectable.

There was no detectable glucosamine synthetase in the buffy coat of the blood of one patient with a leukaemoid white cell count.

The pathologist's grading of inflammation versus the author's grading

It is interesting to note that the negative correlation of glucosamine synthetase levels with the degree of inflammation was slightly closer with the author's grading ($r = -0.30$, Fig. 72) than with the pathologist's grading ($r = -0.21$, Fig. 73). This is probably because, although the pathologists were very experienced, the author was always aware that the grading was to be used for quantitative research and also because most biopsies were graded by the author at a single sitting.

Goblet cell density

Twelve biopsies with a grossly diminished goblet cell density but with a normal epithelial cell density (grades 0-2) had a normal glucosamine synthetase level of 14.87 units (Fig. 74). Thus the glucosamine synthetase level correlates with the density of the epithelial cells rather than with the goblet cell density.

Crypt hypertrophy

The appearance of hyperplastic crypts full of goblet cells was only seen in one sample from the colectomy series and in one follow-up biopsy. The glucosamine synthetase levels in these two samples were 31.6 and 26.8 units respectively, which were very high levels.

Glucosamine synthetase in relation to steroid therapy

Patients taking steroids had higher levels of glucosamine synthetase than patients not taking steroids (Tables 20, 21) but these differences were not statistically significant. It is reasonable to suppose that patients who were being treated with steroids were more likely to be recovering from a recent acute attack of ulcerative colitis than patients who were not receiving steroids (see below).

Glucosamine synthetase levels in serial rectal biopsies in an acute attack of ulcerative colitis

The rise to a peak during recovery from an acute attack of ulcerative colitis

In 24 acute attacks of ulcerative colitis (in 21 patients) in which there was evidence of recovery from the attack, the glucosamine synthetase levels rose to a peak or they were at a peak when first measured. In 21 out of the 24 attacks, the peak was 19.0 units or more; in one attack, it was 17.6 units falling to 13.1 units; in one, it was 13.6 units falling to 6.5 units; and in only one case (patient L. Cof., Fig. 79) was there no evidence of a true peak above a normal level of glucosamine synthetase, this patient showing a rise in the enzyme level consistent with recovery from severe epithelial cell depletion to a normal density. All 15 attacks treated with the intravenous regime showed a peak of 19.0 units or more.

Relationship to steroid therapy

All these patients were treated with steroids and the possibility must be considered that this peak effect is due to steroid therapy. However, 5 patients had glucosamine synthetase levels of 18.9 units or more on their initial readings when they were receiving no steroids, and in two of these patients, and in the patient with a peak of 17.6 units, this initial reading prior to steroid therapy was their peak reading or it was within 1.0 unit of the peak.

Relationship to starvation and the intravenous steroid regime

The intensive intravenous regime which was used to treat 15 of the attacks involved 5 days of parenteral feeding only, apart from sips of water. There were three reasons for thinking that the peak effect was not the result of an empty bowel. First, most of the 9 patients who were treated without the 5-day intravenous regime also showed a peak; secondly, 6 of the 15 patients who were treated intravenously had their peaks more than 14 days after recommencing a normal diet; and, thirdly, some patients were at their peak level before any treatment was instituted.

The patients on the intravenous regime had a more prominent peak than the patients treated only orally, but the attacks of colitis in the patients treated intravenously were more severe and their treatment was more vigorous.

Relationship to epithelial cell density

The peak effect appeared to be independent of epithelial cell density. Some of the patients started from very low glucosamine synthetase levels in the presence of epithelial cell depletion but the peak effect was a rise of glucosamine synthetase activity above the level expected for a normal epithelial cell density.

Serial glucosamine synthetase levels in patients who did not recover from their attack

The peak effect was not seen in the 5 patients who did not recover from their acute attack of ulcerative colitis (Fig. 80). Four of these patients came to panproctocolectomy. The fifth patient (M. Car.) recovered by the 67th day, when there was a peak of 16.9 units.

The peak as a manifestation of recovery

Thus the peak effect in the glucosamine synthetase activity of the rectal mucosa during recovery from an acute attack of ulcerative colitis appears to be a manifestation of recovery. It corresponds to the rise in glucosamine synthetase levels in neonatal rat liver following partial hepatectomy that was shown by Akamatsu and Maeda (1971) (Fig. 12) and confirmed by Bley et al (1973).

More immature cells during the recovery phase may have higher glucosamine synthetase activity than mature cells. Weiser (1973a) showed that the less mature enterocytes of the small intestinal crypts in the rat have higher levels of thymidine kinase than the more mature enterocytes at the tips of the villi.

An increased supply of glucosamine for increased synthesis of glycoprotein may be a requirement for the satisfactory healing of the colonic mucosa in ulcerative colitis. The patients who did not recover may not have done so because they did not produce sufficient mucus to permit mucosal healing.

Glucosamine synthetase levels in Crohn's disease

Inflamed mucosa

When Crohn's disease affects the colon so severely that colectomy becomes necessary, glucosamine synthetase levels in the mucosa are similar to those in ulcerative colitis (Fig. 64). The levels bear the same relationship

to the epithelial cell density as they do in ulcerative colitis (Fig. 68, patients C. A. and A. Tow).

Only 9 rectal biopsies were obtained from patients with Crohn's disease involving the rectum and in only 2 of these was the inflammation moderate or severe (Table 25). The small number of patients in this series is probably because most patients with Crohn's disease of the colon at the Radcliffe Infirmary have a diversionary split ileostomy with an isolated colon (Lee and Truelove, 1975) and it was decided not to study these patients as it would have been difficult to assess the role of an isolated colon.

Uninflamed mucosa

Possibly the most fascinating result of this thesis has been the finding that the glucosamine synthetase levels in the rectal biopsies of 13 patients with Crohn's disease elsewhere in the bowel but sparing the rectum were abnormally high (Table 24), with a mean of 20.55 units which is similar to the levels found during the healing phase of ulcerative colitis. This was not a steroid effect because 7 of the 13 patients were not receiving steroids and their mean level was 20.58 units. Eight of the 13 had never had clinically apparent Crohn's disease distal to the caecum and their mean level was 20.76 units. Six of the 13 patients did not appear to have any clinical evidence of active Crohn's disease and some of the six had not had active disease for a number of years; their mean was 21.58 units. The 5 patients who had not had caecal resections had a lower mean, of 17.7 units, but this need not mean that high glucosamine synthetase levels are related to previous resections: one of these five patients, R. Gr., had a follow-up reading of 31.2 units (Fig. 81).

Eleven out of the 13 patients had entirely normal histological appearances on their rectal biopsies. The other two patients had mild inflammation according to the author's grading but the pathologist considered that both these biopsies were within normal limits.

This finding of abnormal glucosamine synthetase levels in the histologically normal rectal mucosa in patients with Crohn's disease not involving the rectum will need to be confirmed in a larger series but it seems to indicate that the colonic mucosa is always abnormal in Crohn's disease even if its appearances are normal. Crohn's disease has always previously been considered as a patchy disease, although frequently extensive. These results suggest that it is a diffuse disease, even though the overt manifestations may be focal. Even

in the areas which are macroscopically and microscopically normal, the levels of glucosamine synthetase resemble those found in ulcerative colitis during the phase of recovery from an attack. This suggests the possibility that the intact mucosa is reacting against some potentially damaging factor by an enhanced production of mucus, by which it protects itself.

Glucosamine synthetase measurements in the differential diagnosis of ulcerative colitis and Crohn's disease

The starting point which led to this thesis was the difference in the pattern of mucus-secreting cells in ulcerative colitis and Crohn's disease. The measurement of glucosamine synthetase levels in the rectal mucosa has not shown any differences from ulcerative colitis when the mucosa in Crohn's disease shows histological features of the disease. However, high levels of glucosamine synthetase in histologically normal mucosa or in a mucosa with only a mild inflammatory infiltrate may, if persistently recorded, be indicative of Crohn's disease.

In one of the 3 patients in whom it was impossible to decide whether the diagnosis was ulcerative colitis or Crohn's disease (S. Hi., Table 26), three rectal biopsies taken over 3 months had glucosamine synthetase levels of more than 22.0 units; in the third biopsy, the histological appearances were entirely normal. Perhaps this finding supports a diagnosis of Crohn's disease, which was the diagnosis favoured by the clinician; further studies are required to determine whether the measurement of glucosamine synthetase levels can assist in the differential diagnosis of ulcerative colitis and Crohn's disease.

CONCLUSIONS

Glucosamine synthetase levels in the colonic mucosa have been shown to correlate with the overall epithelial cell density although not with the goblet cell density. They are diminished only when the epithelial cells are depleted and not when they are just distorted or damaged. Very severely inflamed mucosa may have a slight but significant glucosamine synthetase activity even when the epithelial cells are completely absent; this is probably due to glucosamine synthetase in the inflammatory infiltrate.

During recovery from an acute attack of ulcerative colitis, the glucosamine synthetase activity of the rectal mucosa usually rises to a peak of at least 1 standard deviation above the mean for normal mucosa. Patients who do not recover from their acute attack usually do not show such a peak.

In Crohn's disease, when the colonic mucosa is inflamed, it has glucosamine synthetase levels which are similar to those found in ulcerative colitis of comparable severity. When the colonic mucosa is histologically normal, it possesses an abnormally high level of glucosamine synthetase, corresponding to that found in the healing phase of ulcerative colitis.

CHAPTER 11

MEMBRANOUS COLITIS

Membranous colitis is most often seen following the use of broad-spectrum antibiotics, especially the recently introduced lincomycin and clindamycin (British Medical Journal, 1974), although it also occurred before the days of antibiotics (Penner and Bernheim, 1939). Major surgery and septicaemia both predispose to the occurrence of membranous colitis (Dearing and Heilman, 1953). The essential pathological feature appears to be mucosal ischaemia in the intestine, affecting most frequently the large intestine but often also the small intestine. The mucosal ischaemia appears to be due to fibrin plugging of the capillaries (Whitehead, 1971). The fibrin plugs probably form during hypotensive episodes, or as part of an Arthur reaction to bacterial antigens, or from disseminated intravascular coagulation. All of these can occur in septicaemia, and abnormal bacterial antigens can arise in the bowel as a result of treatment with broad-spectrum antibiotics. The mucosal ischaemia leads to the typical lesion of dilated glands, which sometimes break down to become ulcerated, together with mucosal inflammation and a membrane of fibrin, mucus and inflammatory cells (Fig. 82).

There is probably a spectrum of colonic mucosal reaction from membranous colitis to typical post-embolic or post-thrombotic ischaemic colitis (Goulston and McGovern, 1965; McGovern and Goulston, 1965).

Patients with membranous colitis frequently have large amounts of mucus in the stools, presumably coming from the goblet cells of the dilated crypts.

PATIENTS STUDIED AND METHODS

Glucosamine synthetase levels were studied in 3 patients with typical membranous colitis and in 2 patients with probable membranous colitis. In each patient, serial biopsies were assayed, by the 4 mg method, as for the patients in an acute attack of ulcerative colitis (Chapter 10).

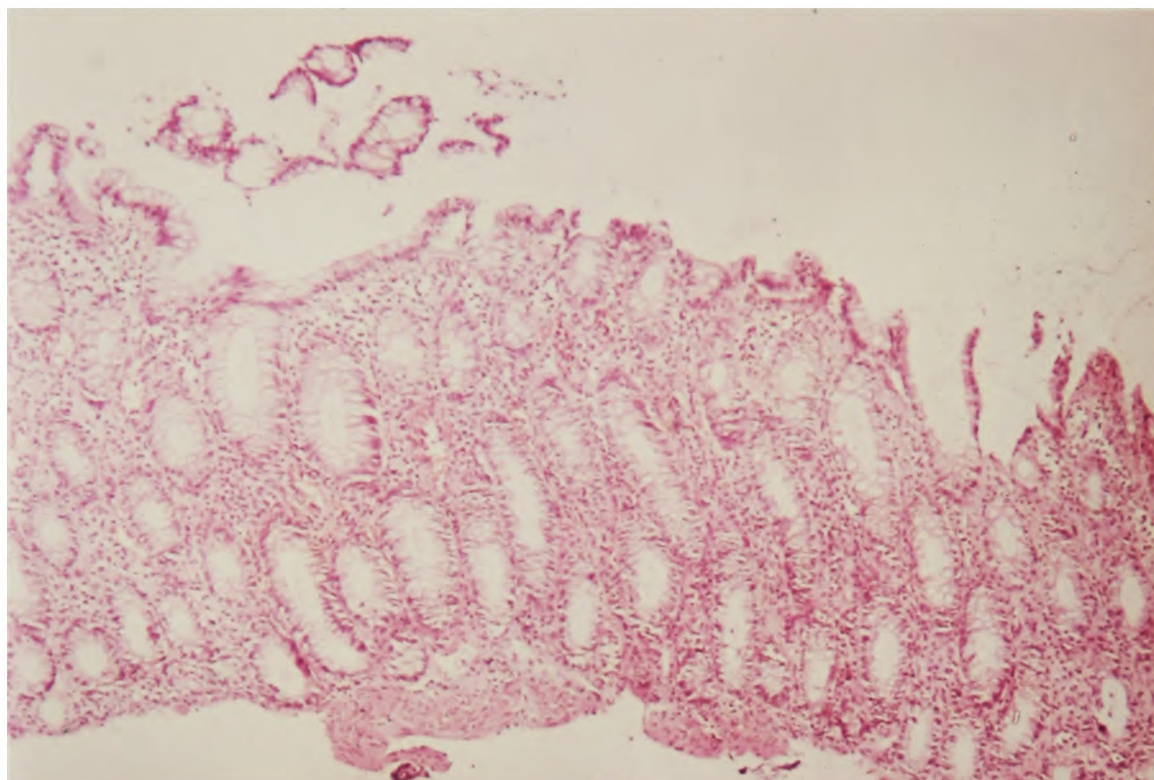


FIG. 82. Rectal biopsy of a patient (M. T.), with membranous colitis.

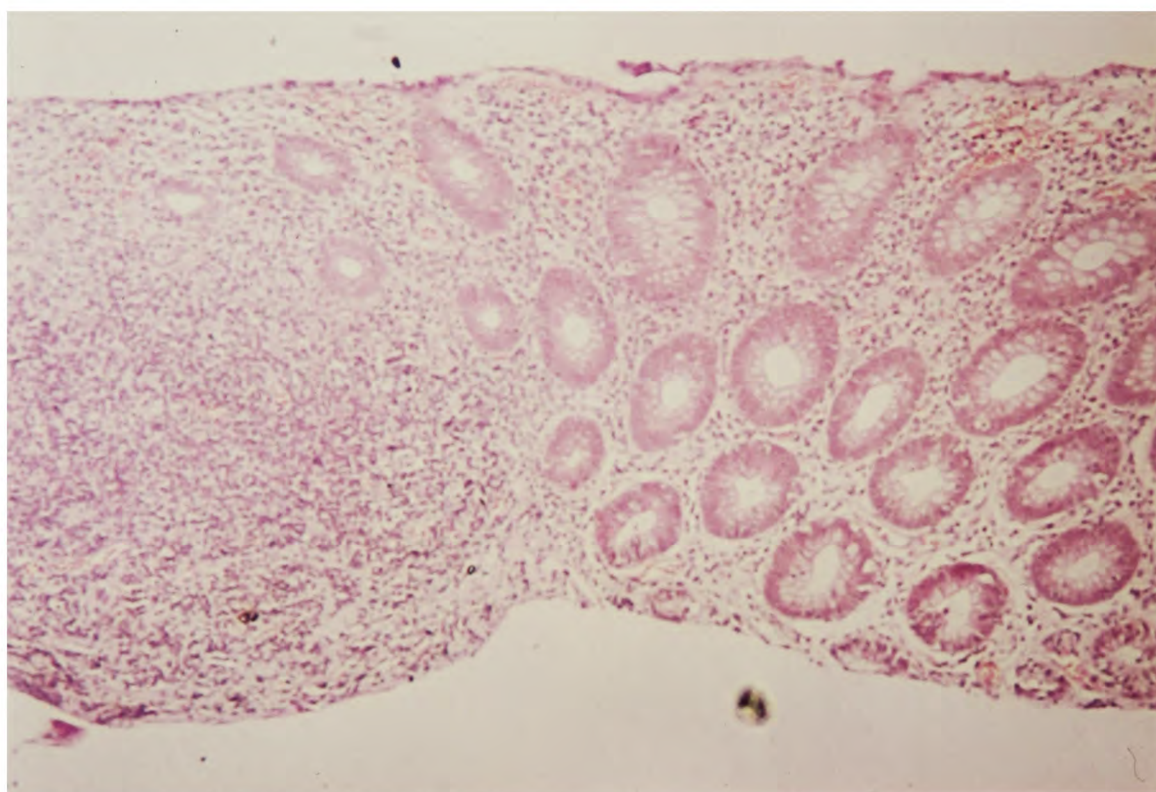


FIG. 83. Rectal biopsy of a patient (E. Bla.) with a history suggestive of membranous colitis.

The three patients with typical membranous colitis had the following histories:-

Q. S. was a 58-year-old Bangladeshi man who developed membranous colitis following a mild upper respiratory tract infection. Close questioning of the patient and his general practitioner confirmed that he had taken no antibiotics.

J. Robn. was a 67-year-old man who had received gentamicin, ampicillin, cloxacillin, lincomycin and cephalothin for a subphrenic abscess following vagotomy and gastrojejunostomy for a perforated duodenal ulcer.

M. T. (whose biopsy is shown in Fig. 82) was a 67-year-old woman who had received cloxacillin for wound infection following an operation for varicose veins.

The two other patients had received broad-spectrum antibiotics and had then developed bloody diarrhoea. Their biopsies were not typical of membranous colitis but they had ischaemic features (Fig. 83). Their histories were as follows:-

E. Bla. (Fig. 83) was a 68-year-old woman who had received clindamycin for a septic toe. The diarrhoea started 2 days after starting the clindamycin.

G. Si. was a 27-year-old man who was treated with tetracycline for acne. After 16 days on tetracycline, he developed bloody diarrhoea.

All five patients complained of excessive amounts of mucus in their stools.

All the patients were treated with systemic steroids and with steroid enemas. All but one were treated with the 5-day intensive intravenous steroid regime as recommended by Truelove and Jewell (1974) for acute attacks of ulcerative colitis, with prednisolone-21-phosphate 40-60 mg daily. The fifth patient, G. Si., was treated with prednisolone 20 mg daily orally and prednisolone retention enemas. All the patients continued to receive oral prednisolone, 20 mg daily, and prednisolone enemas for 6 weeks after the attack.

RESULTS

The glucosamine synthetase levels in the serial rectal biopsies of the 5 patients are shown in Fig. 84. The 3 patients with typical membranous

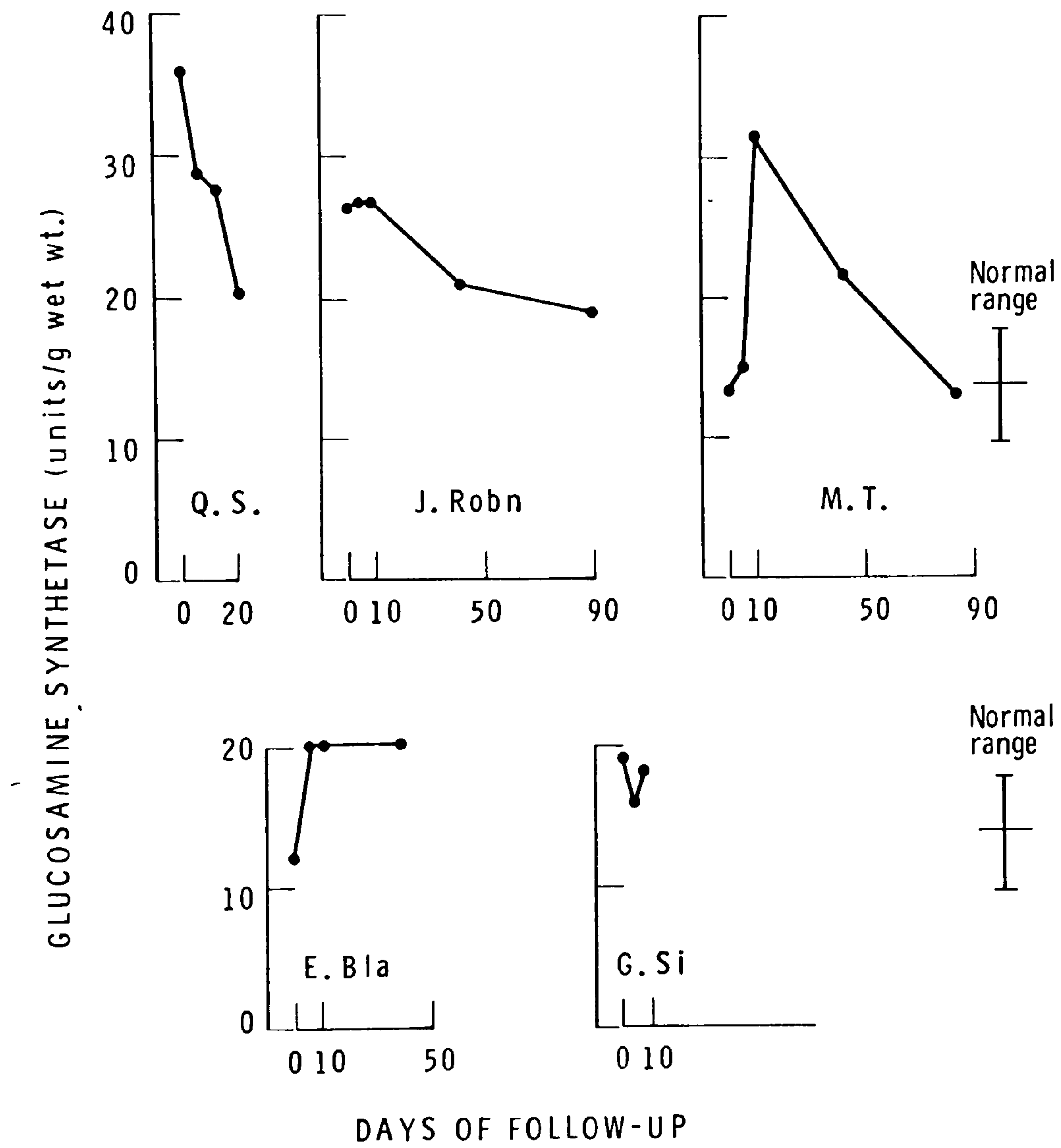


FIG. 84. Glucosamine synthetase levels in serial rectal biopsies of 5 patients with membranous colitis, all of whom were treated with prednisolone 20-60 mg daily.

colitis had glucosamine synthetase levels which rose to peaks of 36.0, 27.0 and 31.7 units respectively, which were extremely high values compared to the normal (13.8 ± 4.0 units). They were high values even compared to the peak levels reached in attacks of ulcerative colitis. The other two patients also had at least one reading above 19.0 units.

DISCUSSION AND CONCLUSIONS

In membranous colitis, the glucosamine synthetase activity of the rectal mucosa appears to rise to very high levels. The levels reached in two of the patients were higher than in all the patients with ulcerative colitis or Crohn's disease reported in Chapter 10. It is tempting to postulate that these high levels are related to the mucus hypersecretion which was a striking feature in these cases of membranous colitis. However, this theory may be unfounded because in one patient (M. T.) the peak occurred more than 5 days after the onset of the attack of membranous colitis.

The peak is not related to steroid therapy because two of the patients (Q. S. and J. Robn.), both with very high peaks, were at their peak levels before treatment was started. The peak effect may be due to healing of the mucosa and be analogous to the peak following an acute attack of ulcerative colitis (Chapter 10). The peak may be higher than in ulcerative colitis because, once the damaging agent has been withdrawn, the bowel in membranous colitis is probably fundamentally normal and capable of a vigorous recovery.

CHAPTER 12

CARCINOMA OF THE COLON

Colectomy specimens for carcinoma of the colon provided normal mucosa from their cut ends for the determination of the normal range of glucosamine synthetase levels in human colonic mucosa (Chapter 9). Glucosamine synthetase was also measured in the tumour tissue in some of the specimens, and in the mucosa immediately adjacent to the tumour in 4 specimens.

TUMOUR TISSUE

Kaufman et al (1971) measured the glucosamine synthetase levels in various tumours in the rat. They found a wide range of glucosamine synthetase levels, from 0.06 to 2.1 μmoles glucosamine synthesized/h/g wet wt, compared to 0.6-0.9 units in liver and 0.13 units in full-thickness small intestine (Table 2). Kaufman et al found higher glucosamine synthetase levels when there was less stroma in the tumour.

PATIENTS STUDIED AND METHODS

Glucosamine synthetase was assayed by the 10 mg method in one portion of the tumour tissue from each of 19 colectomy specimens for carcinoma of the large intestine.

In 4 cases, the piece of mucosa to be assayed was divided in two and one half was sent for histology. In the other 15 cases, the histological sections available were those taken by the pathologist in the routine examinations of the specimen.

The histological sections were examined and the density of the stroma was graded from 0 to 4. In grade 0, there was no stroma; in grade 1, there was a light stroma; in grade 2, there was a moderate stroma; and in grade 3

there was a dense stroma. The morphological features of the tumour, namely the degree of differentiation and the presence of mucus in the tumour, as judged by the author and also as recorded by the pathologist in his report, were also noted.

RESULTS

There was a wide range of glucosamine synthetase activity, from 1.8 to 29.9 units/g wet wt (compared to the range for normal colonic mucosa of 13.8 units ± 4.0, S. D.). There appeared to be no relation to the density of the stroma (Fig. 85).

There was no correlation with the degree of differentiation, the three least differentiated tumours having unremarkable glucosamine synthetase levels of 11.0, 14.7 and 21.0 units.

Mucoid carcinoma

There were 3 specimens of mucoid carcinoma. These had glucosamine synthetase levels of 3.6, 16.3 and 29.9 units.

DISCUSSION AND CONCLUSIONS

Tumour tissue in carcinoma of the colon has a range of glucosamine synthetase levels which is much wider than for normal colonic epithelium. The levels could not be correlated with the density of the stroma, nor with the degree of differentiation, nor with the presence of mucus in the tumour.

