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Dextran Prodrugs of Glucocorticoids for Colon-Specific Drug Delivery

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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To Rangi 🐾
and Anna 😊

Thank you for your laughter and smiles,
you make life such a joy!

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Dextran Prodrugs of Glucocorticoids for Colon-Specific Drug Delivery

Andrew Douglas McLeod

Colitis is currently treated with antiinflammatory glucocorticoids by oral, rectal and intravenous routes of administration. Administration of glucocorticoids by the oral and intravenous routes often leads to adrenosuppression. Colon-specific drug-delivery after oral administration of prodrugs could theoretically increase drug concentrations along the colon relative to systemic drug concentrations, thereby increasing efficacy and decreasing side-effects.

The overall goal of this dissertation research was to synthesize and assess the properties of glucocorticoid-dextran-conjugates as prodrugs for the treatment of colitis. Methylprednisolone and dexamethasone were covalently attached to dextran (molecular weight = 72,600) via succinate and glutarate linker molecules. The dextran conjugates, incubated in a pH 6.8 buffer at 37°C, released glucocorticoid and glucocorticoid-hemiester with half-lives of 75 to 103 hours.

Less than 3 % of the bound-drug was released from the dextran-conjugates during a 160-minute incubation with contents of the small intestine, the site of highest esterase activity. Glucocorticoid was released at a faster rate in cecal and colonic contents than in the upper gastrointestinal tract, presumably by a combination of dextranases and esterases.

The dextran-conjugates and glucocorticoids were administered by infusion to rats. Cecal and colonic tissue concentrations of dexamethasone at steady-state were approximately threefold higher after intragastric administration of dexamethasone-succinate-dextran compared to intragastric dexamethasone. Thus, the dose could theoretically be reduced threefold while retaining the same efficacy. In addition, steady-

state blood concentrations were threefold lower, indicating a potential for decreased systemic toxicity.

The dextran-conjugates were four to ten times more potent than the glucocorticoids in returning colonic fluid absorption to normal in a rat model of colitis induced by 4 % acetic acid. The dextran conjugates caused only mild adrenosuppression in contrast to unconjugated dexamethasone and methylprednisolone.

In summary, the dextran-conjugates were chemically stable, resisted enzymatic-hydrolysis in the upper gastrointestinal tract and released glucocorticoid in the large intestine. The compounds were effective in healing experimental colitis with only minor adrenosuppression.

Date: 11 December, 1992

Thomas N. Tozer
Chairman of Dissertation Committee

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Significance, Objectives and Organization of Dissertation

Significance

Colitis is a chronic disease currently treated with both steroidal and nonsteroidal antiinflammatory drugs by oral, rectal and intravenous routes of administration (Hanauer and Kirsner, 1988). Because of systemic side-effects, administration of glucocorticoids by the oral and intravenous routes is generally reserved for treatment of acute flare-ups (Hanauer & Kirsner, 1988). Administration of steroids by the rectal route is limited because drug is largely confined to the distal region of the colon (Truelove, 1958). Colon-specific drug-delivery after oral administration of prodrugs could theoretically increase drug concentrations along the colon relative to systemic drug concentrations, thereby increasing drug efficacy and decreasing side-effects (McLeod and Tozer, 1992). The ideal colon-specific prodrug is both stable and unabsorbed from the upper gastrointestinal (GI) tract (McLeod et al., 1992). The drug, however, must be completely released and absorbed in the colon (McLeod et al., 1992). Dextran conjugates were recently reported to possess these ideal characteristics (Harboe et al., 1989a; Harboe et al., 1989b; Larsen et al., 1989a) Drug was completely released from the dextran conjugates, presumably by a combination of dextranases and esterases (Larsen et al., 1989a) and absorbed in the colon (Harboe et al., 1989a). The overall goal of this dissertation research was to synthesize and assess the properties of glucocorticoid-dextran-conjugates as possible agents for the treatment of colitis.

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Objectives

The overall goal was divided into six objectives. These were to:

• Synthesize dexamethasone-hemisuccinate and dexamethasone-hemiglutarate. These derivatives, along with methylprednisolone-hemisuccinate were attached to dextran via the diacid linker molecules succinate and glutarate.

• Characterize the hydrolysis kinetics of the hemiesters and dextran conjugates as a function of pH. Incubations at pH 6.8, 37°C were used to estimate the chemical hydrolysis in vivo.

• Measure enzymatic hydrolysis of the glucocorticoid-hemiesters and dextran-conjugates in luminal contents of the rat gastrointestinal tract. The source and type of esterase responsible for the hydrolysis of the hemiesters in the rat large intestine were determined.

• Calculate the permeabilities of the glucocorticoids and glucocorticoid-hemiesters through Caco-2 cell monolayers, an accepted model for the colonic mucosa. These permeability measurements were used to estimate colonic drug absorption in vivo.

• Measure the steady-state pharmacokinetics of the glucocorticoids and dextran-conjugates in rats after subcutaneous and intragastric infusions. Implantable osmotic pumps were used to administer drug solutions to ambulatory rats over a period of 22 hours.

• Assess the efficacy of the glucocorticoids and dextran-conjugates in treating experimental ulcerative colitis in the rat. Gross anatomic, histologic, physiologic and toxicologic assays were used to measure the efficacy and toxicity of the compounds.