THE MUCOSA ADJACENT TO THE TUMOUR

Filipe and Dawson have termed the morphologically normal mucosa immediately adjacent to the tumour in carcinoma of the colon the "transitional" mucosa (Filipe, 1969). In this area of the mucosa, Filipe (1972) found disturbances in the normal pattern of sulphation of the mucus in the goblet cells. Normally the lower two-thirds of the crypts contain sulphated mucus whilst the upper one-third of the crypts and the surface epithelium contain a mixture of sulphated and acidic non-sulphated mucus. In the transitional mucosa, non-sulphated mucus tends to replace sulphated mucus throughout the crypts.

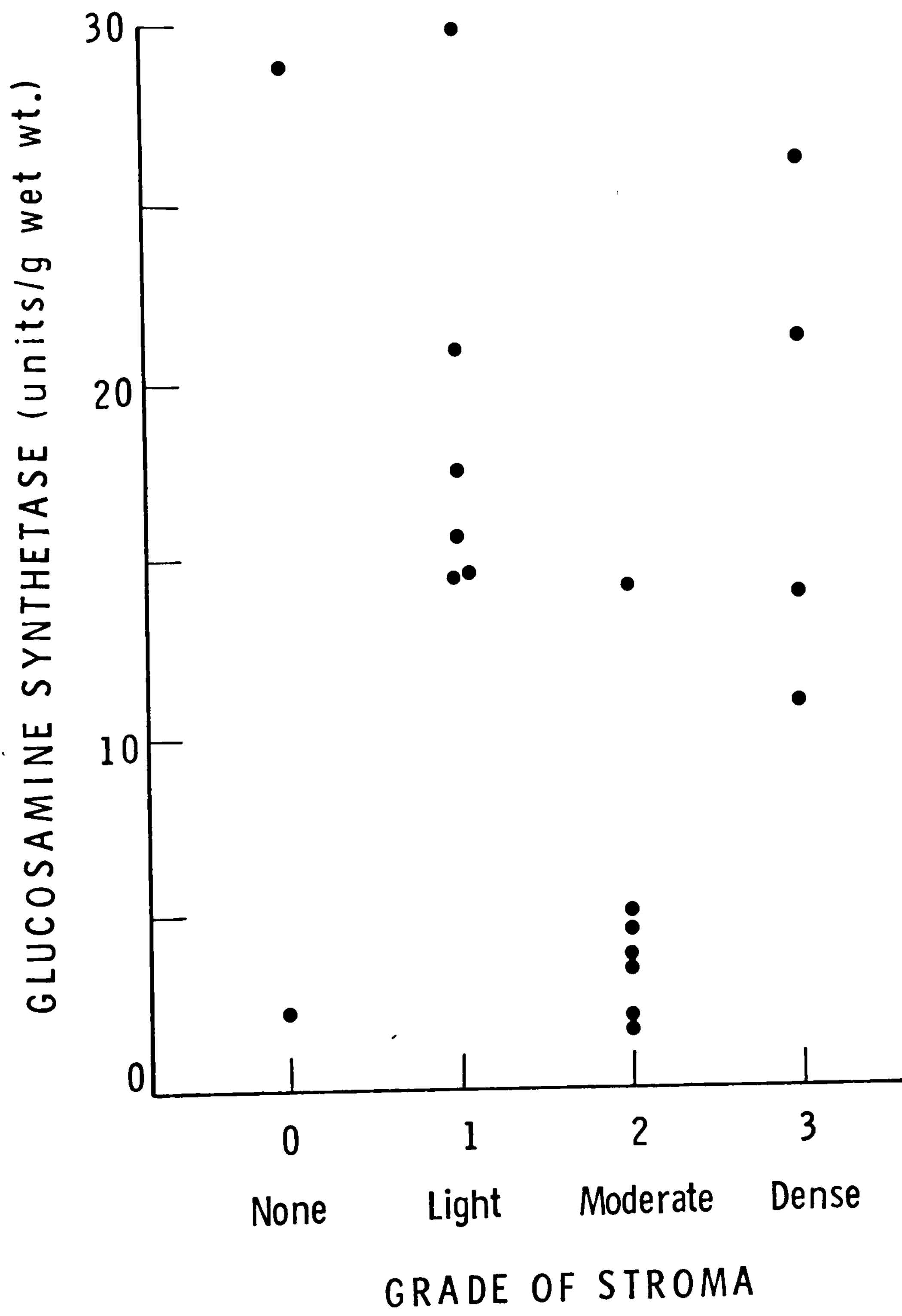


FIG. 85. Glucosamine synthetase in colonic tumour tissue from 19 patients in relation to the density of the fibrous stroma.

Filipe and Cooke (1974) reported finding increased amounts of hexosamine and sialic acid in the transitional mucosa. Marsden and Dawson (1974) measured histochemically the mucosal levels of five enzymes, NADH tetrazolium reductase, glucose-6-phosphate dehydrogenase, succinic dehydrogenase, monoamine oxidase and acid phosphatase and found that the first four enzymes had higher levels than normal in the transitional mucosa.

It was considered worthwhile to study glucosamine synthetase levels in the transitional mucosa, especially as tumours can have abnormal glycoprotein metabolism and, also, tend to arise in hyperplastic mucosa. High glucosamine synthetase levels have been demonstrated in the healing mucosa in ulcerative colitis (Chapter 10) and this disease is frequently complicated by carcinoma of the colon.

PATIENTS STUDIED AND METHODS

In 4 colectomy specimens from patients with carcinoma of the colon, a piece of macroscopically normal mucosa was taken from an area immediately adjacent to the tumour. A second piece was taken on the opposite side of the tumour to the first piece. Two pieces of normal mucosa were taken 10 cm away from the tumour, one proximal and one distal. Each piece was divided in two, one half being assayed for glucosamine synthetase and the other half being used for histochemical studies. The samples assayed for glucosamine synthetase all weighed between 15 and 35 mg. From each specimen, the two samples of adjacent mucosa were pooled and the two samples of distant mucosa were pooled, the glucosamine synthetase being measured in a combined homogenate of each pair of samples, by the 10 mg method.

Histochemical studies

Each sample was stained with PAS to show neutral mucus, with Alcian blue at pH 2.5 to show acidic non-sulphated mucus, and with the high iron-diamine reagent to show sulphated mucus.

RESULTS

The glucosamine synthetase levels in the adjacent and distal samples of mucosa are shown in Table 27. There were no differences between the adjacent mucosa and the mucosa 10 cm away from the tumour.

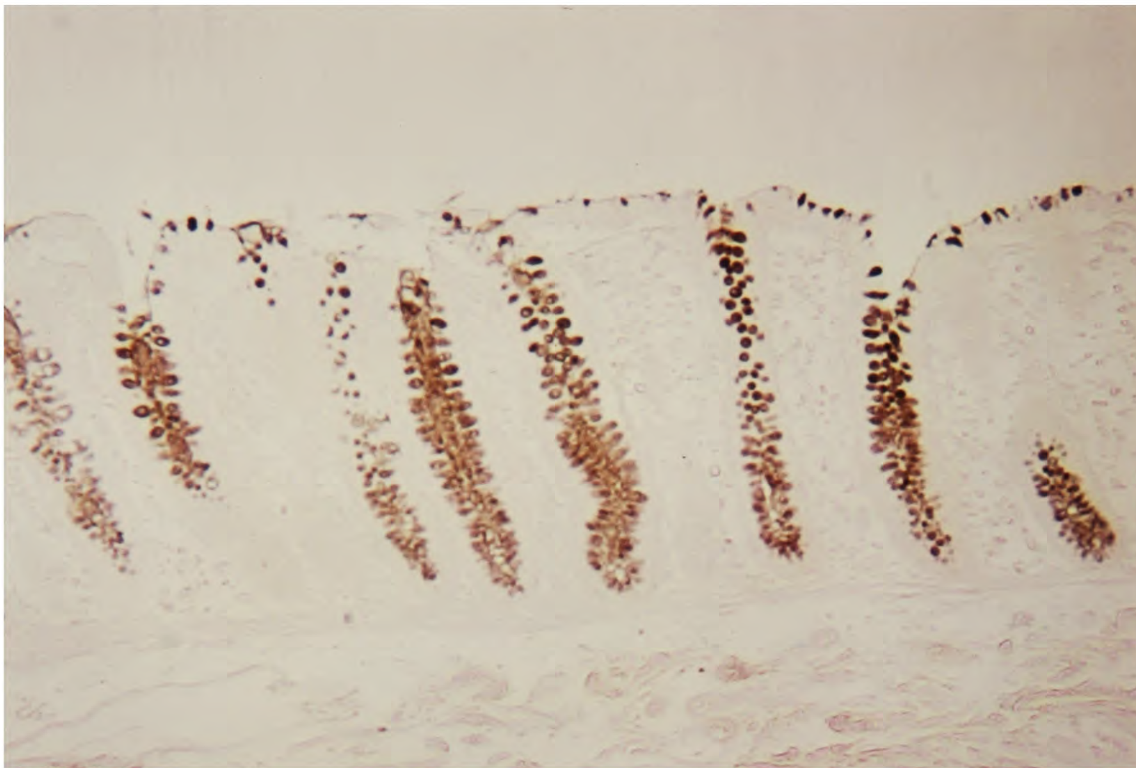


FIG. 86. Normal colonic mucosa stained by high iron-diamine (to show sulphated mucus) and Alcian blue at pH 2.5 (to show acidic non-sulphated mucus).

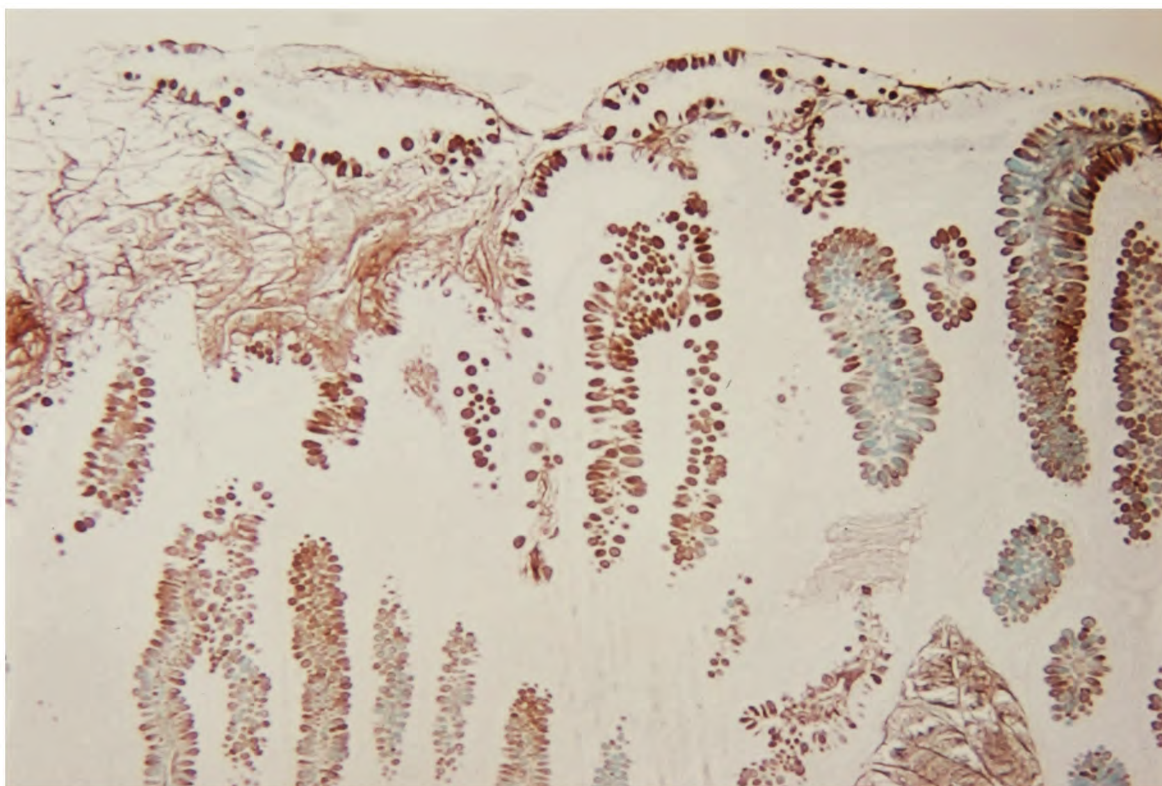


FIG. 87. The "transitional" colonic mucosa: the morphologically normal mucosa immediately adjacent to a tumour, stained with high iron-diamine and Alcian blue at pH 2.5.

TABLE 27. Glucosamine synthetase levels in the colonic mucosa immediately adjacent to a carcinoma and at 10 cm distant from the carcinoma

Patient	Mucosa adjacent to the tumour	Mucosa 10 cm from the tumour
R.Gos.	16.1	19.6
M.Mi.	15.0	12.6
H.N.	19.0	14.4
R.Nu.	11.9	17.0
Mean	15.5	15.9

Histochemistry

The sections of normal mucosa, distant from the tumour, showed sulphated mucus along the entire length of their crypts and in the surface epithelium (Fig. 86).

Although two samples of mucosa adjacent to the tumour were taken from each of 4 colectomy specimens, in only a tiny portion of one specimen was there replacement of the sulphated mucus by acidic non-sulphated mucus. This change was seen best in portions of mucosa which were included in the sections of tumour (Fig. 87).

DISCUSSION AND CONCLUSIONS

The glucosamine synthetase activity of the morphologically normal colonic mucosa immediately adjacent to a carcinoma was normal in the 4 patients in whom it was studied. A study of a larger series of colectomy specimens did not appear to be warranted. The "transitional" mucosa of Filipe and Dawson, in which the sulphated mucus of the colonic goblet cells is replaced by non-sulphated mucus, only appears to be present in a small area of mucosa adjacent to the tumour, an area which is too small to be studied biochemically rather than histochemically.

CHAPTER 13

GASTRIC AND DUODENAL BIOPSIES

The first stage in the work reported in this thesis was the development of an assay for glucosamine synthetase on a sufficiently miniature scale to enable the enzyme to be measured in rectal biopsies. In the event, the degree of miniaturization achieved meant that the assay could be performed with just over 4 mg of tissue, which enabled the method to be used with gastric and duodenal biopsies taken through a fibreoptic endoscope. These biopsies weigh between 3 and 5 mg, so two biopsies from the same area of mucosa had to be pooled in order to be able to perform the assay with a reasonable degree of accuracy.

The mucosal protection theory of peptic ulceration, discussed in Chapter 1, proposes that gastric and duodenal ulcers are a consequence of an impairment in the quality or quantity of the gastric or duodenal mucus, which normally protects the mucosa against the damaging effects of gastric juice. Sander et al (1975) have claimed that gastric ulcers may result from inhibition of the glucosamine synthetase activity of the gastric mucosa. They induced gastric ulcers in rats by restraining them for 3 hours in a cold environment. The glucosamine synthetase levels of the body mucosa fell to 70% of control values, although the glucosamine synthetase activity of the liver also fell, to 25% of control values. The glucosamine synthetase levels returned to normal between 9 and 21 hours after release of the animal from restraint.

The opportunity to measure glucosamine synthetase in the gastric and duodenal mucosa of human patients has allowed the theory of Sander et al to be tested in the clinical situation to which these authors considered that their rat experiments related.

PATIENTS STUDIED AND METHODS

Gastric and duodenal biopsies were taken under direct vision through an Olympus GIF-D panendoscope using the standard biopsy forceps. The patients were selected from amongst those who were having this examination at the Radcliffe Infirmary as part of their clinical management. Thirty-one patients were studied, with the following diagnoses:-

Normal endoscopic appearances. 2 patients with functional abdominal pain.

Oesophageal lesions. 5 patients — 2 with hiatus hernia, 2 with oesophagitis without hiatus hernia, and 1 with a Mallory-Weiss tear.

Gastric ulcer. 6 patients with gastric ulcer alone.

Gastric ulcer + duodenal ulcer. 1 patient.

Gastric ulcer + healing duodenal ulcer. 1 patient.

Suspected gastric ulcer. 1 patient with a gastric ulcer on barium meal not confirmed on endoscopy.

Duodenal ulcer. 7 patients with duodenal ulcer alone.

Previous duodenal ulcer. 2 patients, who had suffered from duodenal ulcer 1 and 2 years previously, respectively.

Atrophic gastritis. 3 patients.

Gastric carcinoma. 1 patient.

Polya partial gastrectomy with a stomal ulcer. 1 patient.

Gastric erosions induced by ulcerogenic drugs. 1 patient with rheumatoid arthritis who was taking aspirin, prednisolone and indomethacin.

Patients were not included in the study if they were taking carbenoxolone sodium or Caved-S (deglycyrrhizinated liquorice, a compound with similar actions to carbenoxolone), nor, expect for the last patient, if they were taking ulcerogenic drugs such as salicylates, corticosteroids, phenbutazones or indomethacin. Patients with intercurrent disease, such as endocrine disorders, myeloproliferative diseases or liver disease, were also excluded.

The sites of the biopsies

Biopsies were taken from three sites in the stomach, namely from the high lesser curve, from the greater curve in the body and from the antrum, and also from the duodenal cap. Occasionally insufficient material was

available from one of the sites for the enzyme assay to be performed. At the beginning of the study, biopsies were taken from only two sites in the stomach, namely the greater curve and the antrum. Biopsies from the high lesser curve were taken following the report of Hossenboccus et al (1975) that there was a lower potential difference across the gastric mucosa in the high lesser curve than in the rest of the stomach. Towards the end of the present study, duodenal biopsies only were taken from patients with duodenal ulcer. The biopsies were always taken well away from any local lesion such as an ulcer, an erosion or a tumour.

Three biopsies were taken from closely adjacent mucosa at each site. One of the biopsies was sent for histological examination and the other two were pooled and assayed for glucosamine synthetase by the 4 mg method. Sometimes less than 4 mg tissue was obtained for the assay, and in these cases the assay was not performed in duplicate (the "2 mg method").

The collection of the biopsies

The biopsies were shaken into normal saline from the opened biopsy forceps of the endoscope. The two biopsies which were to be pooled for the glucosamine synthetase assay were taken with forceps into a bijou bottle containing ice-cold homogenizing buffer. The biopsy for histology was oriented flat on filter paper and placed in formol saline and sent to the Pathology Department for fixation, sectioning, and staining with haematoxylin and eosin.

Blood groups and secretor status

In 27 of the patients, the ABO blood group was determined. In 21 of these patients, the secretor status was also determined from their saliva, which had to be collected before the patient received atropine premedication. These studies were kindly carried out by the Blood Transfusion Department of the Radcliffe Infirmary.

Recording of clinical details

A pro forma was used to record the clinical details and the results. It was similar to that used for rectal biopsies (Fig. 44). Details of the barium meal and the endoscopic findings were recorded, and also the ABO blood group and the secretor status.

Histological assessment

The histological sections were examined and reported by the Radcliffe Infirmary pathologists, usually Dr. R. Whitehead or Dr. J. M. Skinner, and they were also examined by the author, who was unaware of the glucosamine synthetase reading at the time.

RESULTS

Control series

Since there were only 2 patients with entirely normal endoscopic findings, the 5 patients with oesophageal lesions only were included in the control series, to make a total of 7 patients. However, only 2 of these 7 had readings from the high lesser curve. It is notable that few patients with entirely normal endoscopic appearances and with no history of organic disease of the stomach or duodenum are endoscoped at the Radcliffe Infirmary. Roca et al (1975) recruited 10 normal volunteers for endoscopy in order to ascertain the normal histological appearances in the stomach and duodenum, but the use of volunteers was not considered to be warranted in the present study of glucosamine synthetase.

The glucosamine synthetase levels in the 7 patients in the control series are shown in Table 28. In the body and the antrum of the stomach, the mean glucosamine synthetase level was similar, about 10.0 units/g wet wt. In the duodenal cap, the mean level was 14.6 units/g wet wt, which is similar to that of normal colonic mucosa (Chapter 9).

TABLE 28. Glucosamine synthetase levels in gastric and duodenal biopsies of patients with normal endoscopic appearances or oesophageal lesions only

		Glucosamine synthetase (units/g wet wt)			
		Stomach			
Patient	Diagnosis	High lesser curve	Body greater curve	Antrum	Duodenal cap
D.A.	Mallory-Weiss tear		14.3	14.5	14.1
R.Ew.	Hiatus hernia	3.8	4.2	9.7	16.0
J.G.	Oesophagitis		7.6	11.2	17.9
B.H.	Oesophagitis		8.8	6.4	
G.Ma.	Normal	10.0	14.3	10.8	17.0
F.Sa.	Hiatus hernia		12.7	11.9	8.6
M.Sc.	Normal		9.0	9.6	14.1
	Mean	6.9	10.1	10.6	14.6

Correlation between glucosamine synthetase levels in different parts of the stomach and in the duodenum

In the 24 patients in whom readings were made in duplicate in both the body and the antrum, there was a highly significant correlation between these two readings (Fig. 88, $r = 0.75$, $P < 0.001$) despite the different histological features of the two types of mucosa.

In 8 patients in whom the enzyme was assayed in the three sites in the stomach, the correlation between the glucosamine synthetase level in the high lesser curve and the mean of the levels in the body and antrum was far less pronounced (Fig. 89, $r = 0.49$, $0.05 < P < 0.10$).

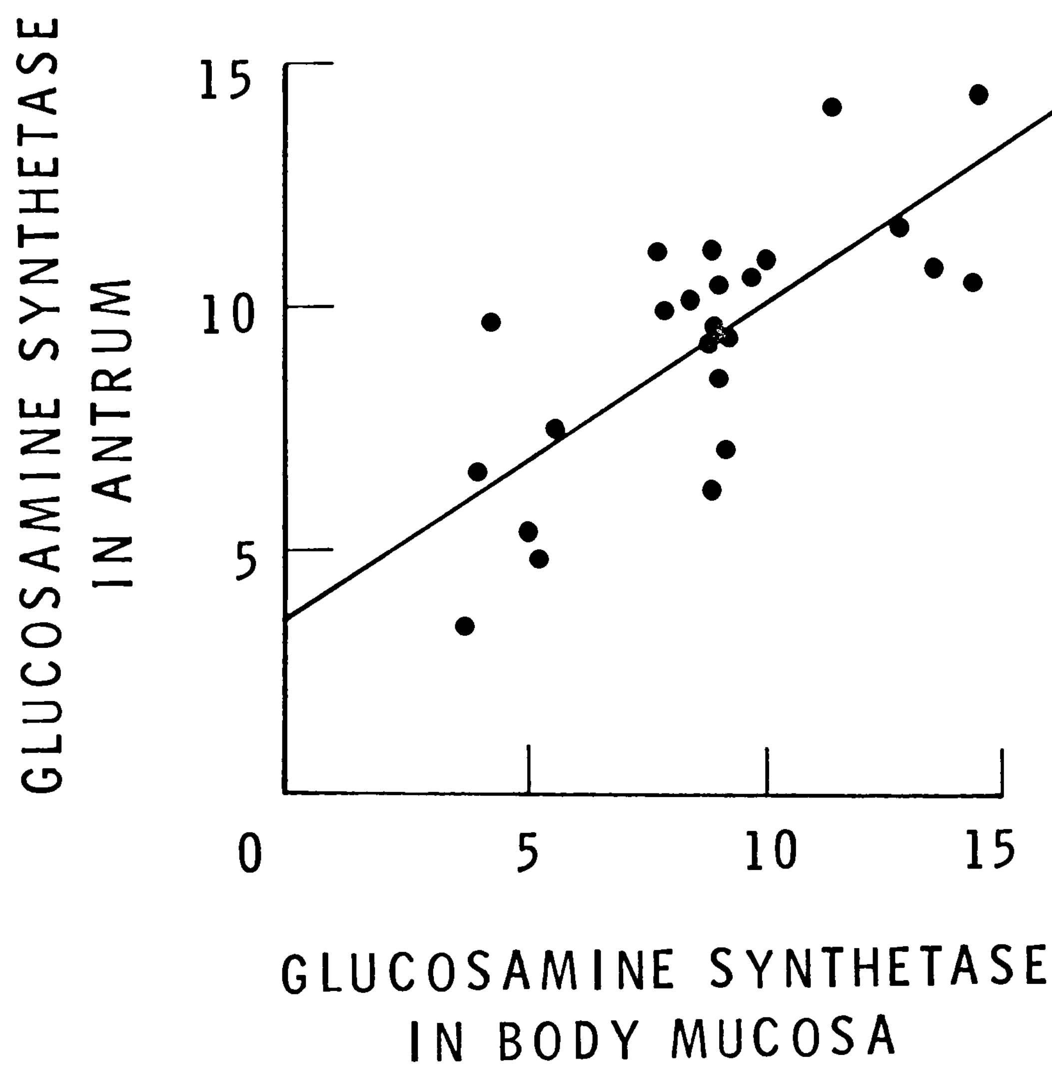
Also, there was a poor correlation between the glucosamine synthetase levels in the antrum and those in the duodenal cap in the 13 patients with no history of duodenal ulcer (Fig. 90, $r = 0.29$, $0.05 < P < 0.10$).

Peptic ulcer: The stomach

Fig. 91 shows the glucosamine synthetase levels in the three sites in the stomach in the control series of patients, in patients with an active duodenal ulcer, in patients with a previous duodenal ulcer, in patients with a gastric ulcer in the same region of the stomach as the biopsy, and in patients with a gastric ulcer in another region of the stomach. In the high lesser curve, the 4 patients with a high lesser curve gastric ulcer had a higher mean glucosamine synthetase level (8.58 units) than the 5 patients with a normal stomach (6.52 units), but this difference was not statistically significant ($P > 0.30$). Only 1 patient had a gastric ulcer on the greater curve, which is an unusual site for benign gastric ulcers, and only 1 patient had an ulcer in the antrum, which is not an uncommon site, and the enzyme levels in these two patients were in the middle of the range for their areas. In the greater curve and in the antrum, patients with gastric ulcers elsewhere in the stomach had glucosamine synthetase levels that were normal. Patients with an active duodenal ulcer also had normal glucosamine synthetase levels that were normal. Patients with an active duodenal ulcer also had normal glucosamine synthetase levels in all parts of the stomach.

Peptic ulcer: The duodenum

In one of the patients with a duodenal ulcer, the biopsy showed pyloric mucosa and this sample was excluded from the series.