Organization

The dissertation begins with a brief description of the anatomy of the gastrointestinal tract. Next, the epidemiology, etiology, pathology, symptoms and treatment of colitis are discussed. Chapter 1 concludes with the history, pharmacology and pharmacokinetics of antiinflammatory glucocorticoids.

Chapter 2 summarizes salient features of the motility of the stomach, small and large intestine with reference to their effects on colon-specific drug delivery. The chapter then defines the drug delivery index (DDI), a means of assessing both efficacy and toxicity of drug released from a delivery system.

The third chapter gives specific examples of colon-specific drug delivery systems, with particular attention to dextran prodrugs. The chapter also describes dextranases and esterases, the enzymes responsible for colon-specific drug release from dextran-prodrugs.

Synthetic methods are outlined in Chapter 4. In addition, this chapter describes the chemical hydrolysis of the glucocorticoid-hemiesters and dextran-conjugates. A model was proposed which includes both ester hydrolysis and acyl migration reactions.

In the fifth chapter, enzyme-mediated hydrolysis of the glucocorticoid-hemiesters and dextran-conjugates in luminal contents of the rat gastrointestinal tract is discussed. The model parameters were fitted to the dextran-conjugate hydrolysis data.

Incubations in vitro indicated that hydrolysis of the dextran-conjugates yields mainly glucocorticoid-hemiesters in the large intestine. Chapter 6 describes the use of intestinal cell-cultures to predict whether the glucocorticoid-hemiesters are well-absorbed from the colon in vivo.

The results of studies in which glucocorticoids and dexamethasone-dextran-conjugates were administered by constant rate infusion to rats are presented in Chapter 7. These results were used to calculate the DDI and bioavailability of dexamethasone from the dextran-conjugates.

The glucocorticoids and dextran-conjugates were tested in a rat model of ulcerative colitis. A number of measures of efficacy and toxicity were used and the results are shown in Chapter 8.

The conclusion section contains a summary of the overall results and a list of topics for future research in this area.

Chapter 1

Colitis and its Treatment

Inflammatory diseases of the colon, colitis, include ulcerative colitis and Crohn's disease. This chapter reviews the condition and methods of its treatment. To gain insight into the nature of the condition and the specific delivery of drugs to the colon, the chapter begins with a brief overview of gastrointestinal anatomy.

Anatomy of the Gastrointestinal Tract

The gastrointestinal (GI) tract is divided into three major organs: the stomach, small intestine and large intestine, Figure 1-1 (Haeberlin and Friend, 1992). The small intestine is further differentiated into the duodenum, jejunum and ileum. Often the jejunum and ileum are approximated by dividing the small intestine into two segments of equal length, the proximal (PSI) and distal (DSI) small intestine. The large intestine is also divided into sections, called the appendix, cecum; ascending (or proximal), traverse, descending and sigmoid colon; and rectum. In rodent species the cecum is disproportionately larger than in humans, Figure 1-2 (Snipes, 1981). Major functions of the stomach include the storage of ingested food, secretion of acid and digestive enzyme and reduction of the food particle size (Haeberlin et al., 1992). The small intestine is primarily involved with the absorption of amino acids and sugars produced as a result of the breakdown of food by digestive enzymes (Haeberlin et al., 1992). The large intestine has three major functions: to reabsorb water and electrolytes and to store fecal matter prior to defecation. The intestinal wall comprises a number of layers, however, the mucosa, submucosa and serosa are the major ones, Figure 1-3. The mucosa contains columnar epithelial cells which have microvilli on the luminal surface. The mucosal surface is folded into villi (crypts in the colon), and further into folds called plicae (haustra in the colon) (Mrsny, 1992).
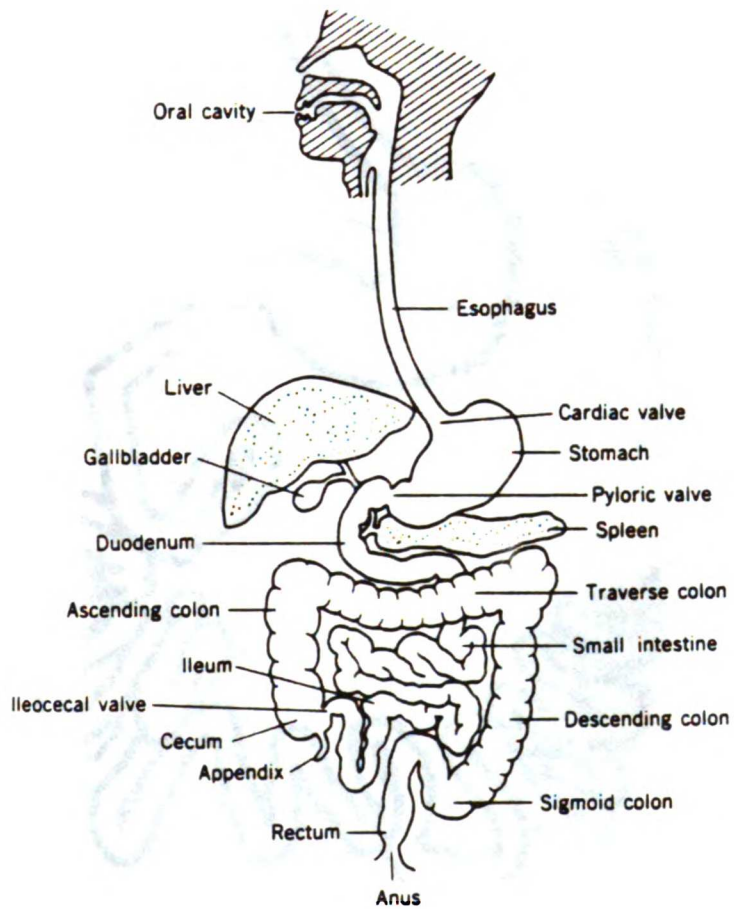
Fig. 1-1   Schematic drawing of the human gastrointestinal tract.