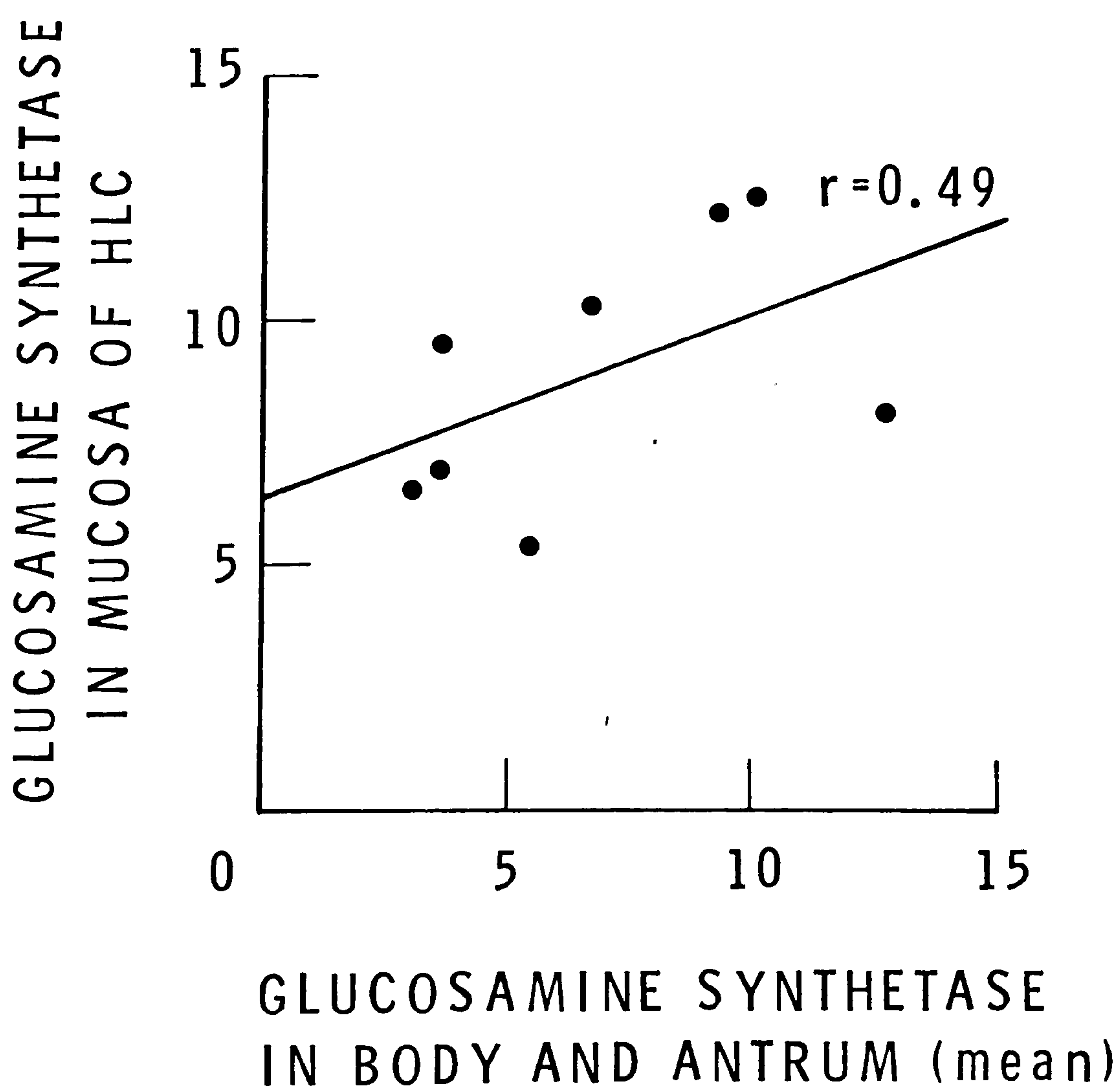


FIG. 88 (above): Correlation between glucosamine synthetase levels in the body and the antrum of the stomach in 24 patients.

FIG. 89 (below): Correlation between glucosamine synthetase levels in the body and antrum of the stomach (mean) and the high lesser curve in 8 patients,

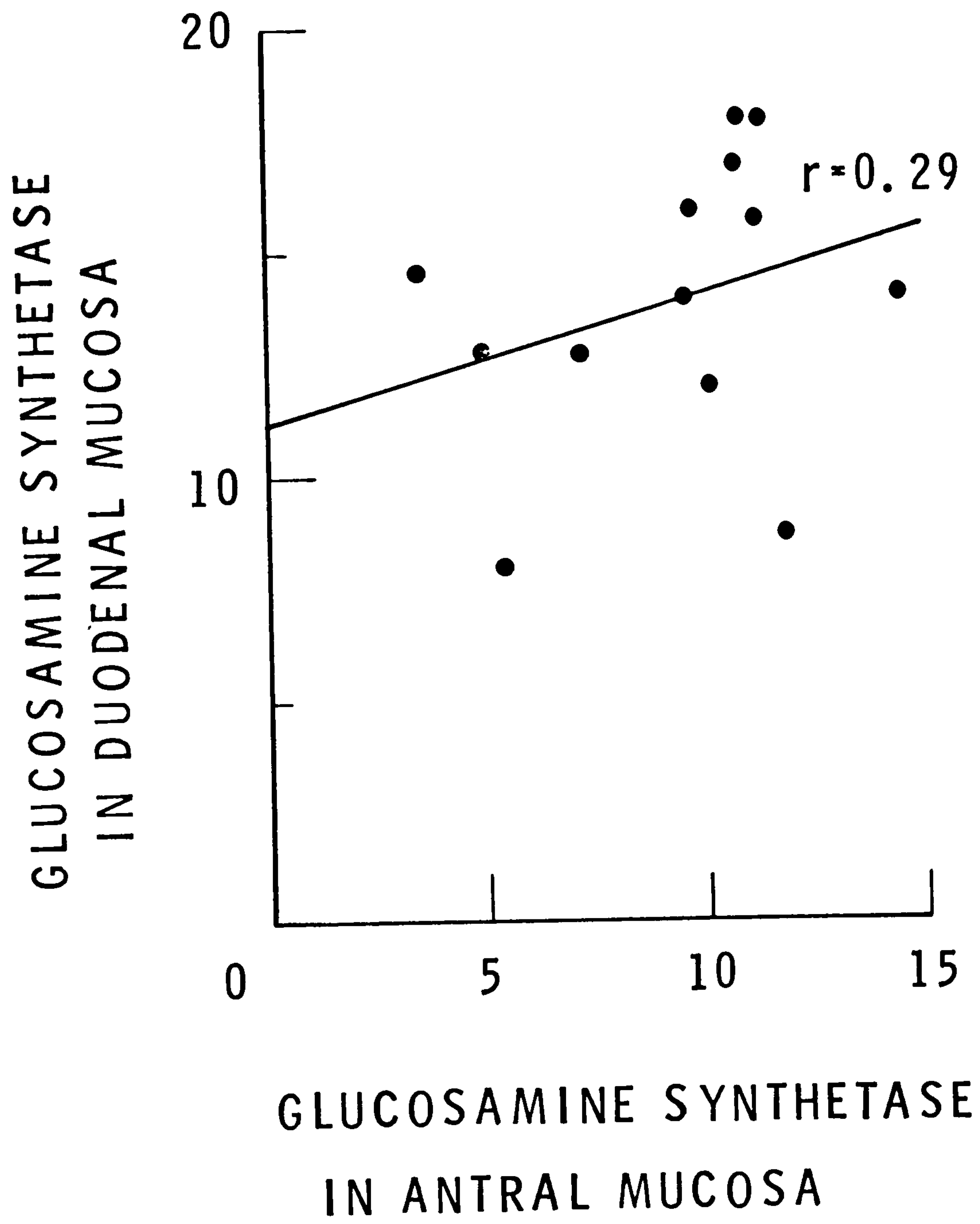


FIG. 90. Correlation between glucosamine synthetase levels in the antrum of the stomach and in the duodenal cap in 13 patients.

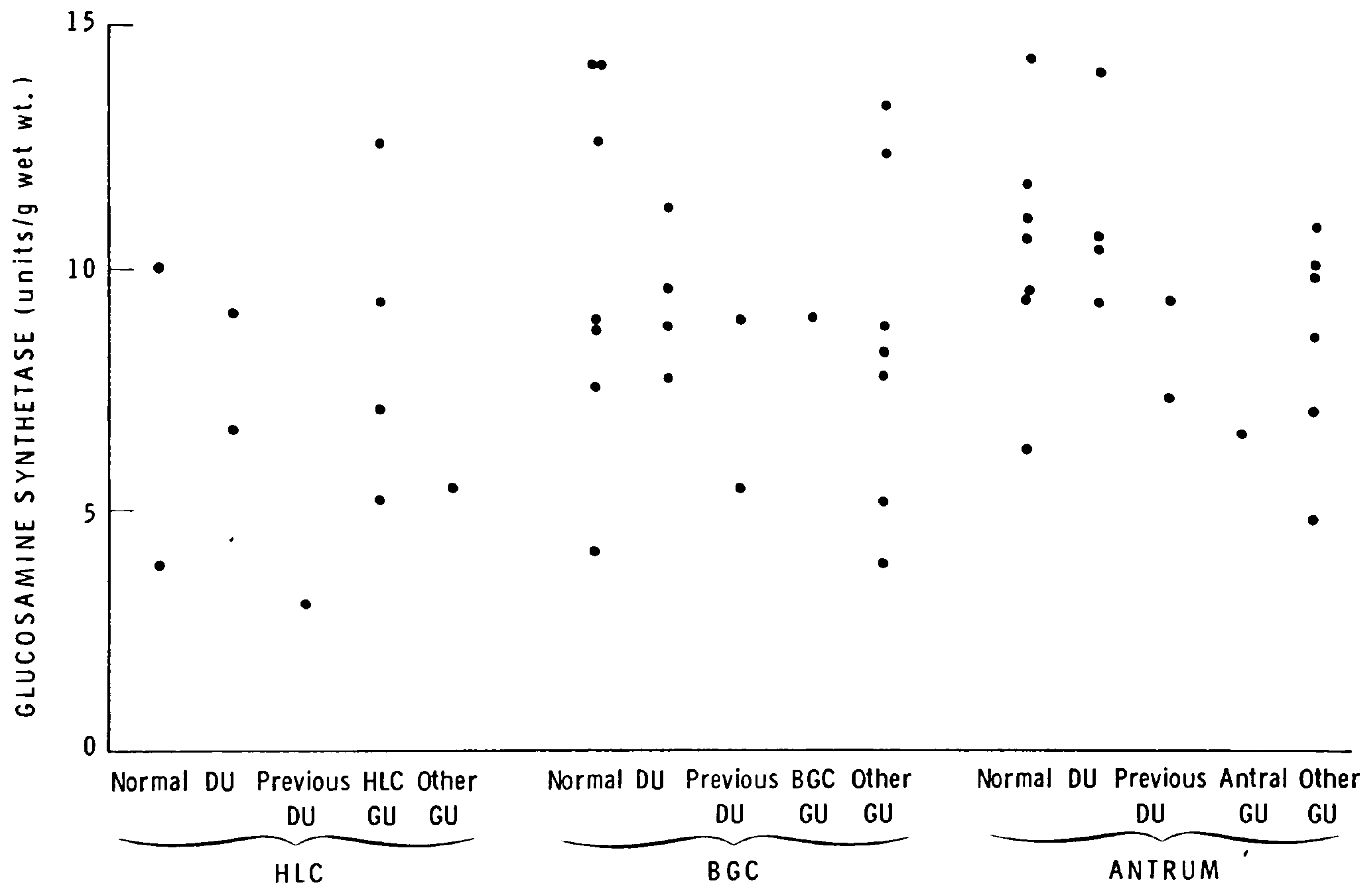


FIG. 91. Glucosamine synthetase levels in three areas of the gastric mucosa—high lesser curve (HLC), body (greater curve) (BGC) and antrum -- in patients with a normal stomach and duodenum, in patients with an active duodenal ulcer (DU), in patients with a previous DU, in patients with a gastric ulcer (GU) in the region of the biopsy, and in patients with a GU elsewhere in the stomach.

Fig. 92 shows the glucosamine synthetase levels in the mucosa of the duodenal cap in 6 patients with a normal stomach and duodenum, in 8 patients with a gastric lesion but a normal duodenum and no history of duodenal ulceration, and in 7 patients with an active duodenal ulcer. The 8 patients with a gastric lesion consisted of 4 with a gastric ulcer, 1 with a suspected but unconfirmed gastric ulcer, and 3 with atrophic gastritis. These 8 patients had duodenal glucosamine synthetase levels which were similar to the 6 patients in the control series.

The 7 patients with an active duodenal ulcer had higher glucosamine synthetase levels in the duodenal cap, with a mean of 18.77 units (S.D. 2.32), than the 14 patients with a normal duodenum, in whom the mean was 14.07 units (S.D. 3.11). This difference was statistically significant ($P < 0.005$).

The 21 biopsies were graded by the author, who was unaware of the diagnosis or the glucosamine synthetase level, according to the scheme of Whitehead et al (1974). They were graded from 0 to 4 with increasing inflammation. In the 14 patients with an endoscopically normal duodenum, 13 had no inflammation or only a mild infiltrate and 1 had moderate inflammation (grade 2). In the 7 patients with a duodenal ulcer, 3 had no inflammation, 3 had a mild infiltrate and 2 had moderate inflammation (grade 2). These 2 biopsies in grade 2 had the highest glucosamine synthetase levels, of 20.9 and 20.8 units, and in the control patient with grade 2 inflammation the level was 17.9 units. Roca et al (1975) have found a mild infiltrate to be common in the duodenal biopsies of normal volunteers.

Patients with a previous duodenal ulcer

The patient with an antral gastric ulcer and a healing duodenal ulcer had a duodenal glucosamine synthetase level of 15.0 units, and the two patients who had had duodenal ulcers in the past had levels of 9.8 and 15.2 units. These were closer to the mean of 14.07 units for patients with a normal duodenum than to the mean of 18.77 units for patients with an active duodenal ulcer.

Atrophic gastritis

Table 29 shows the glucosamine synthetase levels in the body and antrum of the stomach in the 3 patients with atrophic gastritis, and also in the patient with gastric carcinoma, who had histological atrophic gastritis, together with the pathologist's assessment of the biopsy.

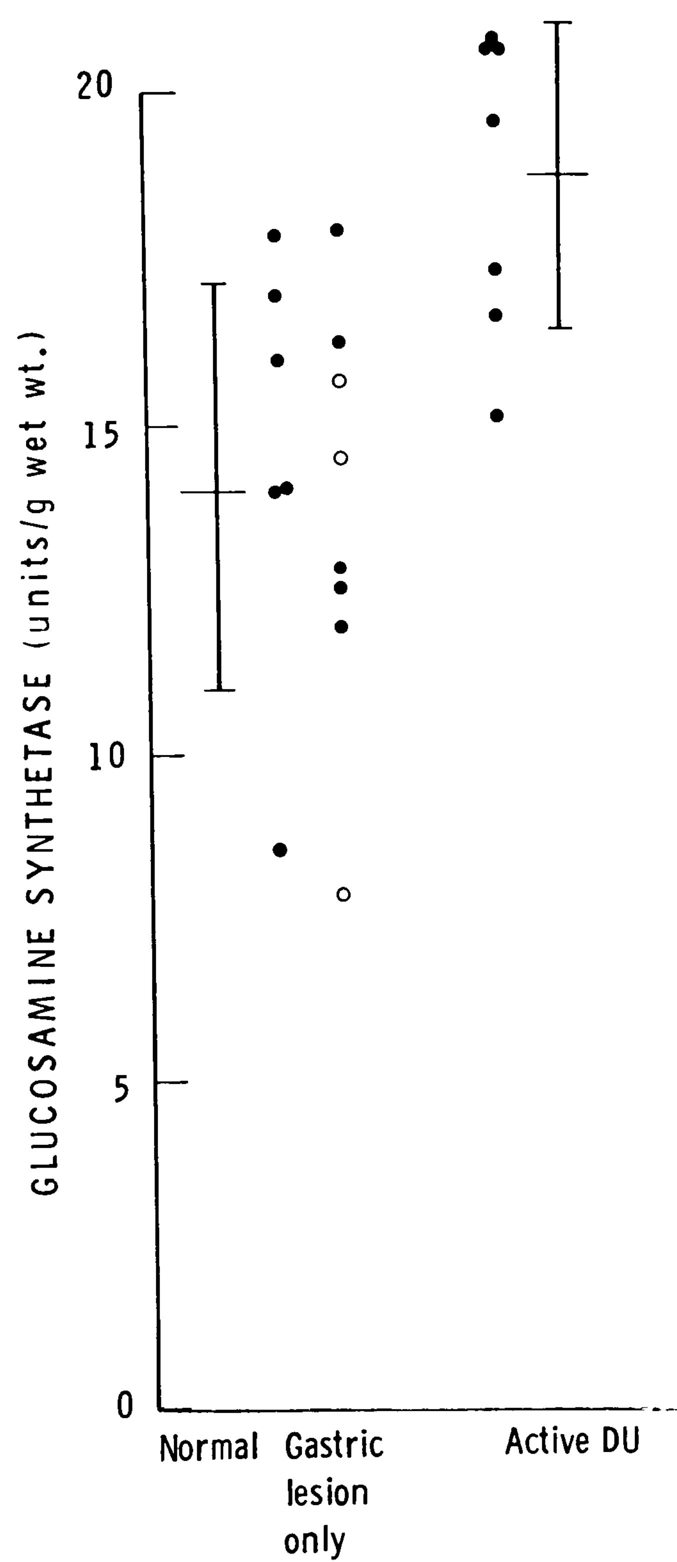


FIG. 92. Glucosamine synthetase levels in the mucosa of the duodenal cap in 6 patients with a normal stomach and duodenum, in 8 patients with a gastric lesion but no duodenal lesion (3 of these with atrophic gastritis shown as open circles), and in 7 patients with an active duodenal ulcer.

TABLE 29. Glucosamine synthetase levels in the gastric mucosa of 3 patients with atrophic gastritis and 1 with carcinoma of the stomach (Pathologist's assessment of the histology)

	Body, greater curve		Antrum	
Patient	Histology	Glucosamine synthetase (units/g wet wt)	Histology	Glucosamine synthetase (units/g wet wt)
M.Ba.	Atrophic + intestinal metaplasia	3.7	Slightly atrophic	3.5
R.Me.	Severely atrophic	8.8	Mildly atrophic	11.2
J.Wil.	Atrophic + intestinal metaplasia	5.0	Atrophic + intestinal metaplasia	5.4
G.Pi. (gastric carcinoma)	Atrophic	9.9	Atrophic + intestinal metaplasia	11.0

The 7 samples which were completely atrophic had a mean glucosamine synthetase level of 6.8 units, compared to 10.4 units for normal body and antral mucosa. Six other gastric biopsies reported as showing mild atrophic gastritis had a mean glucosamine synthetase level of 11.1 units.

Intestinal metaplasia

In addition to the 4 biopsies with atrophic gastritis and intestinal metaplasia (Table 29), one patient with a high lesser curve gastric ulcer had superficial gastritis and intestinal metaplasia in the biopsies from the three regions of the stomach, with glucosamine synthetase levels of 9.3, 13.5 and 11.0 units in the high lesser curve, the body and the antrum respectively.

Polya partial gastrectomy

The one patient who had had a Polya partial gastrectomy 2 years previously for a "benign tumour" (details not available) and who developed a stomal ulcer, had severe superficial gastritis on endoscopic examination, although the histology of the biopsies showed only mild gastritis. The glucosamine synthetase levels in the high lesser curve and in the body of the stomach remnant were 8.2 and 14.7 units respectively.

Gastric erosions induced by ulcerogenic drugs

Biopsies were only taken from the body at the greater curve in this patient with rheumatoid arthritis, taking aspirin, prednisolone and indomethacin, who

had a haematemesis from two large gastric erosions. The glucosamine synthetase level was 6.2 units.

Blood group and secretor status

The glucosamine synthetase levels in the body mucosa of these patients with a normal stomach and the levels in the duodenal cap of the patients with a normal duodenum are shown in Fig. 93 in relation to the ABO blood group and secretor status of those patients in whom they were determined. The numbers are very small but there is no suggestion of any difference between patients of different blood group and secretor status.

DISCUSSION AND CONCLUSIONS

Feasibility of the assay

Although the assay for glucosamine synthetase was miniaturized for use with rectal biopsies and the optimal conditions for the assay were determined in colonic mucosa, the measurement of glucosamine synthetase in gastric and duodenal biopsies obtained through a fibreoptic endoscope has been shown to be feasible.

Normal range

Although it was difficult to find many patients who could be considered as normal, the enzyme levels in the gastric mucosa were similar to those of 2 normal patients in 13 patients with oesophageal or duodenal lesions but with a normal stomach. These are included with the control patients in Table 30.

TABLE 30. Glucosamine synthetase levels in the normal stomach (including patients with abnormalities in the oesophagus and the duodenum) and in the normal duodenum (including patients with abnormalities in the oesophagus and the stomach)

	Stomach			
	High lesser curve	Body, greater curve	Antrum	Duodenal cap
(Number of patients)	(5)	(13)	(13)	(14)
Mean	6.96	9.47	10.46	14.07
S.D.	2.54	3.08	2.30	3.11
S.E.M.	1.14	0.86	0.64	0.83

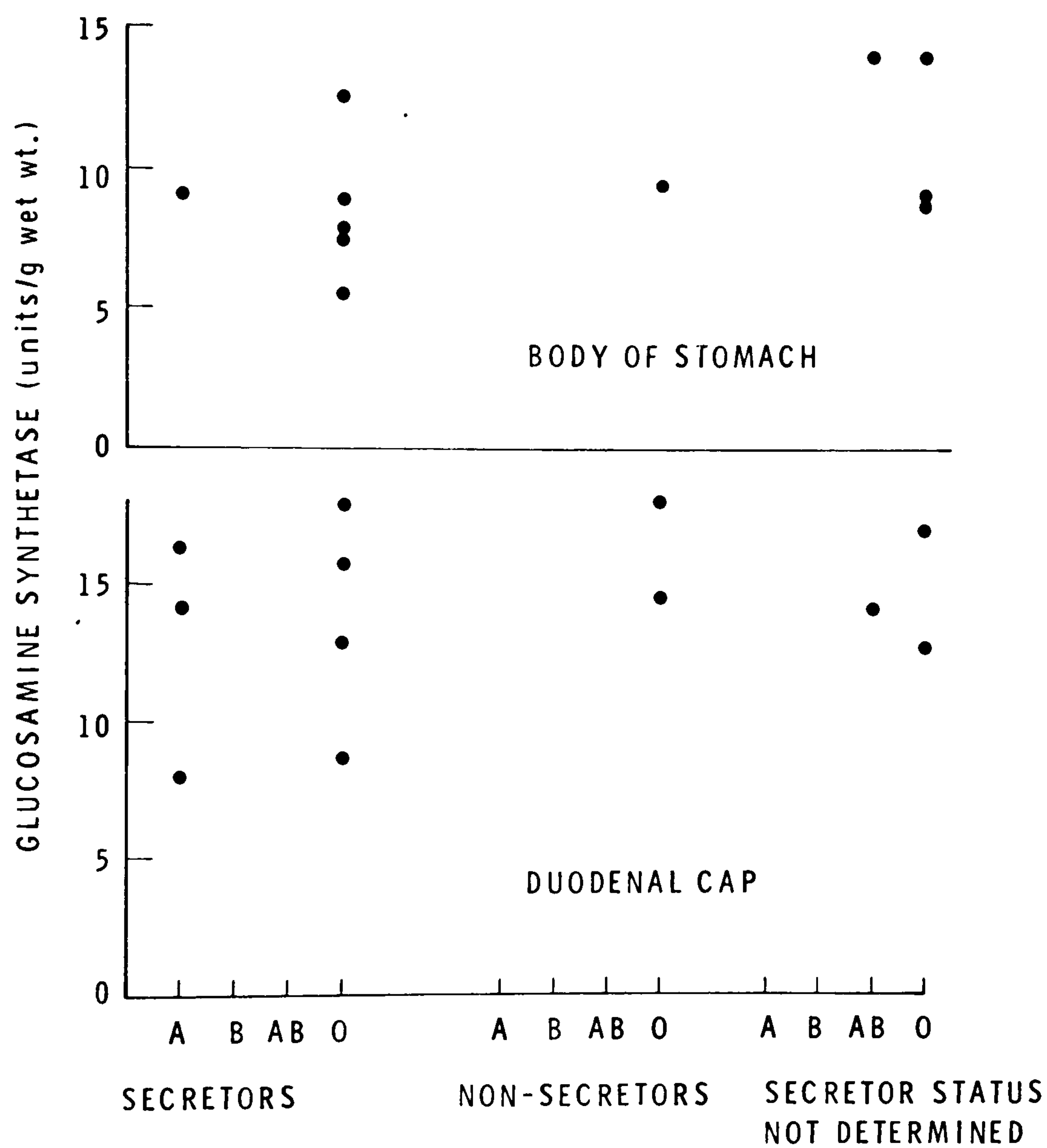


FIG. 93. Glucosamine synthetase levels in the gastric body mucosa in patients with a normal stomach and in the duodenal cap in relation to ABO blood group and secretor status.

Likewise, 8 patients with a gastric lesion, including gastric ulcer and atrophic gastritis, had levels of glucosamine synthetase in the duodenal cap which were similar to the 2 normal patients and to the 5 patients with only oesophageal lesions, and these are included with the control patients in the last column of Table 30.

The glucosamine synthetase level in the normal gastric mucosa is approximately 10.0 units with an S.D. of about 3.0 units, and in the duodenal cap it is approximately 14.0 ± 3.0 units. The gastric levels of the enzyme are roughly one-third less than those in the duodenum. The levels in the duodenal cap are close to the levels in normal colonic mucosa (13.77 ± 3.97 units). The degree of variation between patients is similar in the stomach, the duodenum and the colon. The high lesser curve of the stomach may have lower levels of the enzyme than the body, but in an individual patient there is a good correlation between the glucosamine synthetase levels in all parts of the stomach, despite the varying histological structure. This contrasts with the finding of Johnston et al (1975) that N-acetyl[^{3}H]glucosamine incorporation was similar in the lesser curve and the greater curve but that it was 50% greater in the antrum.

Blood group and secretor status

In a small series, the glucosamine synthetase levels in gastric and duodenal mucosa appeared to bear no relationship to the ABO blood group or the secretor status of the patient. This is not really surprising as the blood group and secretor status are determined by enzymes which direct the addition of the terminal sugars onto the carbohydrate side-chains of the mucus whereas glucosamine synthetase catalyses the first step in the synthesis of many of the sugars, including N-acetylgalactosamine which is the first sugar to be attached to the peptide backbone.

Gastric ulcer

The glucosamine synthetase levels in human patients with gastric ulcer were normal or possibly higher than normal in the region of the ulcer. They were certainly no lower than normal, which contradicts the proposal of Sander et al (1975), based on their results in restrained rats, that impaired glucosamine synthetase levels are an aetiological factor in the development

of gastric ulcers. This illustrates an important feature of modern fibreoptic endoscopy: it enables the oesophageal, gastric and duodenal mucosa of human patients to be sampled under direct vision so that biochemical parameters of the mucosa can be investigated by using methods such as the microassay developed for glucosamine synthetase.

Duodenal ulcer

A significant elevation of glucosamine synthetase levels was found in the duodenal mucosa of patients with duodenal ulcer compared to normals and to patients with only gastric lesions. This may be partly due to the duodenitis which is known to be associated with duodenal ulcer (Whitehead et al, 1974), because inflammatory cells seem to have a small degree of glucosamine synthetase activity (see Chapter 10). On the other hand, the 5 patients with duodenal ulcer and inflammatory grades 0 and 1 still had a mean enzyme level of 17.94 units, which was significantly greater than the mean of the 13 biopsies in grades 0 and 1 of patients with a normal duodenal cap ($P < 0.02$).

The elevated glucosamine synthetase levels in duodenal ulcer correspond to those found in the healing phase of ulcerative colitis and in the morphologically normal mucosa in Crohn's disease (Chapter 10). It would be interesting to see whether patients with a high gastric acid output have high duodenal glucosamine synthetase levels even if they do not have a duodenal ulcer.

Glucosamine synthetase in the duodenal mucosa as a diagnostic test

The measurement of glucosamine synthetase in a duodenal biopsy may prove helpful in clinical practice. A level of 18.0 units or more would be suggestive of an active duodenal ulcer. This could be useful in patients with scarring from previous duodenal ulceration in whom endoscopic assessment of the duodenum may be difficult. One of the patients in this study with a previous duodenal ulcer had a scarred cap but no ulcer was seen and the glucosamine synthetase level was 15.2 units, a normal value. It is possible that the glucosamine synthetase level in the duodenal cap falls as the ulcer heals, but a serial biopsy study would be necessary to see if this were so.

Atrophic gastritis

Gastric glucosamine synthetase levels appear to be slightly lower than normal in atrophic gastritis but the series was too small to draw any firm conclusions.

Although the intestinal mucosa has a higher glucosamine synthetase activity than the gastric mucosa, gastric biopsies with intestinal metaplasia do not appear to have glucosamine synthetase levels corresponding to those in the duodenum or the colon; however, biopsies were obtained from only one patient with intestinal metaplasia without atrophic gastritis. In contrast, Schrager (1974) found that the chemical composition of the mucus in gastric mucosa with intestinal metaplasia resembled that of small intestinal mucus.

Polya partial gastrectomy

In the one patient in whom they were studied, the gastric glucosamine synthetase levels were normal in the high lesser curve but high (14.7 units) close to the stoma, around which the mucosa was seen endoscopically to be inflamed.

Ulcerogenic drugs

The inhibition of glucosamine synthetase by ulcerogenic drugs *in vitro* is discussed in Chapter 15. The opportunity was taken to study a patient who had had a haematemesis from large gastric erosions caused by a combination of three ulcerogenic drugs, namely aspirin, prednisolone and indomethacin. The glucosamine synthetase level in the body mucosa was 6.2 units, or 1.1 standard deviations below the mean, but this is within the normal range. There was, therefore, no appreciable depletion of glucosamine synthetase in this patient who was being treated with three ulcerogenic drugs concurrently.

CHAPTER 14

JEJUNAL BIOPSIES AND COELIAC DISEASE

The jejunal mucosa is biopsied frequently in clinical practice, using the Crosby capsule, a spring-loaded device which the patient swallows and to which is attached a narrow polythene tube which can be made radio-opaque to help position the capsule accurately in the first part of the jejunum, the standard point from which the biopsy is taken. Portions of jejunal biopsies so obtained have been used for the biochemical assay of a number of enzymes. Assays of the disaccharidases (lactase, maltase and sucrase) are used in clinical practice to diagnose conditions of the small intestine, such as hypolactasia (Peña, 1971).

N-Acetyl-β-glucosaminidase is a lysosomal enzyme which has been measured in jejunal biopsies (Mitchell et al, 1974), but no measurements have been made of enzymes involved in glycoprotein biosynthesis in human jejunal biopsies.

This chapter describes measurements of glucosamine synthetase in jejunal biopsies taken from patients with a normal mucosa and from patients with coeliac disease. Coeliac disease is a disease of the small intestinal mucosa caused by an idiosyncratic sensitivity to gluten, which causes atrophy of the villi and, in more severe cases, hypertrophy of the crypts (Dissanayake, 1975). The levels of the disaccharidases in the jejunal biopsy in coeliac disease correlate with the height of the villi (Dissanayake et al, 1974). The crypts of the jejunal mucosa contain more goblet cells than the villi, so that glucosamine synthetase levels might correlate with the crypt size rather than with the villous height.

PATIENTS STUDIED AND METHODS

Glucosamine synthetase was assayed by the 4 mg method (sometimes without duplicates — the "2 mg method") in portions of 36 jejunal biopsies taken from 35 patients (2 biopsies were taken on separate occasions from one of the patients). The patients either had coeliac disease, suspected coeliac disease, or symptoms such as abdominal pain, diarrhoea or iron-deficiency anaemia which required coeliac disease to be excluded. Patients with other serious diseases or any other organic disorder of the gastrointestinal tract, past or present, were excluded, as were patients taking hormonal preparations.

The series included 2 biopsies from one patient with coeliac disease, the first at the time of diagnosis of the disease and the second after 9 months on a gluten-free diet.

In 14 of the biopsies, insufficient mucosa was obtained to perform the enzyme assay in duplicate. These results thus had an error about 8% greater than when the assay was performed in duplicate (Chapter 8).

The collection of the biopsies

The biopsies were obtained from the first part of the jejunum using the Crosby capsule. The biopsy was examined under a dissecting microscope and its appearance noted, and then it was orientated flat on filter paper and divided into one part for histology and one part for the glucosamine synthetase assay. Sometimes a third portion was taken for disaccharidase assays when this was needed for clinical purposes.

Recording of clinical details

A pro forma was used similar to that for rectal biopsies (Fig. 44); the details recorded included the dissecting microscope appearances of the biopsy.

Histology

Portions of each biopsy were sent for routine diagnostic histology. The biopsies which were not normal were classified as showing minor villous changes, moderate partial villous atrophy, severe partial villous atrophy, or total villous atrophy, according to the pathologist's report, usually by Dr. R. Whitehead or Dr. J. M. Skinner, although when the description in the report

did not classify the biopsy clearly into one of these categories it was examined and classified by the author.

RESULTS

There were 14 normal biopsies and 22 biopsies with abnormalities consistent with a clinical diagnosis of coeliac disease. One of the 14 patients with a normal biopsy proved to have carcinoma of the caecum but no organic disease was found in any of the other 13 patients. The results of the glucosamine synthetase estimations are shown in Fig. 94 in relation to the histological appearances of the villi.

The patient in whom the measurements were made in 2 biopsies had glucosamine synthetase levels of 16.2 units in a biopsy with total villous atrophy before treatment and 14.1 units in a biopsy with severe partial villous atrophy after 9 months on a gluten-free diet.

The mean of the glucosamine synthetase levels in the 22 patients with coeliac disease and villous abnormalities was 12.05 units, compared with 17.37 units for the 14 normal biopsies. This difference was statistically significant ($P < 0.01$) although the range of readings was such that little information is conveyed by an individual reading.

Thirteen of the 16 biopsies with total villous atrophy or moderate or severe partial villous atrophy were examined by the author (the other 3 slides were mislaid) and scored 0-3 according to the degree of crypt hypertrophy. At the time of scoring, the author was unaware of the glucosamine synthetase activity of the biopsy. Fig. 95 shows a positive correlation of glucosamine synthetase levels with crypt hypertrophy in this group of biopsies with a similar degree of villous atrophy.

DISCUSSION AND CONCLUSIONS

Normal jejunal mucosa

Glucosamine synthetase can be measured in jejunal biopsies obtained using the Crosby capsule. The normal range, from 14 biopsies, was 17.37 units mean (±1.76, S.E.M.) with a standard deviation of 6.60 units. This was a higher mean than that found in colonic mucosa (13.77) and the variation

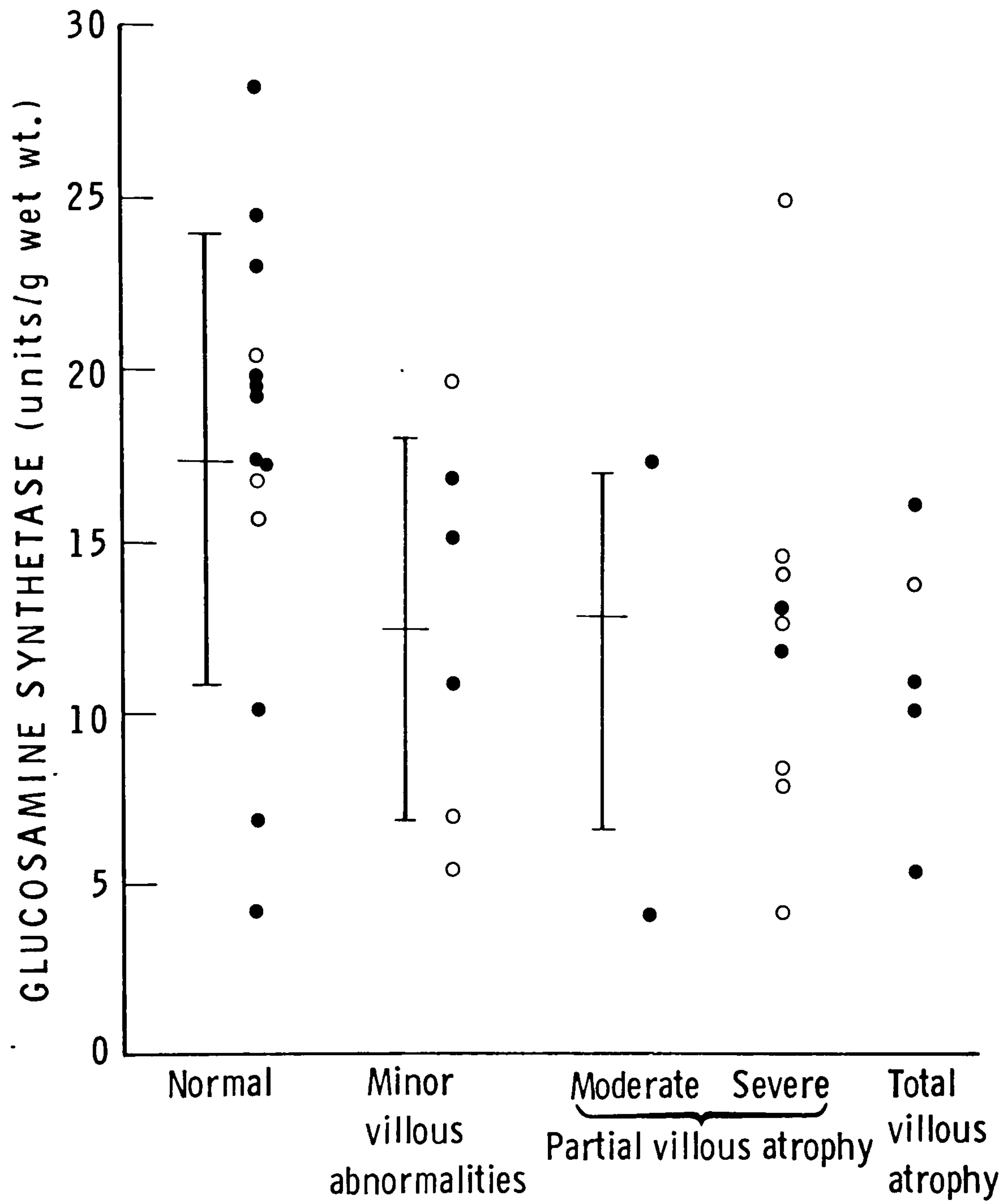


FIG. 94. Glucosamine synthetase levels in 14 normal jejunal biopsies and in 22 biopsies from patients with coeliac disease. [Closed circles indicate duplicated readings. Open circles indicate readings not duplicated.] Mean ± S.D. shown for normals, for mild group, and for moderate and severe partial villous atrophy and total villous atrophy taken together.

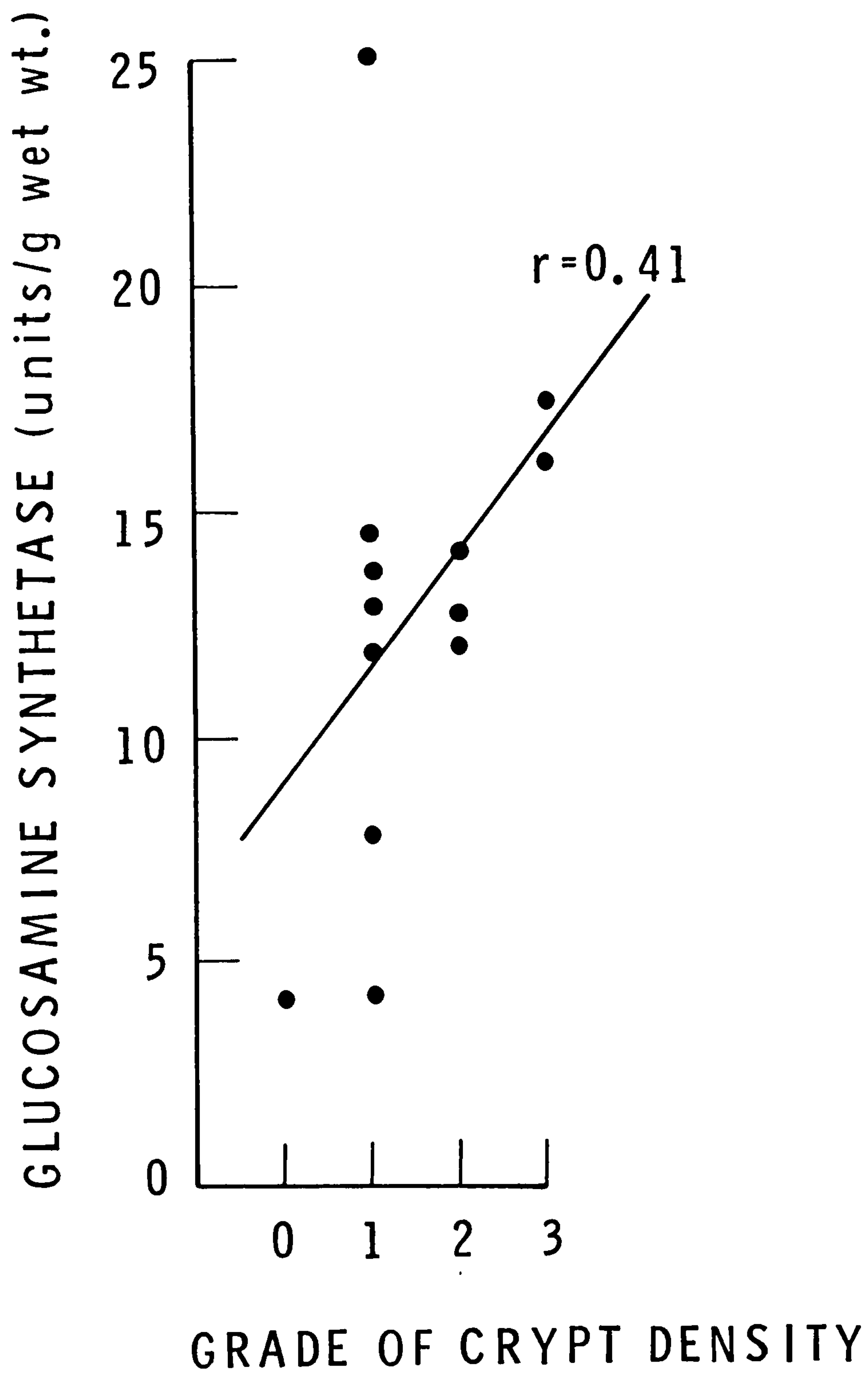


FIG. 95. Glucosamine synthetase levels in 13 jejunal biopsies with total villous atrophy or moderate or severe partial villous atrophy in relation to crypt hypertrophy as scored by the author.

between patients was considerably greater than that of colonic mucosa and that of gastric mucosa and duodenal mucosa. This may be because a jejunal biopsy comprises a variable amount of crypt tissue and glucosamine synthetase may be more abundant in the crypts and at the bases of the villi than at the tips of the villi; the cells containing glucosamine synthetase may be more evenly distributed in the gastric, duodenal and colonic mucosa.

Coeliac disease

In the abnormal jejunal mucosa of coeliac disease, glucosamine synthetase levels are on the whole lower than normal but there is such an overlap that this assay is unhelpful in the diagnosis of coeliac disease and in the quantitation of villous abnormalities.

The negative correlation with villous height was balanced by a positive correlation with crypt density in patients with atrophic or severely stunted villi. Quantitative histology would be needed to ascertain whether glucosamine synthetase correlates with the total epithelial cell density in the jejunum, as it appears to do. However, the wide scatter of results casts doubt on the value of such an exercise. Glucosamine synthetase is not as specific as the disaccharidases as an index of villous height nor as specific as an index of increased cell turnover as the enzymes aspartate carbamoyl transferase and dihydroorotate dehydrogenase, the levels of which are increased in jejunal biopsies in coeliac disease (Clark and Senior, 1969).

CHAPTER 15

THE ULCEROGENIC DRUGS

This chapter describes experiments in which the effects of several ulcerogenic drugs on glucosamine synthetase were studied in vitro.

With the introduction of the anti-inflammatory drugs, the salicylates, the corticosteroids, the phenbutazones and indomethacin, the mucosal protection theory for peptic ulceration was invoked to explain the ulcerogenic effects of these drugs on the assumption that they damaged the gastric and duodenal mucosal barrier. A number of biochemical and histochemical parameters of mucus production have been studied in experimental animals in relation to the ulcerogenic effects of these drugs; these have been discussed in Chapter 1.

Schönhöfer and Perrey (1967) fed salicylates and phenylbutazone to rats and found that the glucosamine synthetase levels in the gastric mucosa were lowered by 84% with salicylate and by 63% with phenylbutazone; there was no reduction in the glucosamine synthetase activity of the liver. Perrey (1968) showed inhibition of glucosamine synthetase in vitro in homogenates of rat mucosa by sodium salicylate (Fig. 96) and by phenylbutazone.

Control systems

The usual practice in studying the ulcerogenic drugs has been to compare the effects of the drug under study with control systems in which the drug is omitted. In general, no attempt has been made to study the effects of other pharmacological compounds which do not appear to cause peptic ulceration. Control experiments of this type were not done in the various studies of the inhibition of mucus production which were described in Chapter 1 (Menguy and Masters, 1963, 1965; Menguy and Desbaillets, 1967a,b; Desbaillets and Menguy, 1967; Kent and Allen, 1968; Lukie and Forstner, 1972b; Robert and Nezamis, 1964; Johansson and Lindquist, 1971; Parke et al, 1975),

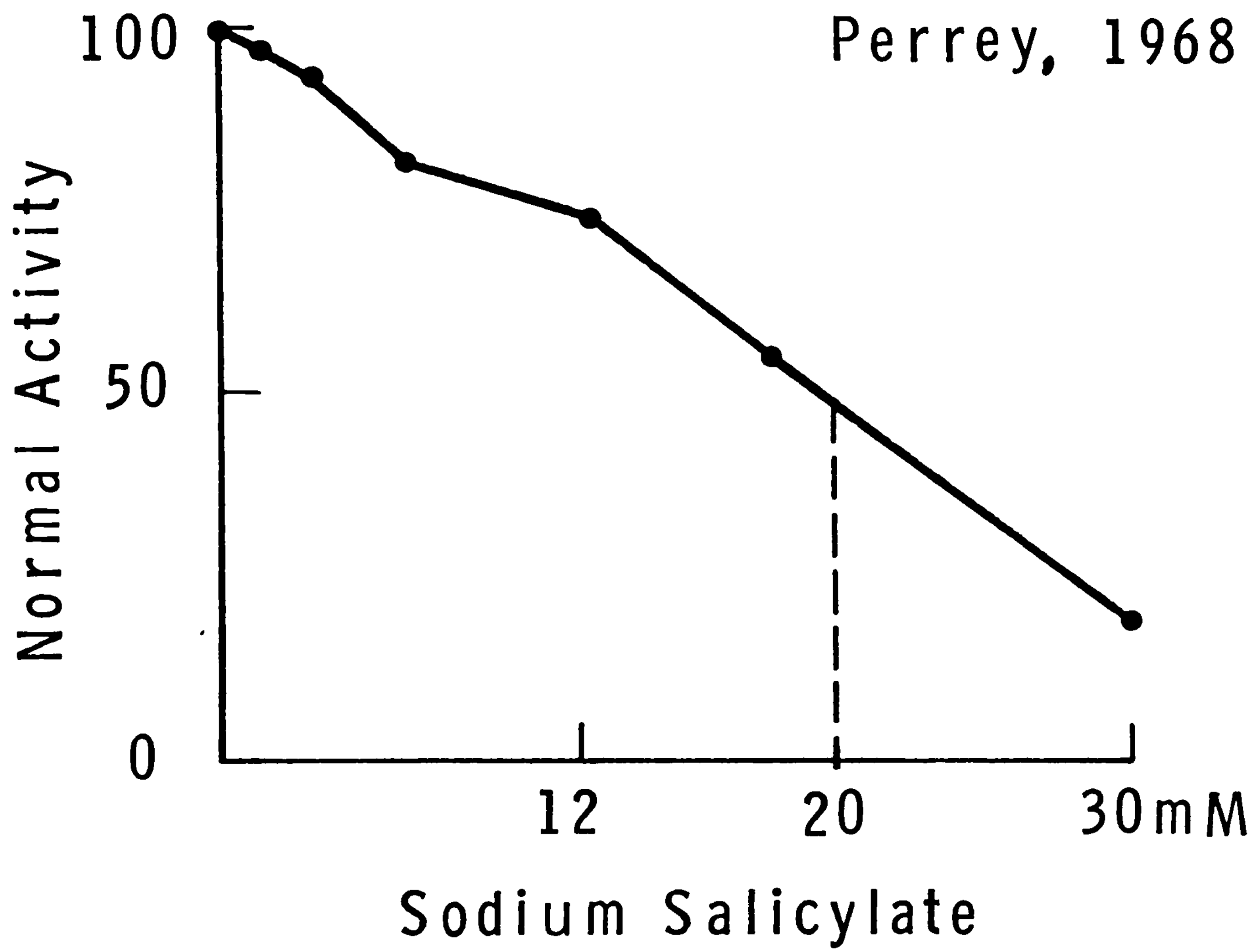


FIG. 96. Inhibition of glucosamine synthetase in homogenates of rat stomach by sodium salicylate *in vitro*. (From Perrey, 1968.)

nor by Perrey (1968) in his studies on the inhibition of glucosamine synthetase in vitro.

Perrey's experiments have been repeated, using homogenates of human gastric and colonic mucosa, with the ulcerogenic drugs acetylsalicylic acid (aspirin), sodium salicylate and hydrocortisone hemisuccinate, and with the non-ulcerogenic analgesic paracetamol and the antibiotic gentamicin.

MATERIALS

Acetylsalicylic acid (B.P.) was obtained from Evans Medical Ltd, Liverpool.

Sodium salicylate (B.P.) was obtained from Evans Medical Ltd.

Hydrocortisone hemisuccinate (injection, B.P., containing sodium phosphates as buffering agents) was obtained from Organon Laboratories, Morden, Surrey.

Paracetamol (B.P.) was obtained from BDH Pharmaceuticals Ltd.

Gentamicin sulphate (Genticin Pure Powder) was obtained from Nicholas Laboratories Ltd.

METHODS

Each drug, apart from gentamicin, was studied in two fresh homogenates of gastric mucosa and in two fresh homogenates of colonic mucosa. Gentamicin was studied in one homogenate of colonic mucosa. The mucosa was obtained from the tumour-free cut ends of surgically resected specimens for carcinoma of the stomach and for carcinoma of the colon. Sufficient tissue was obtained to study all the intended dilutions of a single drug and usually more than one drug was studied with one homogenate.

The mucosa was homogenized in homogenizing buffer as in the 10 mg method for the assay of glucosamine synthetase, except that the concentration of tissue in the homogenate was 50 mg/ml instead of 10 mg/ml.

The substrate-buffer mixture was made up as in the usual glucosamine synthetase assay (Chapter 8) except that the concentrations of the substrates and gentamicin were 5 times those in the usual assay. In other words, the substrate-buffer mixture contained fructose-6-phosphate 200 mM, glutamine 80 mM, and gentamicin 1000 μg/ml.

Solutions of the drugs were all made in the homogenizing buffer (50 mM sodium-phosphate buffer at pH 7.0 containing KCl 100 mM and EDTA 1 mM). Solutions were made containing sodium salicylate, hydrocortisone hemisuccinate, paracetamol and gentamicin at 12.5 mg/ml and acetylsalicylic acid at 6.25 mg/ml (acetylsalicylic acid could not be dissolved at 12.5 mg/ml). By dilution with the homogenizing buffer, solutions of all five drugs were made at 6.25 mg/ml, 2.5 mg/ml and 1.25 mg/ml, and solutions of acetylsalicylic acid, sodium salicylate and hydrocortisone hemisuccinate were made at 0.125 mg/ml and 0.0125 mg/ml.

With each homogenate, two 1.0 ml volumes were made up containing each dilution of the drug under study, as follows:-

0.1 ml homogenate,
0.1 ml substrate-buffer mixture,
0.8 ml solution of the drug.

Two 1.0 ml volumes were also made up containing 0.8 ml homogenizing buffer instead of the drug, to give the uninhibited glucosamine synthetase activity of the homogenate. Since the homogenates and the substrate-buffer mixture used in these drug experiments contained five times the usual concentrations of tissue and substrates, the 1.0 ml incubates contained the same amounts of these as in the usual 10 mg assay which used 0.5 ml homogenate and 0.5 ml substrate-buffer mixture. The 1.0 ml volumes had to be made up in this way for the drug experiments because some of the drugs, namely acetylsalicylic acid and paracetamol, were being studied at the limits of their solubility.