From Haeberlin and Friend, (1992).

esophagus

cardia of stomach

fundus

lesser curvature

greater curvature

pylorus

cecum

duodenum

vermiform appendix

ascending colon

ileum

descending colon

jejunum

rectum

anus
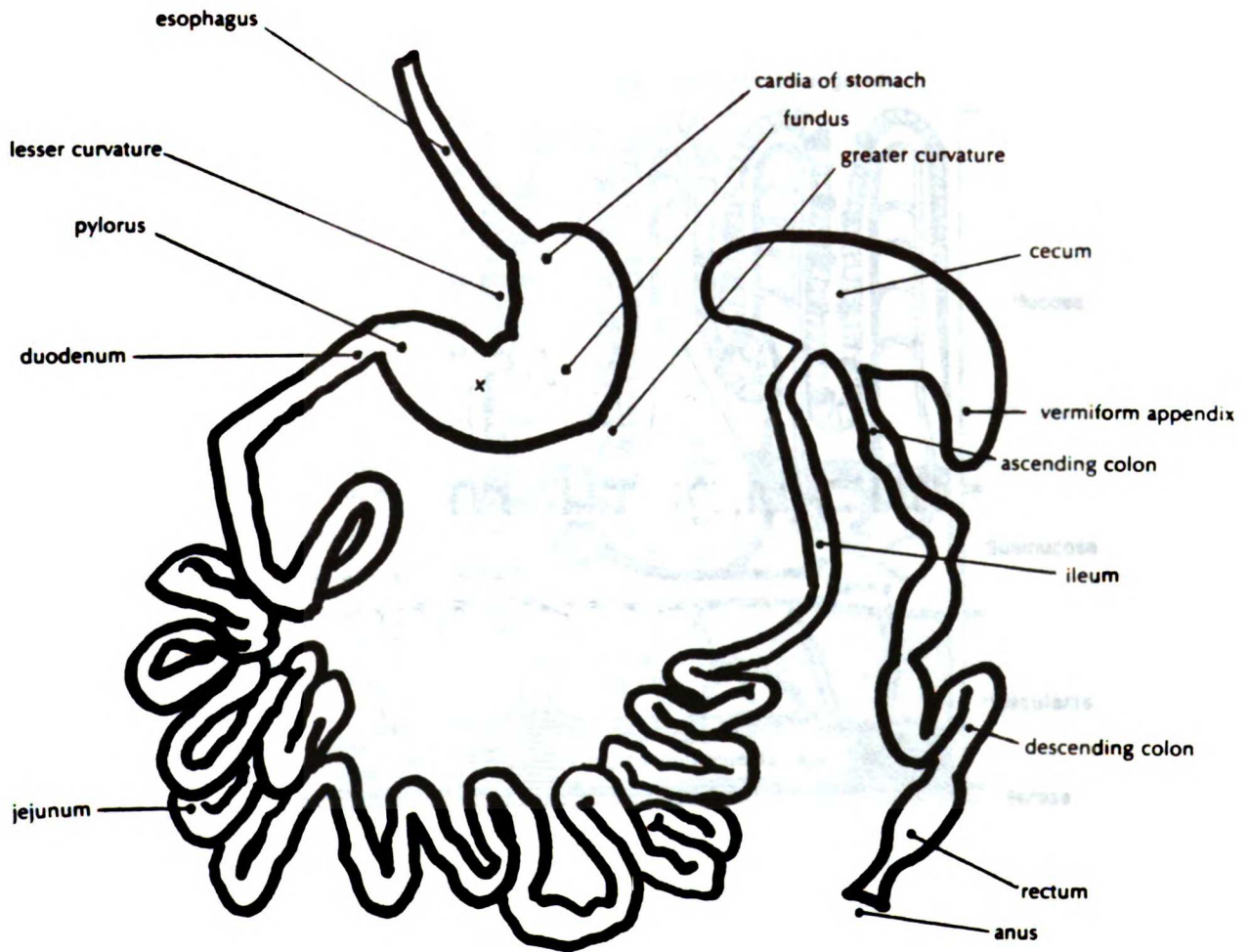
Fig. 1-2   Schematic drawing of the rat gastrointestinal tract. The cannula used for intragastric infusions described in Chapter 7 was placed into the stomach at the point marked with an 'x'. Figure adapted from Wingerd, (1988).
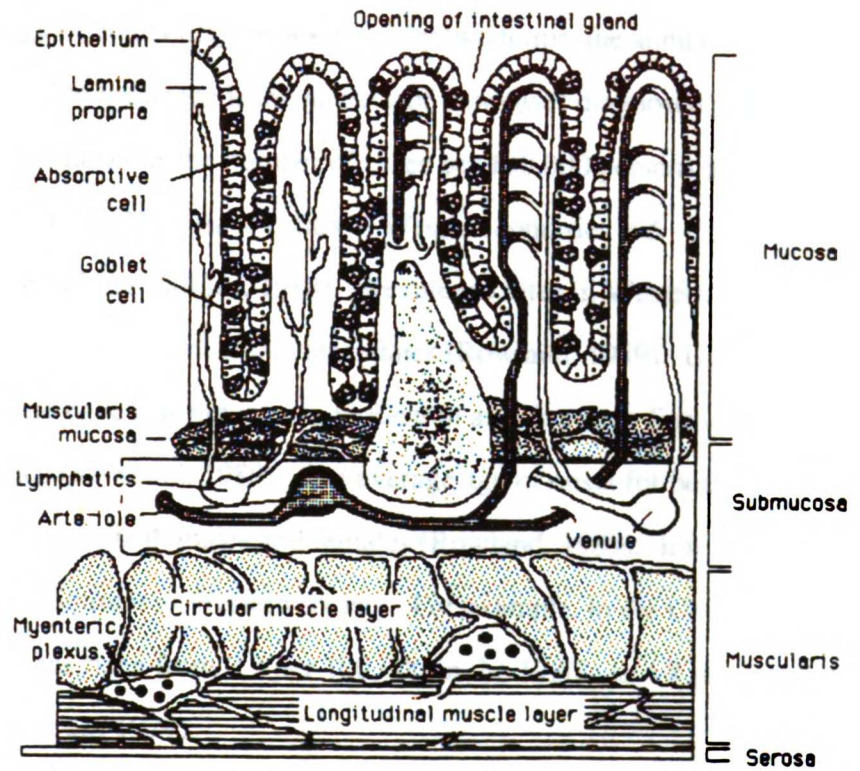
Fig. 1-3   Cross-section of the colon demonstrating associations