The 1.0 ml volumes were incubated for 3 hours at 37° and the reaction was then terminated with 0.05 ml concentrated perchloric acid. The glucosamine synthesized was measured according to the method used in the 10 mg assay (Chapter 8). The duplicated incubates with each dilution of the drugs were read in separate batches with dilute DAB and standards; one sample of each dilution was read in one batch and the second sample of each dilution was read in a second batch.

Control experiments

To determine whether the drug interfered with the colour reaction in the glucosamine assay, two sets of duplicated control samples of 1.0 ml were made up with the highest concentrations of each drug with two out of the

four homogenates studied. The first set of controls consisted of the same components as the test solutions, but perchloric acid was added immediately they were made up, without incubation at 37°. The second set of controls contained 0.1 ml homogenizing buffer instead of 0.1 ml homogenate; these were incubated for 3 hours as for the test solutions. These controls were assayed for glucosamine with the test solutions and they showed whether the drug at the highest concentrations used in the studies gave rise to any colour which was recorded by the Morgan-Elson reaction.

There is the possibility that a drug may interfere with the colour reaction by inhibiting the colour produced by glucosamine. This was investigated by making control solutions of 0.9 ml containing 0.1 ml substrate-buffer mixture and 0.8 ml of the highest concentration of the drug and then adding 0.05 ml perchloric acid. These solutions were stored in the deep freeze with the incubated test solutions and the other control solutions and on the day of assay of the test solutions these 0.9 ml (now 0.95 ml) volumes were thawed and 0.1 ml 2 mM glucosamine was added, so that they had a glucosamine concentration of 0.20 mM (excluding the perchloric acid) and they corresponded to the 0.20 mM glucosamine standards for the glucosamine assay. These drug-glucosamine controls were assayed with the test solutions.

Internal standards

The control experiments with acetylsalicylic acid, sodium salicylate and hydrocortisone hemisuccinate showed that these drugs at the highest concentrations used in the studies gave no colour in the Morgan-Elson reaction themselves. The drug-glucosamine controls gave concentrations of glucosamine between 0.15 and 0.25 mM for expected concentrations of 0.20 mM, which were considered satisfactory.

On the other hand, paracetamol at 10 mg/ml gave a deep yellow colour which interfered with the Morgan-Elson reaction to the equivalent of 3 mM glucosamine. Paracetamol at this concentration also inhibited the development of the colour in the Morgan-Elson reaction, by about 70% mM glucosamine (Fig. 97). Gentamicin gave a slight colour (O.D. less than 0.010) in the

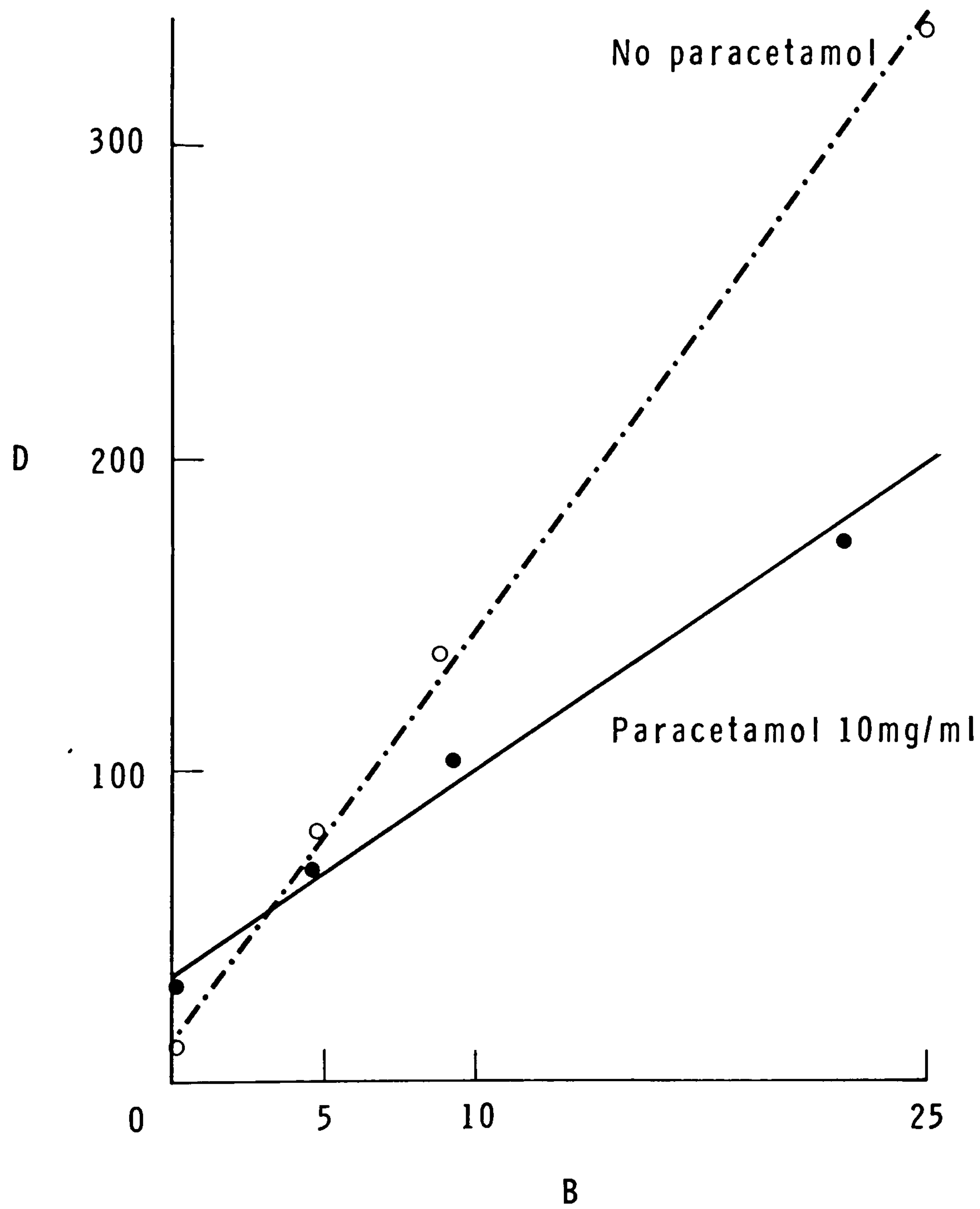


FIG. 97. Optical density X 1000 [=D] at 545 nm in relation to the colour produced by glucosamine solutions in the Morgan-Elson reaction, in the presence of paracetamol 10 mg/ml (closed circles), compared to a similar standard curve with no paracetamol (open circles). [B = 100 X glucosamine concentration in mM.]

Morgan-Elson reaction (it is an oligosaccharide antibiotic) and it too interfered with the colour development by glucosamine.

For these reasons, internal glucosamine standards were used in the assay of glucosamine synthetase in the presence of paracetamol and of gentamicin. The standards were 1.0 ml volumes containing 0.1 ml homogenate, 0.1 ml glucosamine solution in homogenizing buffer, and 0.8 ml of the drug solution. Four standards were used for each concentration of the drug, containing 0.1 ml of 0, 0.50, 1.00 and 2.50 mM glucosamine hydrochloride and giving standards of 0, 0,05, 0,10 and 0.25 mM glucosamine. At each concentration of the drug, it was assumed that the relationship of the optical density of the colour to the concentration of glucosamine was linear. The two duplicates of the test solutions for each of these drugs were assayed by the Morgan-Elson reaction in the same batch of dilute DAB as each other, together with the four standards. For each concentration of the drug, a standard curve was drawn from the results of the four standards, and the glucosamine concentration of the test solutions was read off this standard curve. The glucosamine synthetase activity of the tissue in the presence of the drug was calculated as for the 10 mg enzyme assay. Fig. 97 shows a standard curve for paracetamol 10 mg/ml.

RESULTS

All the drugs, including paracetamol and gentamicin, inhibited glucosamine synthetase in vitro and the degree of inhibition was similar in the gastric and the colonic homogenates (Fig. 98).

The 0.01 mg/ml and 0.1 mg/ml concentrations of acetylsalicylic acid, sodium salicylate and hydrocortisone hemisuccinate did not inhibit the enzyme (Fig. 98). The inhibition by the higher concentrations of these drugs and by paracetamol is shown in Figs. 99-102, giving the concentrations of each drug in mM. For each drug, the uninhibited reading with each homogenate is taken as 100% and the percentage of uninhibited activity is given as a mean for the 2 gastric homogenates and 2 colonic homogenates taken together and the standard error of each mean is also shown.

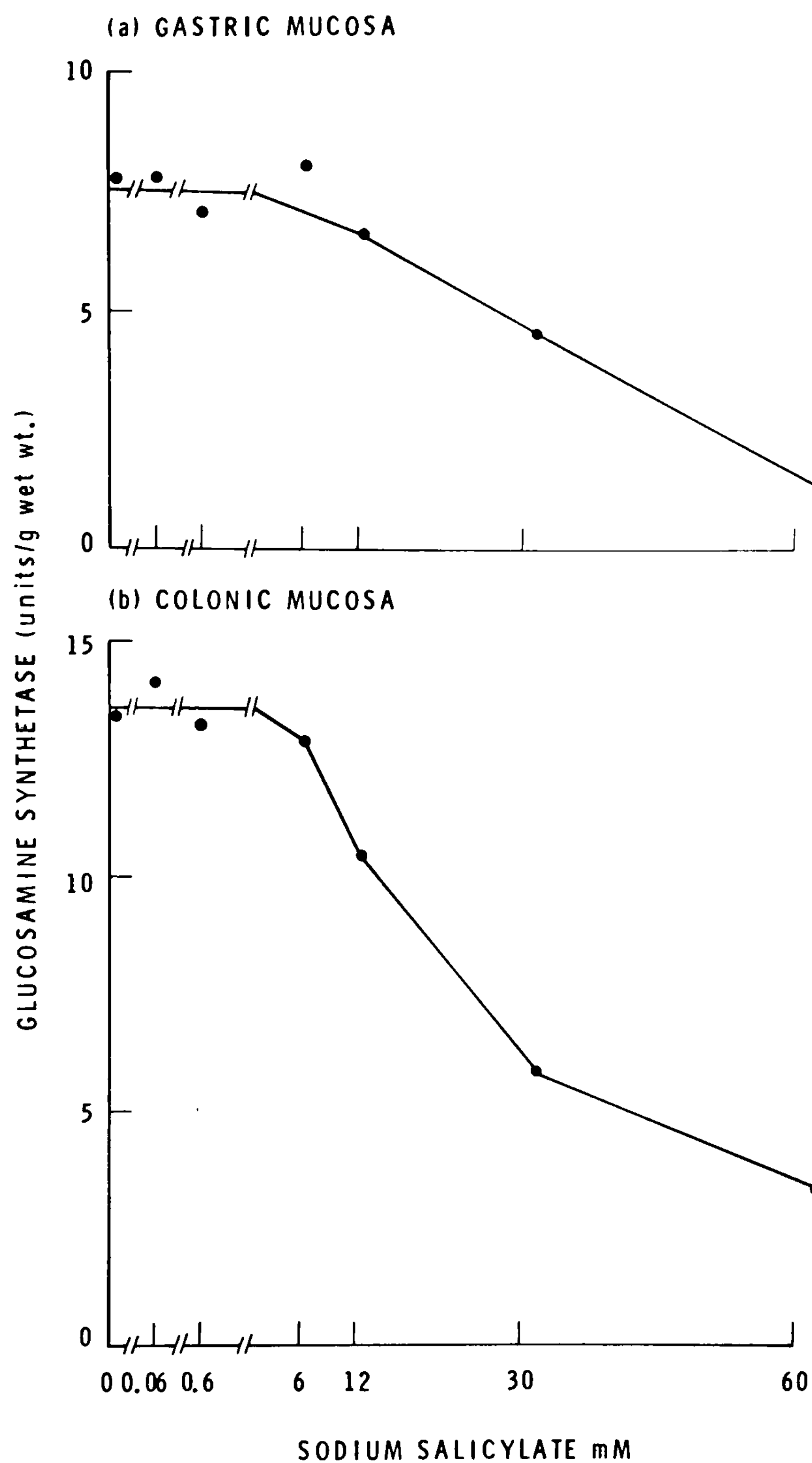
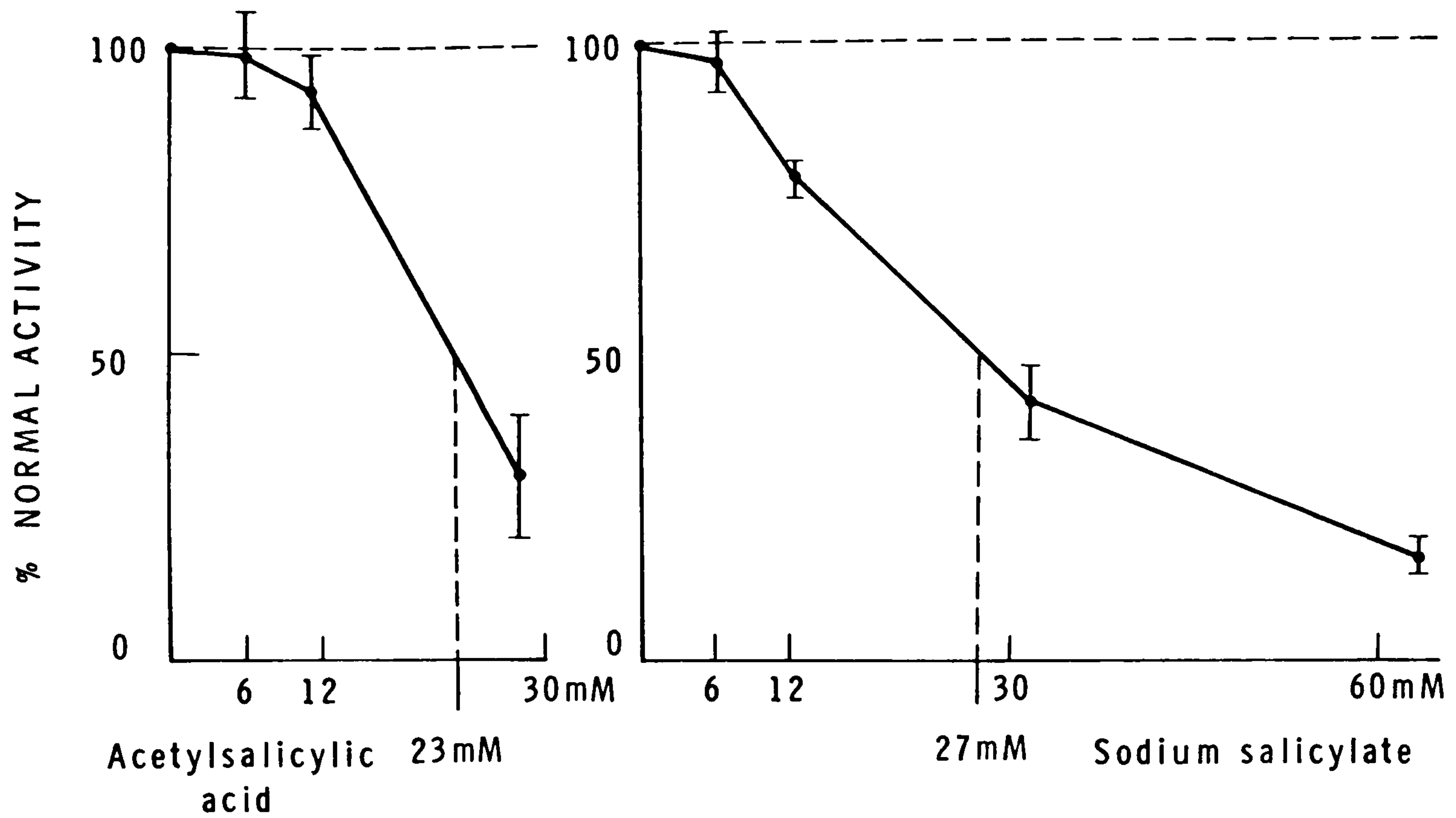
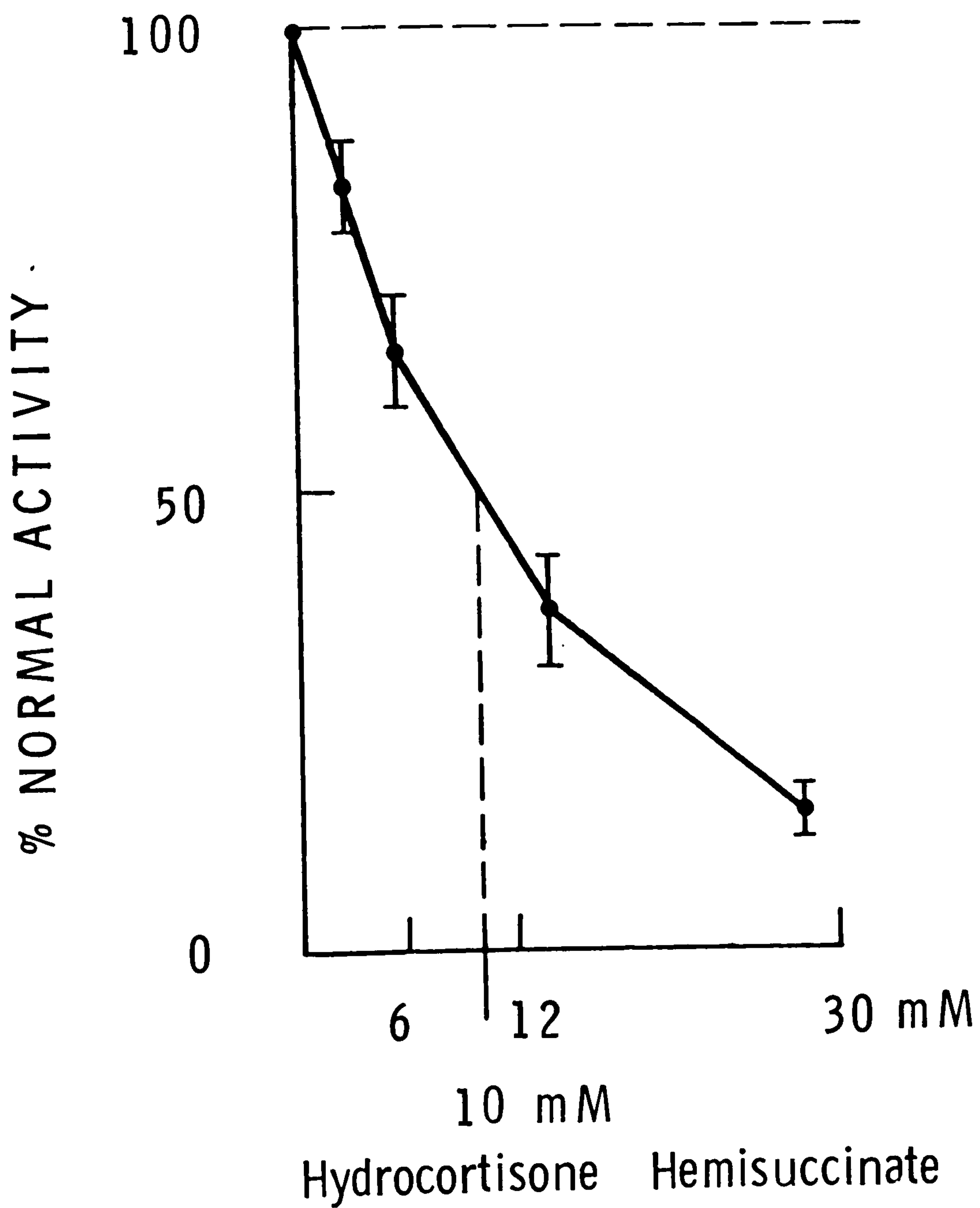


FIG. 98. Inhibition of glucosamine synthetase by sodium salicylate (a) in an homogenate of gastric mucosa and (b) in an homogenate of colonic mucosa.



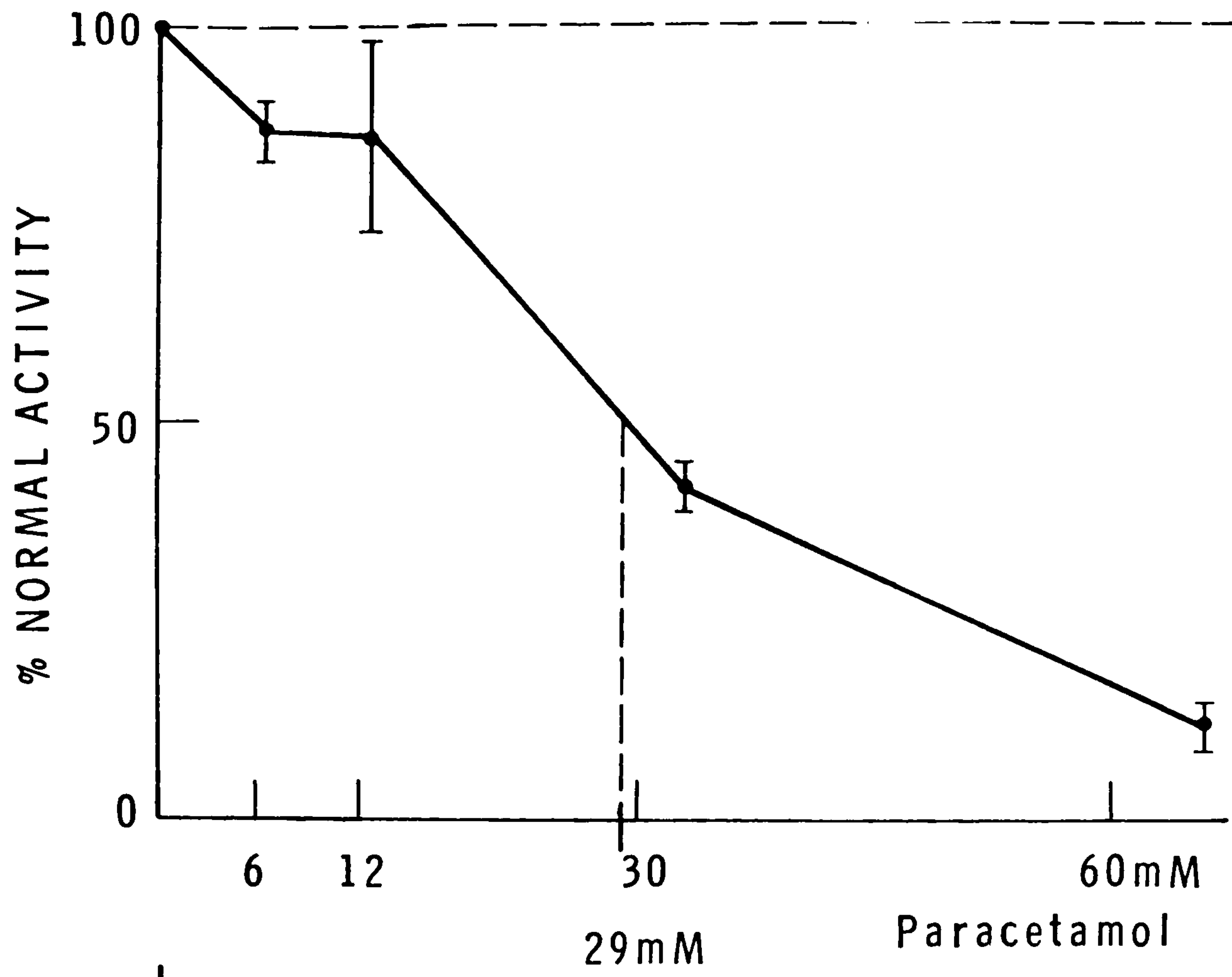


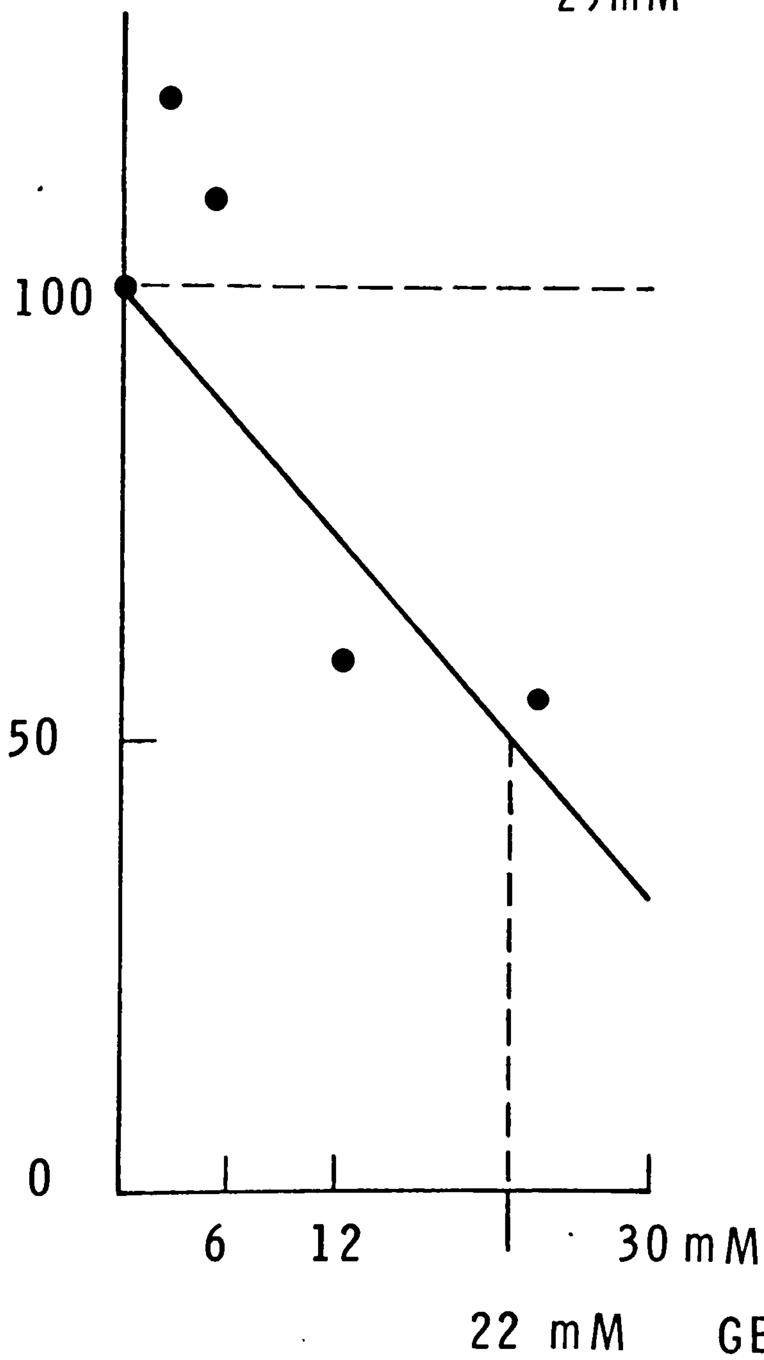
FIGS. 99, 100, 101. Inhibition of glucosamine synthetase in 2 gastric and 2 colonic mucosal homogenates by three ulcerogenic drugs. (Bars show mean ± S.E.M.)

FIG. 99 (above, left): Inhibition by acetylsalicylic acid.

FIG. 100 (above, right): Inhibition by sodium salicylate.

FIG. 101 (below): Inhibition by hydrocortisone hemisuccinate.





FIGS. 102, 103. Inhibition of glucosamine synthetase in 2 gastric and 2 colonic mucosal homogenates by two non-ulcerogenic drugs. (Bars show mean ± S.E.M.)

FIG. 102 (above): Inhibition by paracetamol.

FIG. 103 (below): Inhibition by gentamicin.

Fig. 103 shows the inhibitory effect of gentamicin in the one colonic homogenate in which this drug was studied.

The pH of the drug solutions

The pH of the highest concentration of each drug was measured to see if the inhibition of glucosamine synthetase could be an effect of a change in the incubation conditions to a suboptimal pH (Fig. 26). The drugs were dissolved in a 50 mM sodium-phosphate buffer at pH 7.0. Of the five drugs, only acetylsalicylic acid at its highest concentration (6.25 mg/ml or 35 mM) affected the pH, bringing it down to 5.0 (Table 31).

TABLE 31. The pH of the solutions of the drugs in homogenizing buffer (50 mM sodium-phosphate buffer at pH 7.0, containing 100 mM KCl and 1 mM EDTA) at the highest concentration of each drug used in the studies of the in vitro inhibition of glucosamine synthetase

	Concentration		
Drug	mg/ml	mM	pH
Acetylsalicylic acid	6.25	35	5.0
Sodium salicylate	12.5	79	7.1
Hydrocortisone hemisuccinate	12.5	35	7.1
Paracetamol	12.5	82	7.0
Gentamicin	12.5	30	7.0

DISCUSSION

The similarity between gastric and colonic mucosal homogenates

The similarity between gastric and colonic mucosal homogenates in the patterns of inhibition of glucosamine synthetase by acetylsalicylic acid, sodium salicylate, hydrocortisone hemisuccinate and paracetamol strongly suggests that the enzyme derived from these two sources has similar kinetics. This, together with the similar order of magnitude of glucosamine synthetase levels in gastric and colonic mucosa (Chapter 13), justifies the use with gastric mucosa of the method for assaying glucosamine synthetase developed with colonic mucosa. The enzyme is probably identical in the gastric, duodenal and colonic mucosa.

The inhibition of glucosamine synthetase by ulcerogenic and non-ulcerogenic drugs

With acetylsalicylic acid and sodium salicylate, there was 50% inhibition of glucosamine synthetase at 23 mM and 27 mM respectively (Figs. 99, 100). This corresponded to 50% inhibition by sodium salicylate at 20 mM in Perrey's experiment (Fig. 96). The slightly greater inhibition by acetylsalicylic acid may have been due to the sub-optimal pH of the incubate (5.0) at the highest concentration of the drug, although the difference in inhibition between the two drugs at 30 mM was not statistically significant.

Hydrocortisone was rather more inhibitory than the salicylates, with 50% inhibition at 10 mM.

However, paracetamol also inhibited glucosamine synthetase in gastric and colonic mucosal homogenates, by 50% at 29 mM, which is the same order of inhibition as that induced by sodium salicylate. The inhibition by sodium salicylate was the reason for Perrey (1968) postulating that the inhibition of glucosamine synthetase by sodium salicylate could be the basis of its ulcerogenic effect.

Paracetamol resembles the salicylates in being an analgesic, but there has never been any clinical evidence that it is ulcerogenic. Gentamicin also inhibits glucosamine synthetase to a similar degree as the salicylates, and once again there is no clinical evidence that this drug is ulcerogenic.

CONCLUSIONS

The inhibition of glucosamine synthetase *in vitro* by the ulcerogenic drugs aspirin, sodium salicylate and hydrocortisone is a non-specific effect which is also shown by two non-ulcerogenic drugs, paracetamol and gentamicin.

Studies of the mechanism of drug-induced peptic ulceration should always include an examination of the effects of non-ulcerogenic drugs.

The patterns of inhibition of glucosamine synthetase by each of four drugs is similar in the gastric and colonic mucosa, which suggests that the enzyme is identical in these two parts of the gastrointestinal tract.

SECTION IV

CONCLUSION

CHAPTER 16

THE MEASUREMENT OF GLUCOSAMINE SYNTHETASE IN THE HUMAN GASTROINTESTINAL MUCOSA

This thesis has described the development and application of a microassay for the enzyme glucosamine synthetase in biopsies taken for clinical purposes from the gastrointestinal tract of human patients. In Appendix C, it is shown that the assay can also be used with human percutaneous liver biopsies. The feasibility of this study was at first doubted by several biochemists.

Gastrointestinal mucus has not been a major topic of study in gastroenterology (Fig. 104) despite the considerable number of diseases of the gastrointestinal tract in which an abnormality in mucus secretion is apparent or has been suggested (Chapter 1). In many of these diseases, the exact role of the mucus is in some doubt. Studying the enzymes of mucus biosynthesis in diseased tissue may help to unravel the relationships between mucus and disease.

Glucosamine synthetase

Glucosamine synthetase is the first enzyme in mucus biosynthesis and it is probably a rate-limiting step. This thesis describes the results of its assay in biopsies taken from the stomach, the duodenum, the jejunum and the colon. Its activity in a mucosa has been shown to correlate with the epithelial cell density and the enzyme activity is low in a mucosa denuded of epithelial cells, although inflammatory cells appear to have some slight degree of glucosamine synthetase activity. The enzyme activity is abnormally high in a healing mucosa.

Glucosamine synthetase could be used as an epithelial cell marker. Evans and Burdett (1973) warned of the difficulty of using mucosal homogenates to study enzymes which were also present in the muscularis mucosae,

FIG. 104. A typical month's entry in the "Index Medicus" for Mucus (on the left), compared to the same month's entry for Prostaglandins (on the right).

but glucosamine synthetase is not present in muscle in detectable amounts.

One interesting outcome of the present study has been the finding that the morphologically normal colonic mucosa of patients with Crohn's disease elsewhere in the bowel possesses an enhanced glucosamine synthetase activity similar to that found in the healing phase of ulcerative colitis. This finding suggests the possibility that the entire mucosa is abnormal in Crohn's disease even though the obvious lesions are focal and scattered.

Other lessons from these studies of glucosamine synthetase

The studies with glucosamine synthetase described in this thesis have also demonstrated that animal models of human gastrointestinal disease may not be relevant to the human condition and that instead of making such studies in animals it would be much more fruitful to perform comparable experiments on biopsy samples of human mucosa. This can be illustrated by considering the experiments on restrained rats in relation to the aetiology of human gastric ulcer. These animals show a marked reduction in glucosamine synthetase levels under restraint but no comparable reduction of glucosamine synthetase activity is found in the gastric mucosal biopsies of human patients with a gastric ulcer.

Methods are available for the study of many of the enzymes of glucoprotein synthesis and they have been widely used with rat liver and bovine submaxillary gland. Most of the methods require miniaturization if they are to be used with human biopsies, and the present study has shown that such miniaturization is feasible.

Another lesson from these studies has been the demonstration that a biochemical effect of ulcerogenic drugs, namely the in vitro inhibition of glucosamine synthetase, is also shown by non-ulcerogenic drugs. Studies of the aetiology of drug-induced peptic ulceration should include comparisons with non-ulcerogenic drugs as well as with blank controls.

APPENDICES A — D

Appendix A

DETAILED EXAMINATION OF THE ACCURACY OF THE METHOD

The potential sources of error in the glucosamine synthetase assay which was developed and used in this thesis were listed in Chapter 8 and are discussed in detail in this Appendix.

(a) Cleaning the tissue

Surgically removed tissue was washed in homogenizing buffer. Biopsy specimens weighing 5-20 mg were collected in 3-5 ml homogenizing buffer, which was considered adequate to wash the tissue. All specimens of tissue were oriented flat and blotted on both sides onto filter paper to remove as much as possible of the adherent matter, mainly mucus. Adherent matter remaining probably had no enzyme activity but it may have increased the weight of the tissue to give an erroneously low reading for enzyme activity per g wet wt. Adherent matter may have contributed to the protein content of the tissue when the enzyme activity was measured per g tissue protein.

(b) Drying the tissue

The blotting on filter paper was intended to dry the tissue. Adherent water could have increased the apparent weight of the tissue, giving a falsely low reading of enzyme activity per g wet wt, but the activity per g tissue protein would not have been affected.

(c) Weighing the tissue

There may well have been a small error in the fine Stanton balance.

The weight was measured to the nearest 0.1 mg so that in a biopsy weighing 4.5 mg, which was the minimum required for the 4 mg assay, an error of up to 1% could arise on account of this limitation. In the case of the 2 mg assay on a biopsy weighing 2.5 mg, this error could be 2%. The errors of weighing were greater with the smaller assays.

To eliminate the possibility of tissue adhering to the parafilm in which it was weighed, the parafilm was first weighed together with the tissue, then the tissue was placed in the homogenizer, and then the parafilm alone was weighed. The tissue was removed from the parafilm with the plunger of the homogenizer, to eliminate loss of weighed material by adherence during transfer from the

parafilm to the homogenizer.

The possibility of dehydration during weighing was minimized by weighing the tissue sealed within two layers of parafilm. However, dehydration may have occurred during blotting.

(d) Pipetting

The homogenizing buffer was pipetted into the homogenizer with a glass pipette. The volume was measured to the nearest 0.01 ml, which meant that an error of up to 1% could arise in adding 0.45 ml buffer to a biopsy weighing 4.5 mg when the calculated volume was 0.4455 ml. With a piece of tissue weighing 2.5 mg (in an unduplicated assay), this error could be 2%. This error could have been eliminated in the calculation if 0.1 mg tissue were to have been homogenized in 0.01 ml buffer instead of 0.0099 ml and this could have been taken as a tissue concentration in the homogenate of 9.9 mg/ml instead of 10 mg/ml and the calculation adjusted accordingly. This was not done but usually the error was well under 1%.

Besides the homogenizing buffer, perchloric acid, KOH and DAB were pipetted in glass pipettes, but all other pipettings were performed with Eppendorf pipettes or Finpipettes, always with new tips.

The same Eppendorf pipette was used for the homogenate and for the substrate-buffer mixture, so that in mixing equal volumes there was no error from using different pipettes. The same Eppendorf pipette, or the same Finpipette, without changing the volume adjustment on the Finpipette, was used throughout all estimations of a single batch of incubates. Since volumes were compared to the same volumes of standards (except in the addition of acid and alkali to the standards), no error could arise from using different pipettes.

0.5 ml volumes of deionized water were pipetted into a weighed, sealed container and the weight of each volume was measured on a fine balance. For 17 volumes pipetted with a single 500 μl Eppendorf pipette, using 8 different new tips, the mean weight of water was 0.4986 g with an S.D. of 0.0022 g or 0.4%.

For 25 volumes pipetted with the same Eppendorf pipette using 9 different tips which had been used and washed, the mean weight of water was 0.4770 g ± 0.290 g (6.1%), although for 4 volumes pipetted with each of 5 washed tips the mean S.D. for each tip was 0.0036 (0.8%).

For 14 volumes pipetted with 8 different 1.0 ml graduated glass pipettes, the mean weight of water was 0.4967 ± 0.0058 g (1.2%). All these weighings were performed on the same morning with the same batch of deionized water.

0.1 ml volumes of water were pipetted and weighed similarly. For 6 volumes pipetted with a 100 μl Eppendorf pipette using separate new tips, the mean weight of water was 0.0947 g ± 0.0022 g (2.3%). For 6 volumes pipetted with 6 different 1 ml graduated glass pipettes, the mean weight was 0.1016 g ± 0.0036 g (3.5%).

Thus an Eppendorf pipette, used with new tips, gave more reproducible volumes than 1.0 ml graduated glass pipettes. Used and washed Eppendorf pipette tips gave grossly inaccurate volumes, although a single tip gave a reproducible reading, and reproducibility, rather than absolute volume, was all that was required in the addition of the borate buffer and the acetic anhydride to batches of neutralized incubates.

(e) The composition of the homogenizing buffer

At pH 7.0, the curve for pH versus glucosamine synthetase activity was quite flat and an error of 0.1 pH units would give rise to an error of only 1-2% (Fig. 26).

The homogenizing buffer was diluted 1:1 from double buffer, which was made by weighing calculated amounts of Na_2HPO_4 and NaH_2PO_4 and dissolving them in 1000 ml deionized water. The predicted pH was checked to ensure that it was within 0.1 unit of 7.0 using a pH-meter.

The ionic strength of the incubation medium was not critical to glucosamine synthetase activity (Table 10 and Fig. 108) so that no significant error could be expected as a result of slight errors in the weighing of the components of the homogenizing buffer.

The EDTA concentration of the homogenizing buffer was 1 mM. The effect of a small error in this was not investigated. It was assumed that 1 mM was sufficient EDTA to chelate cations which would inhibit glucosamine synthetase (Table 7) and that small errors in this concentration would not affect the activity of the enzyme.

(f) The composition of the substrate-buffer mixture

Errors of 1-2% in the fructose-6-phosphate concentration could have arisen from errors in the estimated hygroscopic water. Glutamine was non-hygroscopic.

From the matrix of glucosamine synthetase activity at different substrate concentrations (Table 11), it was estimated that an error of 1% (0.2 mM) in the fructose-6-phosphate concentration would give rise to an error of approximately 0.5% in glucosamine synthetase activity. An error of 1% (0.08 mM) in the glutamine concentration would give rise to an error of approximately 0.2% in glucosamine synthetase activity.

Large errors in the gentamicin concentration would have had no noticeable effect on the enzyme estimation (Table 8).

(g) Homogenization

With the method used, the degree of homogenization did not appear to be critical (Table 5).

(h) The temperature of the incubation

The temperature relationship of glucosamine synthetase in human colonic mucosa was studied by incubating two 0.5 ml aliquots of each of two homogenates with 0.5 ml volumes of substrate-buffer mixture at 34°, 37° and 40° for 3 hours (Fig. 105).

An error of 1.0 Celsius degree in the temperature of the incubation would give rise to an error of approximately 4% in the enzyme estimation.

(i) The duration of the incubation

The relationship of glucosamine synthesis to the duration of the incubation was linear to within 20-35% (Fig. 22). An error of 1 min in the duration of the incubation would give rise to an error of approximately 0.5% in the enzyme estimation.

(j) The pH of the neutralized perchloric acid

The perchloric acid was neutralized to a pH of between 6 and 11 using Merck non-bleeding indicator strips.

In the Morgan-Elson reaction, 1 volume of neutralized solution was buffered by an equal volume of 1.12M boric acid/KOH buffer at pH 9.2.

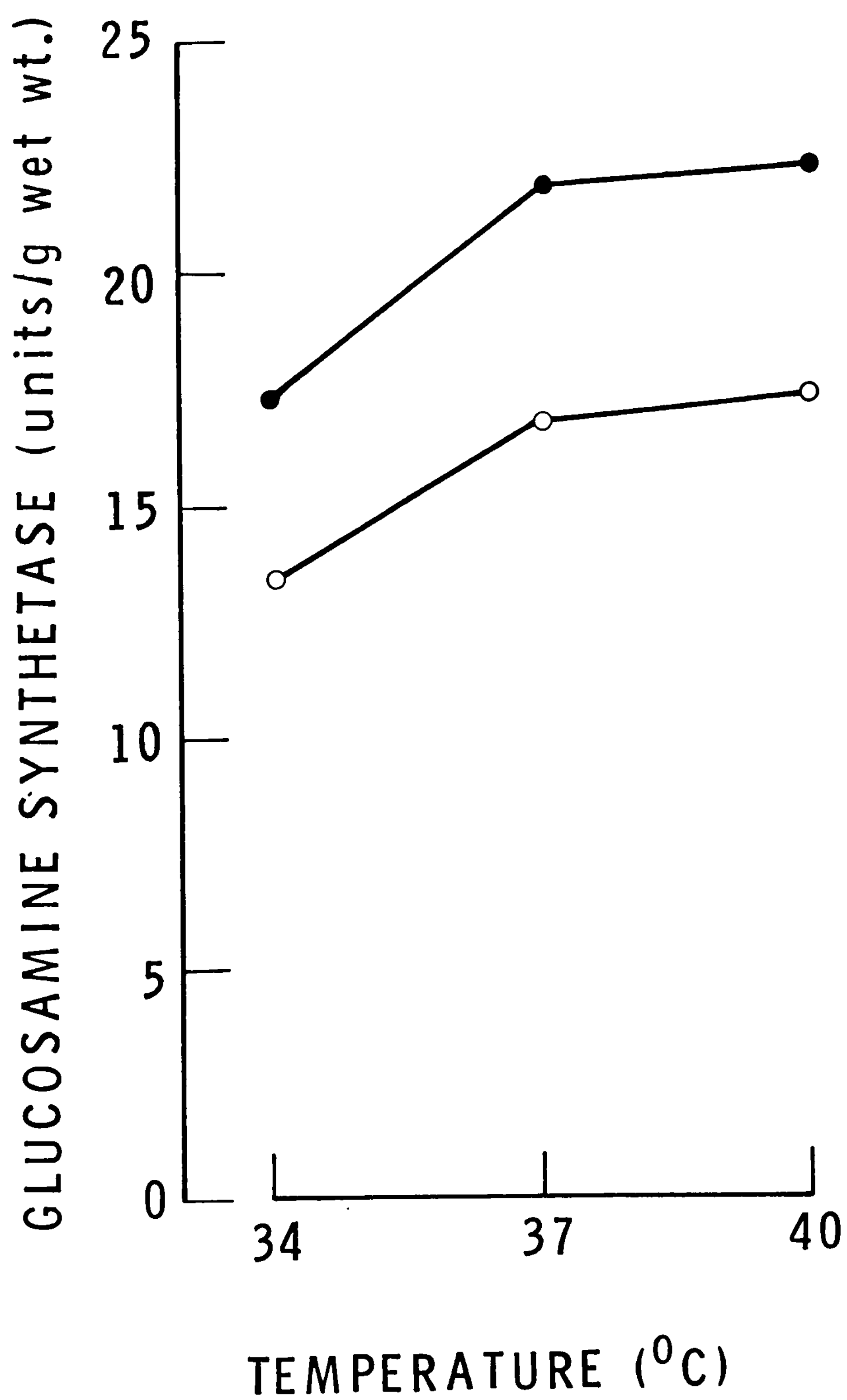


FIG. 105. Glucosamine synthetase activity in two homogenates of normal colonic mucosa, two samples of each homogenate assayed in duplicate at 34°, 37° and 40°.

The effect of inaccurate neutralization was studied by diluting 5.0 ml volumes of a standard solution of 0.20 mM glucosamine with 0.25 ml 71% perchloric acid, 0.35 ml 9M KOH and further volumes of M KOH to give solutions of varying pH values of between 1.8 and 13.0. The supernatants were assayed by the Morgan-Elson reaction with the same batch of dilute DAB. Even with the addition of no M KOH, giving a pH of 1.8, or with the addition of a gross excess of M KOH, giving a pH of 13.0, the borate buffer was sufficiently powerful to keep the pH in the Morgan-Elson reaction between 9 and 10 and the colour loss was only 10-15% at these extreme ranges (Table 32).

TABLE 32. The effect on the colour produced in the Morgan-Elson reaction of the pH of 5.0 ml volumes of a standard solution of glucosamine 0.20 mM, treated with 0.25 ml $HClO_4$, 0.35 ml 9 M KOH and a variable amount of M KOH. D_c is the predicted value of $(D - D_0)$, the O.D. of the colour minus the O.D. of a water-blank (x 1000), calculated from the observed value of D and corrected to the value for the standard when undiluted by acid and alkali

pH		
Standard solution (treated with acid and alkali)	0.5 ml treated standard solution + 0.5 ml borate buffer	D_c
1.8	9.11	215
2.5	9.33	243
9.5	9.34	250
11.4	9.36	232
12.3	9.46	234
13.0	9.73	206

Thus the error of slightly inaccurate neutralization was very small. The standard solutions as used in the method and the 0.20 mM glucosamine in this experiment were not buffered. It did not seem to be necessary. The test solutions were buffered to pH 7.0, which meant that there was even less likelihood of an error due to inaccurate neutralization of the perchloric acid.

(k) The composition of the borate buffer and the acetic anhydride solutions

Since the same stocks of borate buffer and 1.5% acetic anhydride in acetone were used for all samples in a single batch, including the standards, no error could arise as a result of variations in the composition of these solutions.

(l) The composition of the DAB solutions

All samples in a single batch in the Morgan-Elson reaction, including the standards, were assayed with the same solution of dilute DAB so that no error could arise from variations in the composition of the dilute DAB.

(m) The decay of DAB

An error could have arisen due to decay of the chromogenic activity of the dilute DAB solution between its addition to the first tube and its addition to the last tube in a batch. This delay was never more than 12 minutes and it was usually about 8 minutes. On a warm day, an error of up to 1% could have arisen as a result of this, but it could not have been eliminated except by using smaller batches, which would have been inconvenient.

(n) The decay of the colour formed in the Morgan-Elson reaction

This error could have been up to about 1% (Chapter 8). To eliminate it would have meant taking two readings and extrapolating back to zero time, as was done by Winterburn and Phelps (1971a), but this could have introduced a greater error owing to the possibility of summating individual errors of the two readings (see Fig. 17).

(o) The spectrophotometer readings

The optical densities of the spectrophotometer cuvettes were between 0 and 0.020 compared to a cuvette of lower O.D. For each batch of samples assayed, each cell blank was determined twice and the mean of the two readings was taken. The mean cell blank over a number of experiments was 0.008 O.D.

An error of 0.005 in the O.D. of the cuvette would result in an error of 2.5% if the O.D. of the colour produced was 0.200 and 5% if the O.D. of the colour was 0.100. The actual error in the cell blank reading was probably not more than 0.003 on most occasions.

0.5 ml of each of 0.05, 0.20 and 0.40 mM glucosamine standards was assayed in the Morgan-Elson reaction. The colour formed from each standard was measured on the Unicam SP 500 spectrophotometer six times, over a period of no more than 5 minutes. The mean S.D. of the 6 readings on each standard was 4% (3.9-4.1%) (Table 33).

TABLE 33. Reproducibility of the Unicam SP 500 spectrophotometer in reading the colour produced in the Morgan-Elson reaction by one 0.5 ml volume of glucosamine hydrochloride solutions

[Glucosamine] mM	O.D. readings		Mean O.D.	S.D.	
				O.D.	%
0.05	0.071	0.078	0.076	0.003	3.9%
	0.079	0.079			
	0.075	0.073			
0.20	0.250	0.269	0.267	0.011	4.1%
	0.265	0.280			
	0.262	0.278			
0.40	0.579	0.633	0.629	0.026	4.1%
	0.651	0.644			
	0.627	0.642			

(p) The composition of the standard solutions

The same batch of glucosamine hydrochloride was used throughout the work for this thesis. The loss of weight on desiccation was less than 0.3%, both at the beginning of the study and $2\frac{1}{2}$ years later.

For six samples of 0.20 mM glucosamine hydrochloride, each made up separately but on the same day, with two volumes of each sample assayed in the Morgan-Elson reaction and all 12 assays in the same batch with the same dilute DAB solution, the standard deviations of the mean values of each pair of 0.5 ml volumes was 3.1% (Chapter 8).

Some of this error was the error of the Morgan-Elson assay as a whole and some was the error of the spectrophotometer. Two 0.5 ml volumes of each of six different glucosamine standards between 0.05 and 0.50 mM were assayed in the Morgan-Elson reaction in the same batch. The mean S.D of the 3 readings of each of the 6 standards, as a percentage of the mean of each set of 3 readings, was 3%.

Thus the error detected in the variation between standards was of the same order of magnitude as the error in the estimation of glucosamine by the Morgan-Elson reaction. Therefore there was probably no more than 1% error in the variation between standards.

(q) The linearity of the standard curve

To test the linearity of the standard curve as used in the glucosamine synthetase assay, that is with acid-and-alkali treated standards, the values of D_s were taken for each of the acid-and-alkali treated standards in 20 batches with the 10 mg assay and in 20 batches with the 4 mg assay.

It was assumed that D_o, the reading of D for the zero standard, was an accurate reading. D_o was substrated from D_s for each treated standard and a calculated value, D_c, was obtained for $(D - D_o)$ for each standard as if it had been undiluted by acid and alkali.

$$D_c = (D_s - D_o)(1 + 0.90q) \quad \text{for the 10 mg assay, and}$$

$$D_c = (D_s - D_o)(1 + 1.8q') \quad \text{for the 4 mg assay}$$

The mean values of D_c from each of the 20 batches for each of the 0.05, 0.10, 0.20 and 0.30 mM standards were calculated (Table 34). They were also expressed as a proportion of the value of D_c for the 0.05 mM standard.