between mucosal, submucosal, muscularis and serosal components.

From Mrsny, (1992).

Colitis

Epidemiology

The epidemiology of colitis may be described by two terms, the annual incidence rate and the prevalence. The incidence is the number of new cases diagnosed per 100,000 persons per year, and prevalence is the number of cases per 100,000 persons at a given time. The total incidence of both forms of colitis in Western countries is 5 - 10 cases per 100,000 persons (Mendeloff and Calkins, 1988). Prevalence rates in Western societies are generally 12 times higher than the annual incidence rates (Rowland, 1989). Ulcerative colitis and Crohn's disease have low prevalence in Eastern and Southern Europe, Asia, South America and Africa (Rowland, 1989). The average age of onset for both diseases is approximately 15 - 20 years in both males and females (Rowland, 1989). It is believed that colitis is becoming more common in Western society. For example, in the United Kingdom, the incidence has risen from 0.2 per 100,000 in the 1930's to 4.8 per 100,000 in the 1970's (Mayberry et al., 1979).

Etiology

The cause of chronic colitis is not known. A number of mechanisms have been proposed, e.g., immunlogic deficiency, psychological factors and viral and bacterial infections. Today most work is focussed on an immunologic mechanism (Kraft, 1979). Colonic biopsies of some patients show increased levels of eosinophils in the mucosa. Their presence implies that hypersensitivity reactions take part in the local inflammation (Kraft, 1979). There is evidence of increased activation of macrophages as well as increased concentrations of B-cells in the inflamed colon (Kraft, 1979). Arachidonic acid metabolism via the cyclooxygenase and lipoxygenase pathways is increased in colitis (Donowitz, 1985). Glucocorticoids prevent the production of free arachidonic acid from

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membrane phospholipids (Donowitz, 1985). Thus, glucocorticoids block the production of prostaglandins, thromboxanes and prostacyclin. Sulfasalazine and 5-aminosalicylic acid also inhibit both pathways. Cyclooxygenase inhibitors, e.g., indomethacin, are not effective in reducing colonic inflammation (Donowitz, 1985).

Pathology

Ulcerative colitis is always present in the rectum and may spread proximally to involve the colon (Rowland, 1989). Crohn's disease is often discontinuous with the inflamed areas separated by healthy tissue (Rowland, 1989). Histologic changes are usually confined to the mucosa although the submucosa may be involved to a lesser degree. In active colitis, blood vessels are congested and the mucosa is hemorrhaged and edematous. Goblet cells are reduced in number and may disappear altogether (Rowland, 1989). The lamina propria contains large numbers of lymphocytes, plasma cells and eosinophils. Crypt abscesses contain large numbers of neutrophils (Rowland, 1989). Table 1-1 lists the macroscopic and Table 1-2 lists the microscopic features of the two forms of colitis.

Symptoms

Onset may be insidious or sudden. With the former, frequency and intensity of rectal bleeding and bowel movements gradually increases; with sudden onset, symptoms may include fever, uncontrollable bloody diarrhea and abdominal pain (Rowland, 1989). Most patients have alternating exacerbations and remissions, whilst in a small proportion of patients the disease becomes chronic and continuous (Rowland, 1989). Nefzger and Acheson (Nefzger and Acheson, 1963) studied a large group of patients and found that 79 % had diarrhea, 71 % abdominal pain and 55 % rectal bleeding. There is an increased risk of colon cancer in patients with ulcerative colitis (Nefzger et al., 1963). Most cancer cases occur 10 - 19 years after the initial diagnosis of colitis (Hinton, 1966).