The standards did not in fact lie in a straight line (Fig. 106). On the basis that the straight line was drawn visually nearest to the points for the zero and 0.10 mM standards, the assumption of linearity resulted in an overestimate by approximately 3% of the glucosamine concentration of solutions close to the 0.05 mM standard (corresponding to tissue with a glucosamine synthetase activity of about 3.5 units/g wet wt) and an underestimate by approximately 6% of the glucosamine in solutions close to the 0.30 mM standard (corresponding

TABLE 34. Mean values of D_c for glucosamine standards in 20 batches of standards in the 10 mg assay and in 20 batches in the 4 mg assay (see also Fig. 106)

Glucosamine standard		0.05 mM	0.10 mM	0.20 mM	0.30 mM
Mean D_c	10 mg assay	74	142	280	414
	4 mg assay	72	141	258	390
Mean D_c taking D_c for 0.05 mM standard as 5 units	10 mg assay	5.0	9.6	18.9	27.9
	4 mg assay	5.0	9.8	17.9	27.1

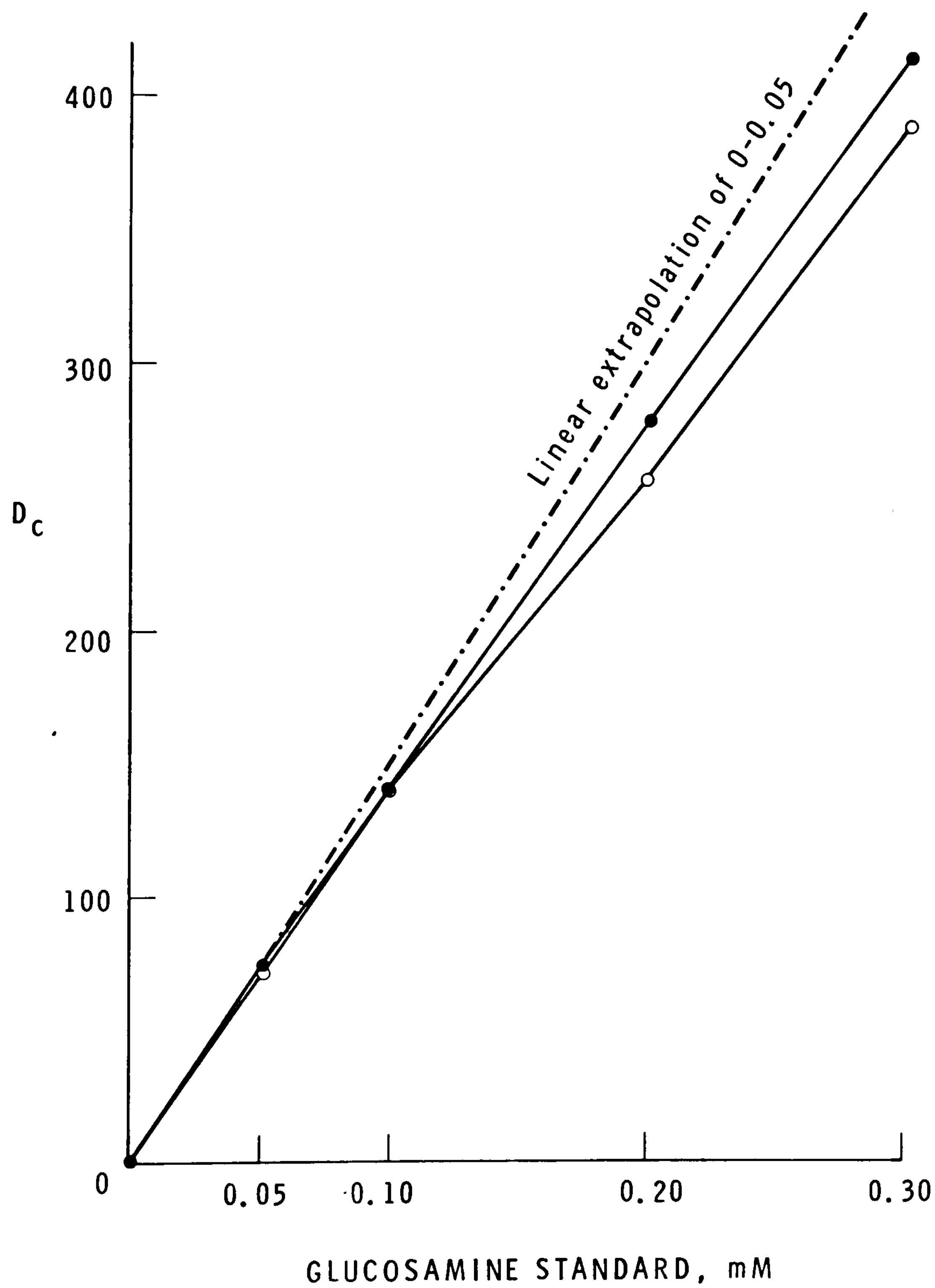


FIG. 106. The linearity of the standard curve in the glucosamine synthetase assay. Calculated values of D_c (= D - D_o) of undiluted glucosamine standards derived from the observed values when diluted with perchloric acid and KOH. Mean values for 20 batches of standards with the 10 mg assay (closed circles) and for 20 batches with the 4 mg assay (open circles). (See also Table 34.)

to tissue with a glucosamine synthetase activity of about 3.5 units/g wet wt) and an underestimate by approximately 6% of the glucosamine in solutions close to the 0.30 mM standard (corresponding to tissue with glucosamine synthetase activity of about 20.0 units).

In a number of instances, mainly with pathological material (see Chapters 10 and 11), the colour produced in the Morgan-Elson reaction was greater than that of the highest standard. It was assumed that the standard curve was linear beyond 0.30 mM glucosamine and this assumption probably accounted for errors, which were underestimates of glucosamine synthetase activity, of up to about 20%.

(r) The accuracy of the standard readings

The non-linearity of the standard curve as derived from 40 sets of standards in Table 34 meant that the expected correlation coefficient of regression was 0.9981, as calculated from the data summarised in that Table. Correlation coefficients were computed for the 40 individual sets of readings.

For the 20 sets of readings with the 10 mg assay, r was 0.9986 ± 0.0024 (S.D.); in other words, the standard readings with the 10 mg assay deviated from the expected curve by an S.D. of approximately 2.5%.

For the 20 sets of readings with the 4 mg assay, r was 0.9965 ± 0.0049. As a proportion of the expected value of r (0.9986), there is a corrected r of 0.9984 ± 0.0049. Thus the standard readings with the 4 mg assay are less accurate than with the 10 mg assay, with an error of about 7% at 1 S.D.

(s) The drawing of the standard curve

The standard curve was always drawn visually. It could have been computed. The error introduced by visual rather than computed drawing of the standard curve was estimated by calculating the expected value of B, B_E, for 20 test samples with the 10 mg assay and for 20 samples with the 4 mg assay, each sample having been read from a separate batch of standards. The observed value of B, B_O, as derived from the visually drawn curve, was expressed as a ratio of B_E.

For the 10 mg assay, B_O was 99.3% ±1.8% (S.D.) of B_E, and for the 4 mg assay, B_O was 99.9 ± 2.5% of B_E. Thus the visually drawn curve gave readings for glucosamine synthetase activity which were usually within 2% of a computed value in the 10 mg assay and within 3% in the 4 mg assay.

Not surprisingly, the ratio $\frac{B_O}{BE}$ deviated from 1.0 by a greater degree when the coefficient of correlation of the five standards in the batch was lower. When the standard readings were closer to linearity, the visually drawn curve was more accurate than when there was a greater error in the standard readings.

(t) The reproducibility of the standard curve

Twenty duplicate pairs of readings, assayed within 24 hours of each other by the 10 mg assay, had a mean difference of 15% ± 12% (S.D.) of the mean of each pair of readings. Twenty duplicate pairs read in the same batch with dilute DAB and standards had a mean variation of 8% ± 6%. The additional degree of variation when the duplicates were read in separate batches was presumably due to errors in reading the standards and in constructing the standard curve. The error in reading the standards was found to have an S.D. of 3% (see (p) above) and in constructing the standard curve the mean correlation coefficient of 20 standard curves with the 10 mg assay was close to that predicted as a result of non-linearity of the readings but the coefficient itself had an S.D. of about 3%.

(u) The delay in commencing the incubation

The incubation was always commenced within 2 hours of removal of the tissue from the body. Biopsy specimens were kept in ice-cold buffer prior to commencement of the incubation. Surgically removed specimens were kept dry at room temperature for up to 30 minutes before the tissue to be assayed could be placed in ice-cold buffer. There was no detectable decay in enzyme activity in unhomogenized tissue in the first 2 hours (Fig. 34).

Once the tissue had been homogenized, the enzyme activity was liable to decay (Figs. 32, 33). Homogenized tissue was kept for no more than 15 minutes prior to commencement of the incubation and this probably eliminated homogenate decay as a source of error.

(v) The storage of the incubated solution and the application of the decay factor

The storage of the incubated solutions at -20° for up to 30 days led to a decay in assayable glucosamine synthetase activity (Figs. 30, 31), which was corrected for by the application of the decay factor f. The source of error was thus in the calculation of the decay factor.

A 20% error in the calculation of the decay factor, taking f as $10^{(0.00169 \pm 0.00034)\,d}$ (in which d = the number of days of storage), results in an error of 2% in the factor itself at 30 days and a 1% error if the incubate was stored for 15 days.

(w) The calculation of enzyme activity

In the calculation of the enzyme activity, x µmoles glucosamine synthesized/h/g wet wt, from B on the standard curve, there were no approximations in the algebra. The enzyme activity was expressed to the nearest 0.1 unit and all calculations were made to one further decimal place, although B from the standard curve was only read to 1 decimal place.

The only important source of error in the calculations was human error in recording the values of D, p and q (or p' and q'), and in working out the results from the observed values.

(x) The linearity of the assay in relation to the glucosamine synthetase concentration

Dilution experiments showed an extremely close correlation between glucosamine synthetase activity as measured by this assay and the concentration of tissue (i.e. of enzyme) in the homogenate, with a coefficient of correlation of 0.999 (Fig. 28).

(y) Glucosamine in unincubated tissue

Up to 1.0 unit/g wet wt could be accounted for by hexosamine in the unincubated tissue when glucosamine synthetase was measured without control readings. It was considered that this was a small error and that control readings of unincubated tissue could be ignored for the sake of improving the miniaturization of the assay.

Error of sampling – The stripping of the mucosa

Any submucosa that was included in the tissue sample would diminish the apparent enzyme activity.

Rectal biopsies were cut in half at right angles to the plane of the mucosa to divide the tissue into one part for the glucosamine synthetase assay and one part for histology. From the histological slides, it was seen that submucosa was only included in the biopsies on a few occasions and always in small amounts.

In surgically removed specimens of colon, the mucosa was stripped from the submucosa by blunt dissection. Small amounts of submucosa were seen to be included with the mucosa. A histological section of a piece of stripped mucosa confirmed that a depth of mucosa was obtained which corresponded to that of a rectal biopsy (Fig. 14). The glucosamine synthetase of normal rectal biopsies was similar to that of the stripped normal mucosa (Chapter 9).

Appendix B

SOME KINETIC PARAMETERS OF GLUCOSAMINE SYNTHETASE IN HUMAN COLONIC MUCOSA

From the experiments described in Chapter 8 in the determination of the optimum conditions of activity of glucosamine synthetase in the human colonic mucosa, experiments which were performed in order to miniaturize the assay of the enzyme, it has been possible to derive some kinetic parameters of the enzyme.

pH dependence

The pH optimum was on a broad band from 6.5 to 7.5 (Fig. 26). This is similar to the pH dependence of glucosamine synthetase from the bovine tracheal mucosa (Ellis and Sommar, 1972). Winterburn and Phelps (1971a) found that the pH optimum of purified glucosamine synthetase from rat liver was 7.5, with a sharp drop below 7.0 (Fig. 25, a), although in the presence of fructose-6-phosphate the curve of pH dependence was almost flat between 6.5 and 7.5 (Fig. 25, b).

Temperature dependence

The temperature dependence was determined between 34° and 40° (Fig. 105). There appears to be an increase in enzyme activity from 34° to 37°, and a further increase, but with much less of a gradient, from 37° to 40°.

Substrate kinetics

Winterburn and Phelps (1971a) found that fructose-6-phosphate was saturating to glucosamine synthetase in rat liver at 3 mM and that glutamine was saturating at 6 mM. In human colonic mucosa, the saturating concentrations of the two substrates have been found to be approximately 20 mM for fructose-6-phosphate and approximately 10 mM for glutamine.

Both substrates are inhibitory to the enzyme in human colonic mucosa in concentrations of more than about 20 mM for fructose-6-phosphate and more than about 15 mM for glutamine (Fig. 107). Ellis and Sommar (1971) found that glutamine inhibited glucosamine synthetase in the bovine tracheal mucosa at greater than 5 mM but that glutamine at 20 mM did not inhibit the enzyme in rat liver.

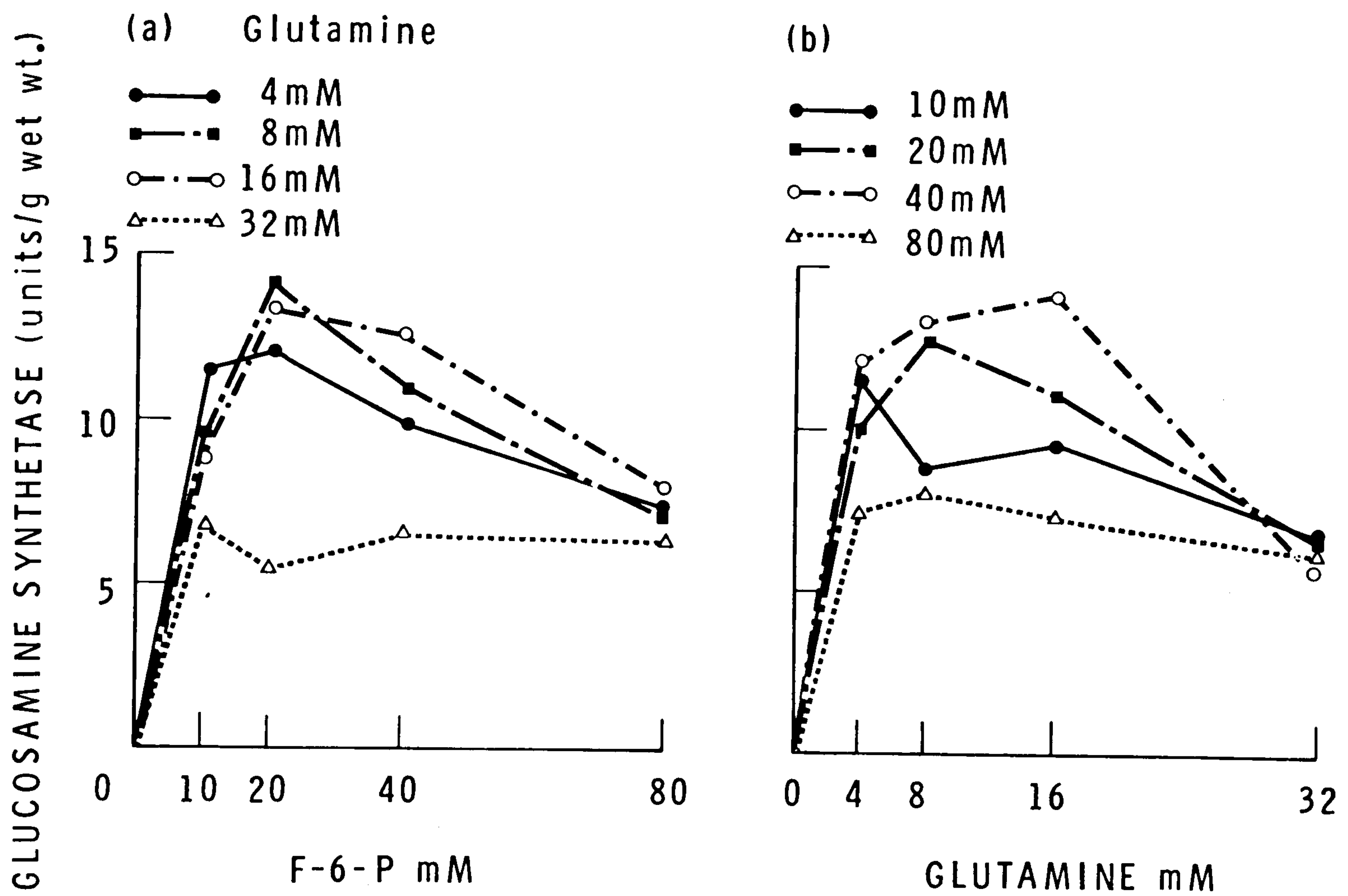


FIG. 107. Glucosamine synthetase activity of an homogenate of normal colonic mucosa in relation to substrate concentrations (4 X 4 matrix), pH 7.0, incubated for 3 hours at 37°.
(a) Effect of fructose-6-phosphate concentration at several concentrations of glutamine. (b) Effect of glutamine concentration at several concentrations of fructose-6-phosphate.

It is noteworthy that the velocity of glucosamine synthetase in human colonic mucosa appears to tend to a rate that is half of the maximum velocity as the concentrations of both substrates are increased above their optimum molarities (Fig. 107).

The matrices of substrate concentrations that were used in the determination of the optimum concentrations for glucosamine synthetase were not large enough to plot Lineweaver-Burk and secondary plots for this two-substrate enzyme reaction. The experiments were performed to miniaturize an assay for glucosamine synthetase and the determination of the kinetic characteristics of the enzyme was not carried out. A very crude homogenate was under study.

The effect of the ionic strength

At pH 7.5, KCl did not noticeably affect the glucosamine synthetase activity of human colonic mucosa, up to an ionic strength of 0.26 (Table 10).

The effect of NaCl in concentrations of up to 170 mM was studied with the 2500 rpm supernatants of two homogenates of normal human colonic mucosa, at pH 7.0 in 50 mM sodium-phosphate buffer containing KCl 100 mM and EDTA 1 mM, by the 10 mg method of assay. The tissue was homogenized without added NaCl. The glucosamine synthesis at different ionic strengths was measured in pairs of samples in the same batches with dilute DAB.

The activity of the enzyme appeared to decline with increasing ionic strength (Fig. 108). Winterburn and Phelps (1971a) found that there was a maximum of activity of rat liver glucosamine synthetase at an ionic strength of 0.18-0.25 and then a decline above this. Human colonic glucosamine synthetase did not show any marked dependency on ionic strength down to 0.12 and it was not studied at ionic strengths below this.

CONCLUSION

Glucosamine synthetase was studied in the human colonic mucosa, obtained from the histologically normal cut ends of surgical colectomy specimens of patients with carcinoma of the colon. The pH dependence and the substrate kinetics differed from those found by Winterburn and Phelps and others for glucosamine synthetase from the rat liver.

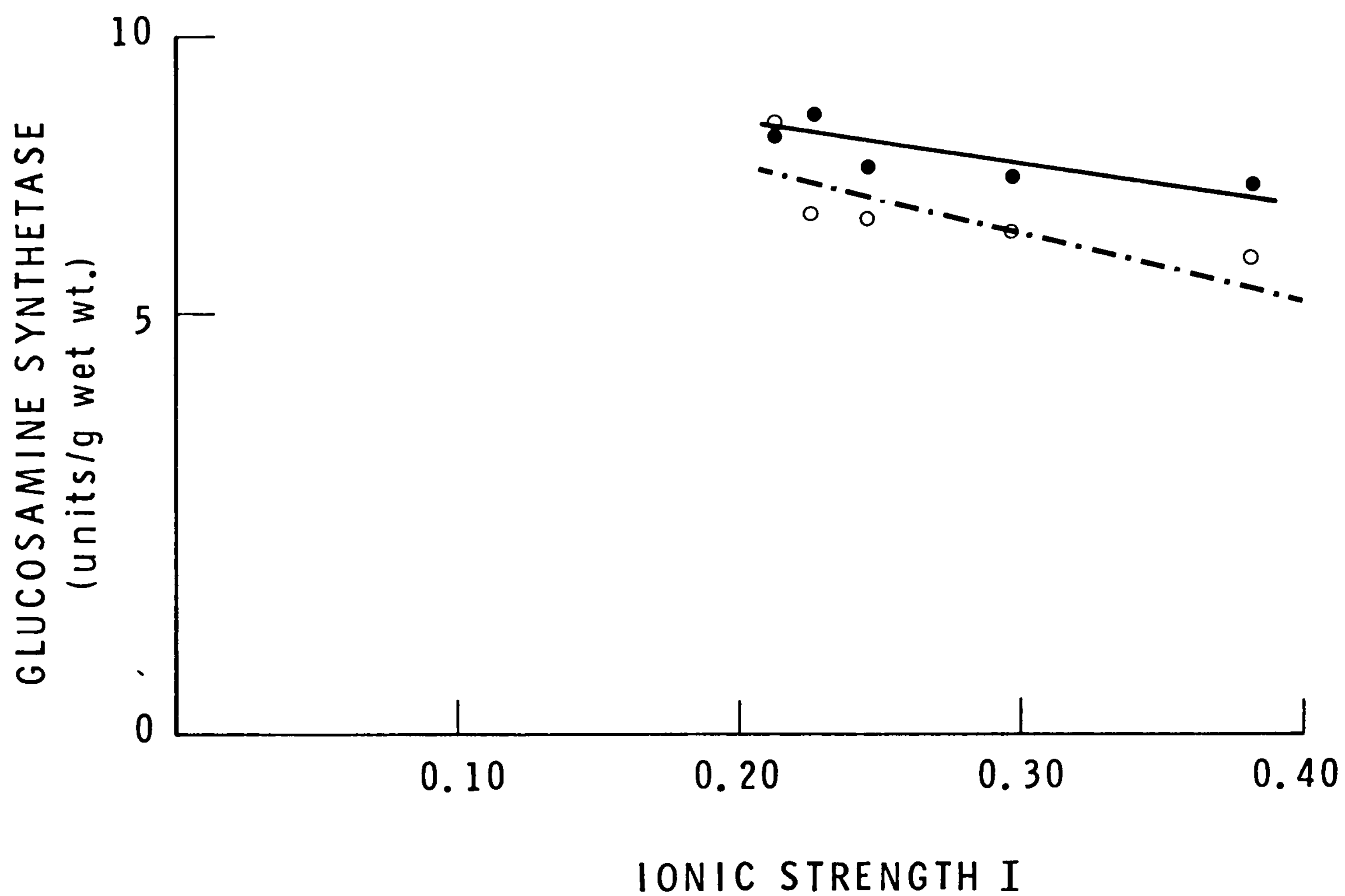


FIG. 108. Glucosamine synthetase activity in relation to increasing ionic strength with added sodium chloride in the supernatants of 2 homogenates of normal colonic mucosa.

Appendix C

CLINICAL STUDIES WHICH HAVE NOT YET BEEN COMPLETED

(i) CARBENOXOLONE SODIUM

This account describes briefly the results of the measurement of the gastric and duodenal glucosamine synthetase levels in two patients with gastric ulcer, before and after treatment with carbenoxolone sodium, and in two other patients who were already being treated with carbenoxolone.

The healing effect of carbenoxolone sodium in human gastric ulcer has been clearly demonstrated (Doll et al, 1962) and this drug appears to increase the amount of mucus in the stomach (Goodier et al, 1967). Johnston et al (1975) have shown a doubling of N-acetyl[^{3}H]glucosamine incorporation into the gastric glycoprotein of rats and ferrets in response to 7 days of carbenoxolone therapy but no effect on acetylglucosamine incorporation in the duodenum. Johnston et al also showed a 2-to-3-fold increase in N-acetyl[^{3}H]glucosamine incorporation into human gastric biopsies obtained endoscopically, before and after treatment with carbenoxolone, in 2 patients with gastric ulcer and in 1 other patient with oesophago-gastric abnormalities.

On the other hand, Croft (1973) reported a lowering by carbenoxolone of the rate of turnover of the gastric mucosa in one patient with a gastric ulcer and atrophic gastritis, and Klein et al (1975) found that carbenoxolone treatment lowered the rate of incorporation of [^{3}H]methyl-thymidine into the gastric biopsies of human patients and prolonged the mean generation time of the mucosa.

Because of the relationship of glucosamine synthetase to mucus synthesis and to the healing of the mucosa (Chapter 10), it was decided to study the effect of carbenoxolone therapy on the glucosamine synthetise levels in gastric and duodenal biopsies of patients with gastric ulcer.

PATIENTS STUDIED

To date, only 2 patients have been studied. The intention is also to study control patients not taking carbenoxolone, but there have been no patients yet in this group. It is intended to match control patients with the treated group, according to age, sex and site of the ulcer, so the study will not be blind.

There has been a low rate of recruitment into this study. The patients have to have a gastric ulcer which appears radiologically and endoscopically to be benign, to have had no surgery on the upper gastrointestinal tract, and to have no other serious diseases, although a concurrent duodenal ulcer does not exclude the patient from the study. In view of the risks of carbenoxolone in causing sodium retention, potassium depletion and oedema, the patients must be under 60 years of age and with no history of chest or heart disease. One patient recruited into the study developed oedema 10 days after starting carbenoxolone, which had to be discontinued.

With the study requiring a second endoscopy, the performance of the first endoscopic examination must turn out to be fairly easy, the patient must live close to Oxford, and he must be willing to undergo the second endoscopy, it being explained to him that this is a study of the effect of a drug that is used to help heal his ulcer.

In addition, patients are excluded if they are taking hormonal preparations or ulcerogenic drugs or if they have received carbenoxolone, Caved-S (deglycyrrhizinated liquorice, which is related to carbenoxolone) or gefarnate (which may have a healing effect in gastric ulcer — Newcombe et al, 1970) in the previous 3 months.

METHODS

Biopsies are taken from the high lesser curve, from the greater curve and from the antrum in the stomach, and from the duodenal cap, at each of two endoscopic examinations of each patient. These biopsies are assayed for glucosamine synthetase by the 4 mg method, as with the gastric and duodenal biopsies in the patients described in Chapter 13. The biopsies at the initial endoscopy in the two patients studied so far are included in the series in Chapter 13.

Following the first examination, the patient is prescribed carbenoxolone sodium 100 mg four times daily for two weeks, at which time the second examination is performed, the patient having been questioned to ensure that he has taken the carbenoxolone regularly.

In addition to the 2 patients studied in this way, results are available of the glucosamine synthetase levels in the gastric and duodenal biopsies of two

patients who were excluded from the series in Chapter 13 because they were receiving carbenoxolone. One of these patients (A. E.) had on the high lesser curve the scar of a gastric ulcer that had been seen at endoscopy 2 months previously, when there had also been a duodenal ulcer which had healed by the time the biopsies were taken. The other patient (J. Pa.) had a healing gastric ulcer high on the posterior wall of the stomach.

RESULTS

Glucosamine synthetase before and after treatment with carbenoxolone

Fig. 109 shows the glucosamine synthetase levels in the gastric and duodenal biopsies before and after 2 weeks' treatment with carbenoxolone in the 2 patients in whom this was studied. The first patient (L. Sm.) had a gastric ulcer high on the posterior wall of the stomach. The second patient (L. W.) had a lesser curve gastric ulcer and also a duodenal ulcer. For the gastric biopsies from the three sites in the 2 patients, there was a rise in the glucosamine synthetase level from a mean of 10.36 units to 13.53 units. There was no apparent rise in the duodenal biopsies; in fact, the mean value fell, but the results were complicated by the presence of a duodenal ulcer in the patient L. W.

TABLE 35. Glucosamine synthetase levels in gastric and duodenal biopsies of 4 patients with gastric ulcer treated with carbenoxolone sodium (unduplicated readings in square brackets)

		Carbenoxolone therapy		Glucosamine synthetase (units/g wet wt)			
				Stomach			
Patient	Diagnosis (before treatment)	Daily dose	Duration	High lesser curve	Body, greater curve	Antrum	Duodenal cap
L. Sm.	G. U.	400 mg	2 wks	13.8	13.6	16.9	18.8
L. W.	G. U. + D. U.	400 mg	2 wks	[10.5]	15.7	9.2	11.8
A. E.	G. U. + D. U.	150 mg	7 wks	3.0	10.4	[12.5]	10.5
J. Pa.	G. U.	300 mg	5 wks		7.4	9.3	[13.3]
Mean				8.82	11.77	11.90	13.64
Normal mean (Table 30)				6.96	9.47	10.46	14.07
Mean for all gastric biopsies					11.33		
Normal series					9.48		

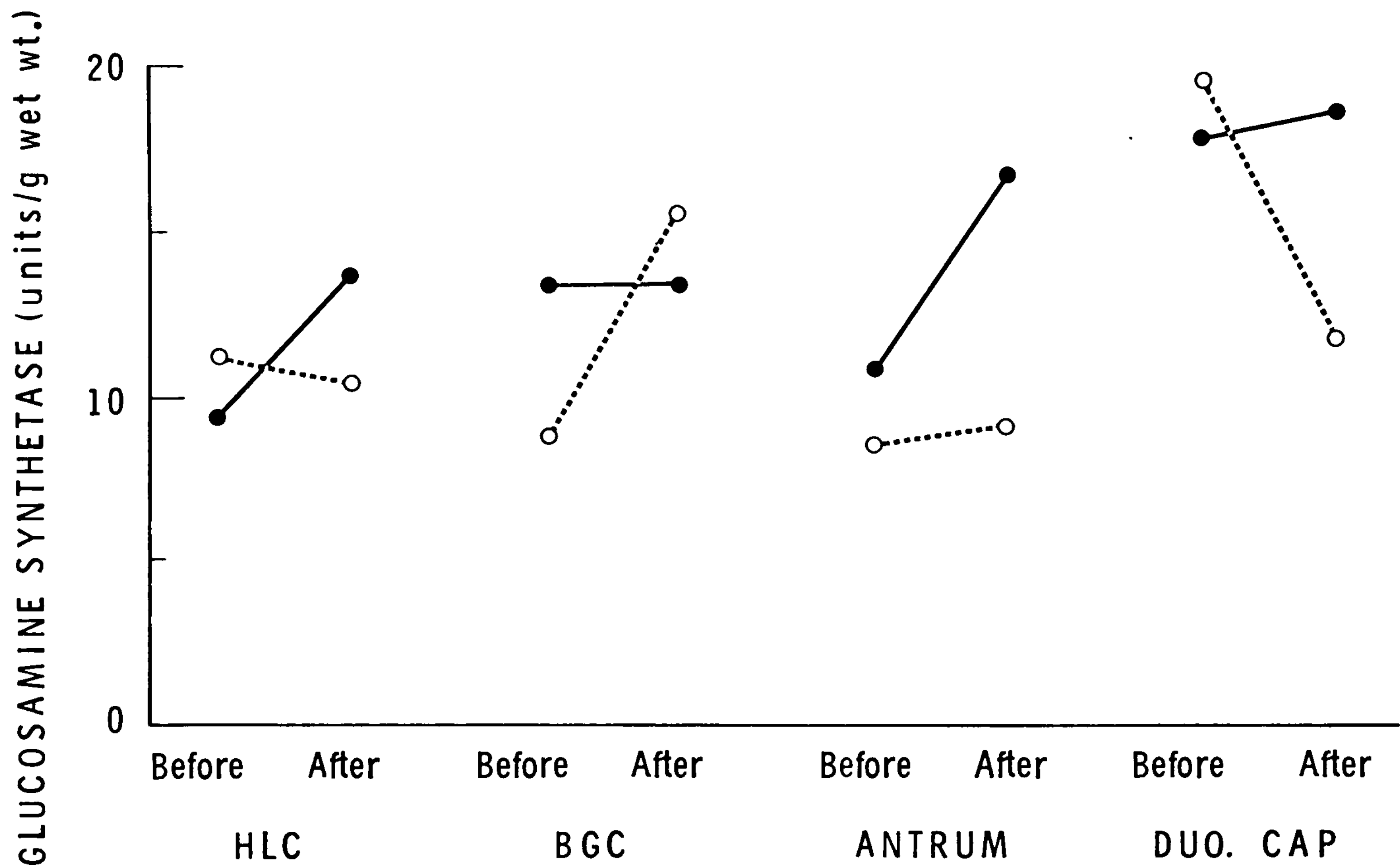


FIG. 109. Glucosamine synthetase levels in gastric and duodenal biopsies of 2 patients with a gastric ulcer before and after treatment for 2 weeks with carbenoxolone sodium 100 mg q.d.s. Closed circles: patient L.Sm. Open circles: patient L.W. (who also had a duodenal ulcer). [HLC = high lesser curve; BGC = body, greater curve.]

Glucosamine synthetase in patients taking carbenoxolone

Table 35 gives the glucosamine synthetase readings in the gastric and duodenal biopsies in the 2 patients studied before and after carbenoxolone therapy and in the 2 patients in whom the enzyme levels were only measured in biopsies taken at a single endoscopic examination when they had been taking carbenoxolone. The results are compared with the normal mean for each region (from Table 30). There was a slight elevation of glucosamine synthetase levels in the gastric mucosa, but not in the duodenal mucosa, after carbenoxolone therapy.

DISCUSSION AND CONCLUSIONS

From the few results to date, there is a suggestion that the glucosamine synthetase levels in the gastric mucosa, but not in the duodenal mucosa, may rise following carbenoxolone therapy. The matched control patients which it is intended to study should show whether this is a real difference due to the drug or whether it is simply due to the healing of the ulcer, as occurs in the healing phase of ulcerative colitis (Chapter 10).

(ii) GLUCOSAMINE SYNTHETASE IN LIVER BIOPSIES

This study, although incomplete, has been included because it shows that the glucosamine synthetase microassay which was developed for use with rectal biopsies can also give results with portions of percutaneous liver biopsies and with surgical wedge liver biopsies. The study has been delayed mainly by the difficulty in obtaining normal liver tissue as control material.

Glucosamine synthetase was first identified in animals in the rat liver by Pogell (1956) and most of the kinetic studies on this enzyme have been performed with liver tissue (Winterburn and Phelps, 1971a,b,c). The glycoproteins produced in the liver are mainly the serum glycoproteins. Heparin, a mucopolysaccharide containing hexosamine, is also synthesized in the liver.

Alcoholic liver disease

Histochemically, the fibrous tissue which is found in the liver contains acid mucopolysaccharides (Galambos, 1966). Galambos and Shapira (1973) measured the glycosaminoglycans in human livers at post-mortem, finding increased amounts, mainly of chondroitin-4-sulphate and chondroitin-6-sulphate, in 7 patients with alcoholic hepatitis compared with 10 normal patients. On the other hand, in 10 patients with cirrhosis there was no increase in glycosaminoglycans.

Proline hydroxylase, a key enzyme in colagen synthesis, has been measured in human liver biopsies and elevated levels were found in alcoholic hepatitis and in cirrhosis (McGee et al, 1974).

It was decided to study glucosamine synthetase in human liver biopsies in patients with alcoholic liver disease, since acetylglucosamine and acetylgalactosamine are important components of the glucosaminoglycans.

Schafer et al (1974) have shown inhibition by ethanol of ^{14}C-glucosamine incorporation into slices of rat liver cultured in Krebs medium.

Obstructive jaundice

Wound healing is inhibited in patients with jaundice (Lee, 1974) and Than et al (1974) have shown a 70% inhibition of skin prolyl hydroxylase in obstructive jaundice, although there have been no reports of the measurement of this enzyme in the liver in obstructive jaundice. It was decided to study glucosamine synthetase levels in the liver in obstructive jaundice, using wedge biopsies obtained at laparotomy.

PATIENTS STUDIED AND METHODS

Alcoholic liver disease

Portions of the diagnostic percutaneous liver biopsy in 7 patients with alcoholic liver disease have been assayed for glucosamine synthetase using the 4 mg method developed for rectal biopsies. In 2 cases, insufficient material was available for duplicated readings. The biopsies have been obtained using the Klatskin needle, which has a diameter of 1.5-2.0 mm and produces a larger biopsy than the conventional Menghini needle which is shorter and has a diameter of only 0.75-1.0 mm. The quantity of tissue available for the glucosamine synthetase assay is limited by the requirement for an adequate sample of tissue for diagnostic histology and of a certain amount for electron microscopy which was being performed on these biopsies.

Obstructive jaundice

Wedge liver biopsies have been taken at operation in 4 patients undergoing laparotomy for obstructive jaundice. The biopsies were placed in a dry bottle, which was sent immediately to the laboratory. The biopsies weighed more than 100 mg. Part was sent for histology and a portion weighing about 20 mg was homogenized and assayed for glucosamine synthetase by the 10 mg method.

Normal liver

It is intended to use as a control series wedge biopsies from patients undergoing gastric surgery for peptic ulcer, patients with no history of liver disease nor of an excessive alcohol intake. The prothrombin time is checked prior to operation, but to date only one such biopsy has been obtained.

RESULTS

In the 7 patients with alcoholic liver disease, the glucosamine synthetase levels have ranged from 2.4 to 9.5 units/g wet wt.

With the biopsies from the patients with obstructive jaundice, the bilirubin in the tissue can interfere with the colour reaction in the glucosamine assay. However, after 2 weeks in the deep freeze, the incubates, containing perchloric acid, become colourless with a green deposit, suggesting that the perchloric acid may be oxidizing the bilirubin. Provided the incubates are

stored for this length of time, it is possible to measure the glucosamine synthesized without interference with the colour. The glucosamine synthetase levels in the 4 patients with obstructive jaundice that have been studied have been between 4.4 and 5.9 units/g wet wt.

The single biopsy from a patient having a vagotomy for duodenal ulcer had a glucosamine synthetase level of 6.5 units/g wet wt.

A surprising finding has been a reading of 1.2 units/g wet wt for the homogenate of one of the biopsies with obstructive jaundice which was not incubated at 37°. This means that there is a detectable amount of hexosamine in the liver and that unincubated samples of the homogenate may need to be assayed for glucosamine as part of the estimation of glucosamine synthetase activity. This would increase the volume of tissue required for the estimation of the enzyme activity and this question needs further study.

DISCUSSION AND CONCLUSIONS

Despite the fact that the microassay for glucosamine synthetase described in this thesis has been developed in relation to the enzyme in the colonic mucosa, it has proved possible to measure the enzyme levels in portions of percutaneous liver biopsies.

Sufficient material is available in surgical wedge biopsies of the liver to study the incubation conditions most suitable for the hepatic enzyme, which may well differ from those appropriate to the enzyme in the colonic mucosa. With optimum concentrations of substrates and an optimum pH, the sensitivity of the assay might be improved, and interesting studies of glucosamine synthetase levels in the human liver in various pathological conditions may become possible. The preliminary studies described in this Appendix, on alcoholic liver disease and on obstructive jaundice, could be developed into larger series.

Appendix D

AN ATTEMPT TO ASSAY GLUCOSAMINE-6-PHOSPHATE N-ACETYLASE

Glucosamine-6-phosphate N-acetylase (acetyl coenzyme A-glucosamine-6-phosphate N-acetyltransferase, Ec. 2. 3. 1. 4) catalyses the N-acetylation of glucosamine-6-phosphate, which is the next step after the glucosamine synthetase reaction in the biosynthesis of the glycoproteins (Fig. 8). Kent (1970) has suggested that this may also be a rate-limiting step in mucus synthesis. This Appendix describes an attempt to assay this enzyme in homogenates of human colonic mucosa.

Glucosamine-6-phosphate N-acetylase was first identified by Chou and Soodak in pigeon liver in 1952. Leloir and Cardini (1953) demonstrated a requirement for ATP and Mg^{++} in glucosamine-6-phosphate acetylase in _Neurospora crassa_, which was confirmed for the mammalian enzyme by Davidson et al (1957) in a study in which they identified the enzyme in human liver. Pasternak in 1961 detected the enzyme in the bovine intestinal mucosa. Izumi (1965) found that, in rat liver, glucosamine-6-phosphate acetylase was only in the soluble fraction. Kent and Allen (1968) reported that the enzyme appeared to be inhibited by salicylate but this seemed to be an inhibition of acetyl coenzyme A formation rather than of the enzyme itself.

Corfield (1973) calculated the _in vivo_ maximum rate of acetylation of glucosamine-6-phosphate in rat liver as 1 mmole/h/g wet wt of tissue. Davidson et al (1957) found that the V_{max} of glucosamine-6-phosphate acetylase in human liver was 0.4 mmole/h/mg protein in a crude homogenate although it was 5 mmoles/h/mg protein in a purified preparation.

Kent and Draper (1968) described two methods of assay of the enzyme, one method being the incubation of the tissue homogenate with glucosamine-6-phosphate and acetyl CoA, and the other method being similar except that acetyl CoA was formed in the incubation from sodium acetate and coenzyme A (which replaced exogenous acetyl CoA in the incubate). Acetylglucosamine phosphate formed in the reaction was measured colorimetrically by the Morgan-Elson reaction.

Corfield (1973) made a detailed study of glucosamine-6-phosphate N-acetylase and he described four methods of assay, two of which were similar to the methods of Kent and Draper, the third was an incubation of glucosamine-6-phosphate with acetyl CoA but quantitation was by the appearance of free CoA as measured by the liberation of thiol by the DTNB reagent, and the fourth method involved radioisotopes using [1-^{14}C] glucosamine-6-phosphate.

The Morgan-Elson reaction was already being used in the current work in the assay of glucosamine synthetase, and it was therefore decided to attempt to study glucosamine-6-phosphate N-acetylase by one of the first two methods of Corfield, in which the glucosamine-6-phosphate which was acetylated was estimated by the Morgan-Elson reaction. The method using sodium acetate and coenzyme A was selected because it gave twice as high a yield of acetylglucosamine as the method using exogenous acetyl CoA. Corfield thought this may have been due to impurities which depleted the commercial acetyl CoA.

MATERIALS

Adenosine triphosphate disodium (ATP) (crystalline grade) was obtained from the Sigma Chemical Co.

Coenzyme A, lithium salt (grade 1-L from yeast) was obtained from the Sigma Chemical Co.

Glucosamine-6-phosphate, disodium salt (grade 1), was obtained from the Sigma Chemical Co.

Magnesium chloride (Analar grade) was obtained from BDH Chemicals Ltd.

Sodium acetate trihydrate (Analar grade) was obtained from BDH Chemicals Ltd.

Other chemicals used were from the same sources as for glucosamine synthetase (Chapter 8).

METHODS

Glucosamine-6-phosphate N-acetylase was studied in homogenates of human colonic mucosa obtained from the cut ends of surgical colectomy specimens for carcinoma of the colon. The second method of Corfield (1973) was followed.

The tissue was homogenized in "acetylase buffer", which contained tris-HCl 75 mM at pH 7.4, $MgCl_2$ 1.5 mM and EDTA 1.5 mM. The buffer was

actually made up in 8-fold strength and diluted with water. The experiment was performed with two homogenates of colonic mucosa. In the first experiment, the homogenate contained 350 mg tissue per ml; in the second experiment, it contained 100 mg/ml. The homogenate was centrifuged at 2500 rpm for 15 minutes and the supernatant was taken. Dilutions of the supernatant were made with the buffer, so that the concentrations of tissue studied, when 0.3 ml substrate-buffer mixture had been added to 0.2 ml homogenate, were 140, 70, 45 and 35 mg/ml in the first experiment and 40. 20 and 10 mg/ml in the second experiment.

0.5 ml volumes to be incubated were made up by adding 0.3 ml of a substrate-buffer mixture to 0.2 ml of the homogenate. The substrate-buffer mixture contained 1 part of "8-fold acetylase buffer" (tris-HCl 600 mM at pH 7.4, $MgCl_2$ 12 mM and EDTA 12 mM), 1 part of sodium acetate 133 mM, 2 parts of ATP 13.3 mM, 2 parts of coenzyme A 33 mM, and 2 parts of glucosamine-6-phosphate 16.6 mM. The 0.5 ml volumes thus contained tris-HCl 75 mM, $MgCl_2$ 1.5 mM, EDTA 1.5 mM, sodium acetate 10 mM, ATP 2 mM, coenzyme A 5 mM, and glucosamine-6-phosphate 2.5 mM (the homogenate also contained the components of the "acetylase buffer").

The substrate-buffer mixture was made up of a few minutes before the incubation commenced and, as soon as it had been added to the homogenate, the 0.5 ml volumes were placed in a water-bath at 37°. The reaction was terminated after 30 minutes by the addition of 0.025 ml concentrated perchloric acid (this was the only point of departure from the method of Corfield, who terminated the reaction with trichloracetic acid). The activity of glucosamine-6-phosphate acetylase falls off rapidly after 30-40 minutes (Corfield, 1973).

As controls, 0.2 ml buffer replaced the homogenate in one sample in each experiment, and one 0.5 ml volume containing the highest concentration of homogenate in each experiment was not incubated but perchloric acid was added as soon as it was made up.

Following the addition of perchloric acid, the tubes were centrifuged to precipitate the protein, 0.3 ml supernatant taken, 0.02 ml 8M KOH added, and then a further volume of M KOH to neutrality, using Merck non-bleeding indicator strips. Potassium perchlorate was precipitated by centrifugation, 0.2 ml of the solution was taken into a screw-cap tube, 0.2 ml borate buffer

added, and then 0.05 ml water (replacing acetic anhydride in the assay of unacetylated glucosamine). The tubes were placed in a boiling water-bath for 3 minutes and then in an ice water-bath for 5 minutes. Acetylglucosamine was assayed by the addition of dilute DAB, as in the glucosamine assay, and the colour formed after 20 minutes incubation at 37° was read in a spectrophotometer at 545 nm. Standards consisted of solutions of glucosamine hydrochloride which were heated with borate buffer and acetic anhydride in acetone, to form acetylglucosamine.

RESULTS AND CONCLUSION

The optical density of the colour produced by the aqueous controls was between 0.047 and 0.053 and that produced by the unincubated homogenates was between 0.059 and 0.074. The test solutions gave O.D.'s of between 0.035 and 0.095 which bore no relationship to the concentration of tissue in the homogenate. A difference of about 0.070 above control values would have represented 0.05 mM acetylglucosamine. For a tissue concentration in the incubate of 100 mg/ml, this would have been equivalent to a rate of synthesis of acetylglucosamine of 1 μmole/h/g wet wt or 0.01 μmole/h/mg protein, well below the rates of activity of the enzyme found by Corfield (1973) and Davidson et al (1957) in the liver.

It was concluded that glucosamine-6-phosphate N-acetylase could not be measured in crude homogenates of human colonic mucosa by this method.

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ABSTRACT

Glucosamine synthetase (L-glutamine-D-fructose-6-phosphate aminotransferase, EC.2.6.1.16) is the cytoplasmic enzyme which catalyses the synthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. It is a rate-limiting step in the biosynthesis of UDP-N-acetylglucosamine and probably also of gastrointestinal mucus, which is glycoprotein in composition. Glycoproteins consist of a protein backbone on which are assembled complex carbohydrate side-chains. The first sugar to be attached to the peptide chain in gastrointestinal mucus is N-acetylgalactosamine, and other important sugars are N-acetylglucosamine and the sialic acids. These particular sugars are supplied to the glycoprotein molecule from UDP-N-acetylglucosamine or from sugar nucleotides which are formed from UDP-N-acetylglucosamine.

Glucosamine synthetase has previously been measured in experimental animals but there is no published work on its activity in human tissue. The present thesis describes a method for the measurement of glucosamine synthetase in the human gastrointestinal mucosa. The thesis describes the steps which were necessary to make the method applicable for use with small biopsy specimens. It then deals with the application of this method to various diseases of the gastrointestinal tract, in particular, to disorders in which it has been suggested that there may be abnormalities of mucus production.

The enzyme is assayed by incubating an homogenate of the tissue at 37° with fructose-6-phosphate and glutamine and measuring the synthesized glucosamine colorimetrically by the Morgan-Elson reaction. Rectal biopsies, taken through a sigmoidoscope, weigh between 5 and 30 mg, and gastric biopsies taken through a gastroscope are even smaller. The assay was miniaturized so that only 4 mg tissue were required. This was done by scaling down volumes

from methods described in the literature and by adjusting the pH and the substrate concentrations to give the maximum yield of glucosamine. These studies were performed with homogenates of the dissected mucosa from the histologically normal cut ends of colectomy specimens for carcinoma. It was found that the optimum concentration of fructose-6-phosphate was 20 mM, that the optimum glutamine concentration was 8 mM, and that the optimum pH was 7.0. The incubation time selected was 3 hours, and gentamicin 100 μg/ml was added to inhibit bacterial contamination. The requirement for 1 mM EDTA was confirmed.

The reproducibility of this microassay was demonstrated by a standard deviation of 10-15% in multiple readings of the same tissue homogenate. The units of enzyme activity adopted were μmoles glucosamine synthesized per hour per g wet weight, which was shown to correlate closely with enzyme activity per g tissue protein ($r = 0.90$).

The requirement for only 4 mg tissue meant that only half of a rectal biopsy needed to be used; the other half could be used for diagnostic histology. When mucosa from surgically resected specimens was assayed, larger volumes were used in the assay, which then required a minimum of 10 mg tissue, but the smaller volumes used with 4 mg tissue in fact gave rise to little additional error.

The studies in the development of the microassay showed relationships to pH and substrate concentrations that differed from those of glucosamine synthetase in the rat liver, suggesting that the enzyme in the human colonic mucosa was not the same molecule (Appendix B).

A normal range for glucosamine synthetase levels in the human colonic mucosa was obtained from the rectal biopsies of 20 patients with the irritable colon syndrome and from the normal cut ends of colectomy specimens of 22 patients with carcinoma of the colon and 3 patients with localized diverticulitis. The range was 13.77 ± 3.97 (S.D.) units. The levels were similar in all regions of the large intestine and there were no differences with regard to age and sex. There was no glucosamine synthetase activity detectable in the submucosa.

In the irritable colon syndrome, five patients who complained of excessive mucus in their stools had normal glucosamine synthetase levels.

Mucosal glucosamine synthetase levels were lower than normal in panproctocolectomy specimens for ulcerative colitis and Crohn's disease. There was a close correlation between the glucosamine synthetase level and the overall epithelial cell density; the levels were diminished when there was a general depletion of the epithelial cells but not when there was just distortion of the epithelial glands or damage to the surface layer. Provided the overall epithelial cell density was normal, the glucosamine synthetase levels were normal even when the goblet cells themselves were diminished. Severely inflamed mucosa had a slight glucosamine synthetase activity even when the epithelial cells were completely absent, probably due to enzyme activity in the inflammatory cells.

In 15 patients in an acute attack of ulcerative colitis who recovered from the attack, the glucosamine synthetase levels in serial rectal biopsies rose to a peak of 23.94 ± 4.12 units and then fell to normal. Five other patients who did not recover from their acute attack did not shown such a peak.

In the morphologically normal rectal mucosa of 13 patients with Crohn's disease elsewhere in the bowel, the glucosamine synthetase levels were 20.55 ± 5.20 units, a highly significant elevation above the normal ($P < 0.001$). This suggests that the entire colonic mucosa is abnormal in Crohn's disease, even though the obvious lesions are focal.

In 3 patients with membranous colitis, the glucosamine synthetase levels rose to 36.0, 31.7 and 27.0 units respectively, which were extremely high values.

The enzyme was studied in the tumour tissue of 10 cases of carcinoma of the colon. The range of levels was very wide (1.8-29.9 units) but there was no correlation with any histological feature. In 4 of the patients, the glucosamine synthetase levels were also measured in the histologically normal mucosa immediately adjacent to the tumour; these levels were normal.

By pooling two adjacent biopsies in each assay, it was possible to measure the enzyme in biopsies taken through a panendoscope from various sites in the stomach and from the duodenal cap. This was done in 7 patients with normal endoscopic appearances or with lesions confined to the oesophagus and in 24 patients with various disorders of the stomach and duodenum. The glucosamine synthetase level in normal gastric mucosa was found to be approximately 10.0 ± 3.0 units in all parts of the stomach. No significant differences were

found between patients with a gastric ulcer and patients with a normal stomach. In the duodenal cap, the glucosamine synthetase levels were 14.07 ± 3.11 units in 14 patients with a normal duodenum and 18.77 ± 2.32 units in 7 patients with an active duodenal ulcer, a highly significant difference ($P < 0.002$). There was no apparent relationship between the glucosamine synthetase level in the stomach or duodenum and the ABO blood group or the secretor status.

The enzyme was also measured in portions of jejunal biopsies obtained with the Crosby capsule. In 14 patients with a normal jejunum, the levels were 17.37 ± 6.60 units. In 22 biopsies from patients with coeliac disease the levels had a lower mean of 12.05 units but there was a large overlap with the normal range.

The glucosamine synthetase microassay was shown to be feasible with percutaneous liver biopsies, giving levels of the order of 3-10 units (Appendix C).

An attempt was made to miniaturize an assay for another key enzyme in mucus synthesis, glucosamine-6-phosphate N-acetylase, but this was unsuccessful (Appendix D).

The in vitro inhibition of glucosamine synthetase in gastric mucosal homogenates by sodium salicylate and phenylbutazone has been reported by Perrey (1968) and adduced as a possible mechanism for the ulcerogenic effect of these drugs. The experiments of Perrey were repeated in gastric and colonic mucosal homogenates for the ulcerogenic drugs acetylsalicylic acid, sodium salicylate and hydrocortisone hemisuccinate and inhibition of glucosamine synthetase was confirmed. However, a similar degree of inhibition was also shown by paracetamol and by gentamicin, which are non-ulcerogenic drugs. This indicates that experiments to demonstrate the mechanism of drug-induced peptic ulceration should include comparisons with non-ulcerogenic drugs.