Table 1-1

Macroscopic Pathology of the Large Intestine in Ulcerative Colitis and Crohn's Disease[a]

Ulcerative Colitis	Crohn's Disease
Disease in continuity	Disease discontinuous
Rectum always involved	Rectum involved in 50%
Terminal ileum involved in 10%	Terminal ileum involved in 30%
Mucosa granular and ulcerated but not fissured	Mucosa discretely ulcerated, cobblestoned and fissured
Vascular	Not vascular
Serosa normal	Serosa inflamed
Colon shortened because of muscle changes, fibrous strictures rare	Colon shortened because of fibrosis, fibrous strictures common
No fistulas	Fistulas in 10%
Inflammatory polyposis common	Inflammatory polyposis uncommon
Anal lesions in 25%; fissure, excoriation and occasional fistula	Anal lesions in 75%; fistulas, fissure and ulceration severe

[a] Table from Morson and Dawson (1979).

Table 1-2

Microscopic Pathology of the Large Intestine in Ulcerative Colitis and Crohn's Disease[a]

Ulcerative Colitis	Crohn's Disease
Mucosal and submucosal inflammation only	Transmural inflammation
Width of submucosa normal or reduced	Width of submucosa normal or increased
Increased vascularity	Marked edema
Focal lymphoid hyperplasia limited to mucosa and superficial submucosa	Focal lymphoid hyperplasia in all layers of bowel wall
Crypt abscesses common	Crypt abscesses infrequent
Loss of goblet cells	Normal populations of goblet cells
Paneth cell metaplasia common	Paneth cell metaplasia rare
Granulomas absent	Granulomas in bowel and nodes in 70%
Precancerous changes seen	Precancerous changes not seen
Non-specific inflammation of anal lesions	Sarcoid foci of anal lesions

[a] Table from Morson and Dawson (1979).

Treatment

Antiinflammatory drugs are the main treatment for ulcerative colitis and Crohn's disease (Hanauer and Kirsner, 1988). These drugs include glucocorticoids, sulfasalazine, olsalazine and 5-aminosalicylic acid (Hanauer et al., 1988). Glucocorticoids are frequently used by both local and systemic routes (Haynes, 1990). The major disadvantage with chronic administration of systemic glucocorticoids is the development of severe side-effects (Haynes, 1990).

Glucocorticoids

History

The significance of the adrenal glands was recognized by Addison in the mid-19th Century (Addison, 1855). In pioneering experiments Brown-Séquard found that the adrenal glands were essential to maintain life (Brown-Séquard, 1856). By the 1930's the cortex, rather than the medulla, was widely accepted as the crucial section of the adrenal gland for this essential role (Haynes, 1990). Before long the glucose and mineral regulating effects of adrenocortical extracts were recognized. Thus, adrenocortical hormones were classified into two groups; the glucocorticoids, which regulate carbohydrate metabolism, and the mineralocorticoids, which regulate electrolyte balance. Although Addison first described the syndrome of hypocorticism, now known as Addison's disease in the 1850's, it wasn't until 1932 when Cushing described the syndrome of hypercorticism (Cushing, 1932). These early case reports by Cushing laid the foundation for today's knowledge of the effect of glucocorticoid overdosage and the resulting side-effects. In 1942, organic chemists managed to isolate, crystallize and determine the structures of 28 adrenal steroids (Reichstein and Shoppee, 1943). The structure and stereochemistry of hydrocortisone are shown in Figure 1-4. Cortisone was isolated in the 1950's and heralded a wave of basic research into the pharmacology of the

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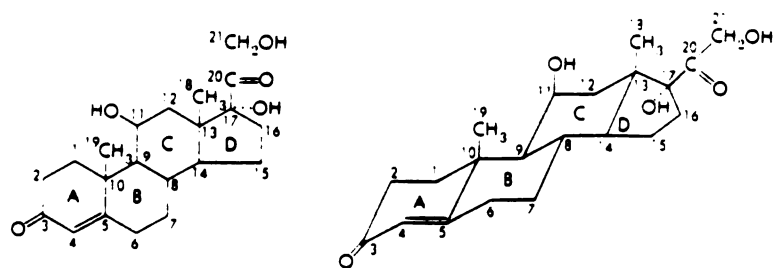
Fig. 1-4   Structure of glucocorticoids as typified by hydrocortisone.

From Haynes, (1990).

Table 1-3
Relative Potencies and Equivalent Doses of Glucocorticoids[a]

Compound	Relative Anti-inflammatory Potency	Relative Sodium Retaining Potency	Duration of Action[b]	Approximate Equivalent Dose (mg)[c]
Hydrocortisone	1	1	S	20
Prednisone	4	0.8	I	5
Prednisolone	4	0.8	I	5
Methylprednisolone	5	0.5	I	4
Fludrocortisone	10	125	S	-
11-Desoxycortisone	0	0	-	-
Cortisone	0.8	0.8	S	25
Corticosterone	0.35	15	S	-
Triamcinolone	5	0	I	4
Paramethasone	10	0	L	2
Betamethasone	25	0	L	0.75
Dexamethasone	25	0	L	0.75

[a] From (Haynes et al., 1980).
[b] S = Short or 8- to 12-hour biologic half-life; I = intermediate or 12- to 36-hour half-life; L = long or 36- to 72-hour biologic half life. (Rose and Saccar, 1978).
[c] These dose relationships apply only to oral or intravenous administration; relative potencies may differ greatly when injected intramuscularly or into joint spaces.

glucocorticoids (Haynes and Murad, 1980). Many of today's glucocorticoids were first synthesized during this period along with the development of glucocorticoid structure-activity relationships (Haynes et al., 1980). Most synthetic glucocorticoids were made with the hope of reducing toxicity. This ideal, however, has yet to be realized.

Pharmacology

The pharmacologic effects of glucocorticoids are numerous. These include alterations in the distribution of carbohydrate, fat and protein. The immune, cardiovascular and nervous systems are also affected by glucocorticoids (Haynes, 1990).

At a molecular level, glucocorticoid reacts with receptor proteins present in the cytosol of sensitive cells to form a steroid-receptor complex (Pratt, 1987). This complex then undergoes a conformational change and passes into the nucleus where it uses zinc fingers to binds to DNA (Luisi et al., 1991). Next the complex affects the synthesis of mRNA for proteins which perform numerous cellular functions (Hollenberg et al., 1987). While glucocorticoids have anabolic effects in certain tissues, e.g., liver, other tissues such as lymphoid cells, undergo catabolism. Table 1-3 compares the antiinflammatory and mineralocorticoid properties of both natural and synthetic glucocorticoids.

Immunosuppression

Addison's disease increases mass of the lymphoid tissues, whereas Cushing's syndrome results in lymphocytopenia and decreased lymphoid tissue mass (Haynes, 1990). Within 1 - 3 hours of administering glucocorticoids to rodents there is a marked effect on lymphocytes. The nuclei become pyknotic and disintegrate which results in cell death. After discontinuing glucocorticoid administration the cells begin to be replaced from the bone marrow and eventually levels return to normal (Haynes et al., 1980).

Glucocorticoids also cause immunosuppression by blocking the immune process at a number of key steps, Figure 1-5 (Dinarello and Mier, 1987). Macrophage cells
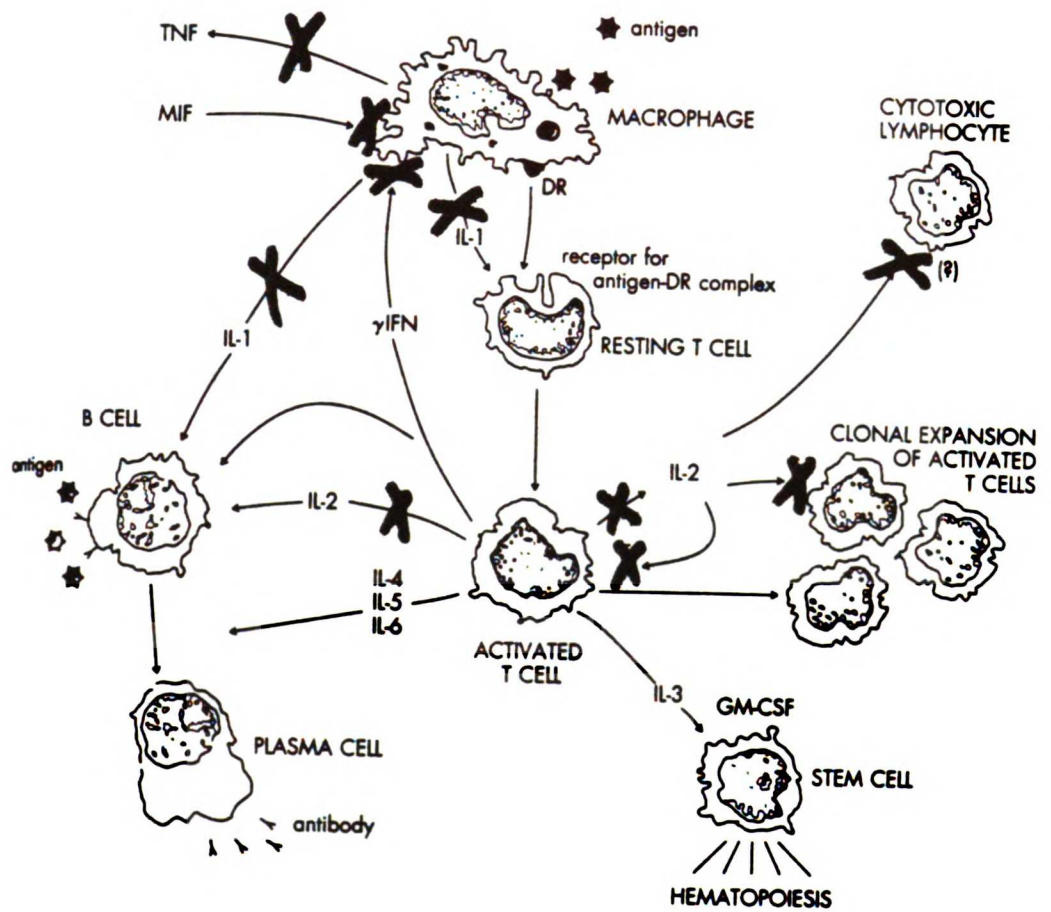
Fig. 1-5   Sites of action of glucocorticoids in the immune response during

antigenic challenge and inflammation.

X = inhibitory effects of glucocorticoids.

Abbreviations: TNF, tumor necrosis factor; MIF, macrophage migration-

inhibitory factor; IL, interleukin; γIFN, gamma interferon;

GM-CSF, granulocyte-macrophage colony-stimulating factor;

From Haynes, (1990), adapted from Dinarello and Mier, (1987).

phagocytose antigen and subsequently present the processed antigen to T-cells. Thus, macrophages have an important role at the beginning of the immune response. Macrophages remain at the site of inflammation when they are exposed to macrophage migration-inhibitory factor (MIF). Glucocorticoids block the action of MIF on macrophages, thereby promoting the movement of macrophages out of the inflamed area (Cupps and Fauci, 1982). Activated T-cells use gamma interferon (γIFN) to facilitate the processing and display of antigen by macrophages. Glucocorticoids block the action of γIFN on macrophages, thus inhibiting this pathway (Mokoena and Gordon, 1985). Interleukin-1 (IL-1) is produced by macrophages and causes the activation of resting T-cells. Once activated, T-cells release a variety of lymphokines, e.g., IL-2, IL-3, IL-4, IL-5, IL-6, γIFN, granulocyte-macrophage colony-stimulating factor (GM-CSF), Figure 1-5. Glucocorticoids block the release of IL-1 from macrophages, consequently T-cells are not activated and the cascade of cytokine release does not take place (Lew et al., 1988). Although the glucocorticoids interact at many (and currently not well understood) sites, the end result is simple: glucocorticoids substantially decrease immunoresponsiveness.

Antiinflammatory Effect

Hydrocortisone and synthetic analogs prevent or reduce the development of local heat, redness, swelling and pain of inflammation (Haynes, 1990). The glucocorticoids inhibit both early phenomena (edema, capillary dilatation, fibrin deposition, leukocyte migration and phagocytosis), and later processes (fibroblast and capillary proliferation and deposition of collagen) (Haynes, 1990). Thus, glucocorticoids have potential utility for the treatment of both acute and chronic inflammation (Swartz and Dluhy, 1979). Antiinflammatory activity occurs locally, because those glucocorticoids that do not require metabolic activation are very effective after topical application to the skin, nasal mucosa, lung and eye (Haynes, 1990).

Pharmacokinetics

Absorption and Distribution

Water-soluble esters of glucocorticoids have been prepared for parenteral administration when rapid effect is desired (Melby and Silber, 1961). Examples include sodium salts of phosphate and hemisuccinate esters (Anderson et al., 1985). Prolonged action is obtained through the use of lipophilic esters, e.g., acetate. These esters are often used as depot injections into muscle and joints (Fariss et al., 1978). In plasma > 90 % of hydrocortisone is reversibly bound to plasma proteins (Swartz et al., 1979). The glycoprotein, transcortin, binds endogenous glucocorticoids with high affinity but low capacity (Haynes, 1990). Albumin binds glucocorticoids with low affinity, however it has a high binding capacity (Haynes, 1990). Binding to transcortin is easily saturated and accounts for the non-linear plasma protein binding of many glucocorticoids, e.g., cortisone, hydrocortisone, prednisone and prednisolone (Haughey and Jusko, 1992). Some glucocorticoids do not appreciably bind to transcortin, e.g., dexamethasone, therefore exhibit linear plasma protein binding to albumin in the pharmacologic range of concentrations (Rohdewald et al., 1987).

Metabolism

All glucocorticoids have a double bond at the 4,5 position and a ketone group at C3, Figure 1-4. Loss of the 4,5-double bond by reduction leads to an inactive metabolite (Haynes, 1990). Reduction of the 3-ketone group to a 3-hydroxyl is a common phase-I metabolic pathway (Haynes, 1990). Most of the 3-hydroxyl metabolites are conjugated with sulfuric or glucuronic acid to form water soluble phase-II metabolites (Haynes, 1990).

Toxicity

Ulcerative colitis and Crohn's disease are chronic diseases that are often treated with antiinflammatory drugs for decades. Long-term systemic administration of glucocorticoids in colitis patients has an increased risk of side-effects. Two categories of

toxicity are recognized. The first is caused by the continued use of high doses of glucocorticoids and the second from too rapid withdrawal of glucocorticoid therapy.

Chronic overdosing with glucocorticoids causes dramatic changes, known as Cushing's syndrome, in the physical appearance of the patient. Redistribution of fat deposits to the face and back give the features called 'moon face' and 'buffalo hump' (Haynes, 1990). In addition glucocorticoids cause osteoporosis by increasing bone resorption and decreasing calcium absorption from the GI tract. In some patients the vertebrae and ribs become weakened to the point of fracture (Haynes, 1990). Glucocorticoid-induced psychoses are a considerable problem and may develop into suicidal tendencies (Haynes, 1990). Other systemic-side effects include fluid and electrolyte disturbances, hypertension, hyperglycemia, increased susceptibility to infections, myopathy and peptic ulcers.

Chronic overdosing causes the body to stop producing endogenous glucocorticoid. This condition is known as adrenosuppression (Haynes, 1990) and is a source of toxicity when therapy is discontinued. Too-rapid withdrawl of exogenous glucocorticoid may result in a withdrawl syndrome having symptoms of fever, malaise, and soreness in the muscles and joints. Adrenal and pituitary functions may take 9 months to recover to normal and the patients may require exogenous glucocorticoids during stressful situations for 1 to 2 years thereafter (Graber et al., 1965).

Potential Advantage of Colon-Specific Delivery of Glucocorticoids

Topical administration of glucocorticoids inhibits both inflammatory and immunologic processes, however, systemic administration leads to severe side-effects. Colon-specific delivery of glucocorticoids may lower the dose needed to treat colitis, the lower dose may reduce systemic side-effects.

Chapter 2

Kinetic Perspectives in Colon-Specific Drug Delivery

This chapter describes kinetic aspects of the GI tract. It begins with a discussion of gastric, small intestinal and colonic motility. The factors that influence motility, namely, food, type of food ingested and pharmacologic agents, are presented. The kinetics of colon-specific delivery are examined.

Physiologic Determinants of Delivery

Gastric Emptying

After oral administration of a dosage form, the stomach is the first organ encountered. The functions of the stomach are to;

1. Temporarily store ingested food (Barker et al., 1979).

2. Reduce particle size of contents (Meyer, 1987).

3. Gradually release contents into the small intestine.

The kinetics of these processes are now examined as they relate to colon-specific delivery after oral administration.

The stomach is a variable-volume container. The proximal stomach relaxes to accommodate successively more food as a meal is consumed. The intraluminal pressure remains essentially constant, even though the volume may increase considerably (Meyer, 1987). The proximal stomach has been reported to be not involved in the mixing process in that the center of the contents in this part of the stomach retains neutral pH for a considerable period (Meyer, 1987). Figure 2-1, taken from an early radiographic study, demonstrates that stratification can occur in the stomach. In another study, the proximal
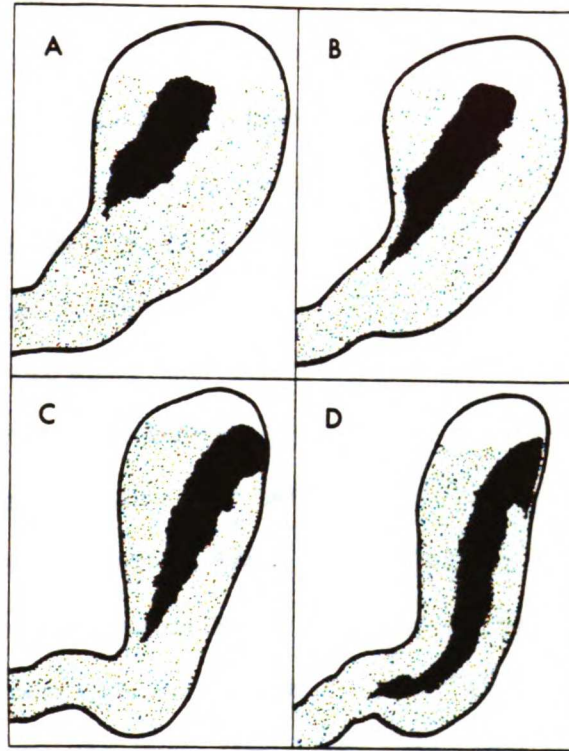
Fig. 2-1   Tracings of x-ray photographs of the stomach of a young man. Immediately

before A, he ate 200 g of fried meatballs, then 50 g of meatballs mixed with

powdered barium sulfate, and finally another 100 g of meatballs without

contrast medium. B, 10 min. later. C, 35 min. later. D, 64 min. later. The

layers remain stratified in the body of the stomach, but mixing occurs in the

antrum. From Davenport, 1982.

contents were observed to become well mixed using radiolabeled polyethylene glycol as a nonabsorbable marker (Malagelada et al., 1976). A solid dosage form taken after a meal is emptied last and consequently is delayed from reaching the colon, suggesting that stratification does indeed occur.

Particle size reduction is an important function of the distal stomach (antrum), and is the result of coordinated muscular contractions. The pyloric sphincter closes at the onset of terminal antral contraction, trapping the contents in the terminal antrum. Next the antrum forcefully contracts, grinding the solids together. The antral content is unable to pass through the pylorus, and is retropelled back into the main body of the stomach. This series of movements is repeated many times, and eventually grinds the contents into smaller fragments (Kelly, 1981). It has been hypothesized that the particle size of the contents must be reduced to approximately 1 mm before the contents can pass through the pyloric sphincter (Mazzotta and Malagelada, 1981). This particle size limit is extremely important when studying large, enteric-coated dosage forms such as tablets and capsules. Enteric coated tablets do not disintegrate in the stomach and are too large to pass through the pyloric sphincter if given with food. They remain in the stomach until virtually all the food is emptied (Kelly, 1981; Hinder and Kelly, 1977). It has been hypothesized that small multi-unit enteric coated pellets are preferable to large single units, as they are more reproducibly emptied from the stomach (Bogentoft et al., 1978). The graph in Figure 2-2 follows the gastric emptying of radiolabelled pellets administered after a light meal, and follows apparent zero order kinetics.

A lag time has been used (Siegel et al., 1988) to account for the delay between meal consumption and the onset of gastric emptying.

Variability of Gastric Emptying

The stomach has the ability to selectively discharge its contents into the duodenum on the basis of particle size (Meyer et al., 1985). Liquids are emptied at a faster rate than
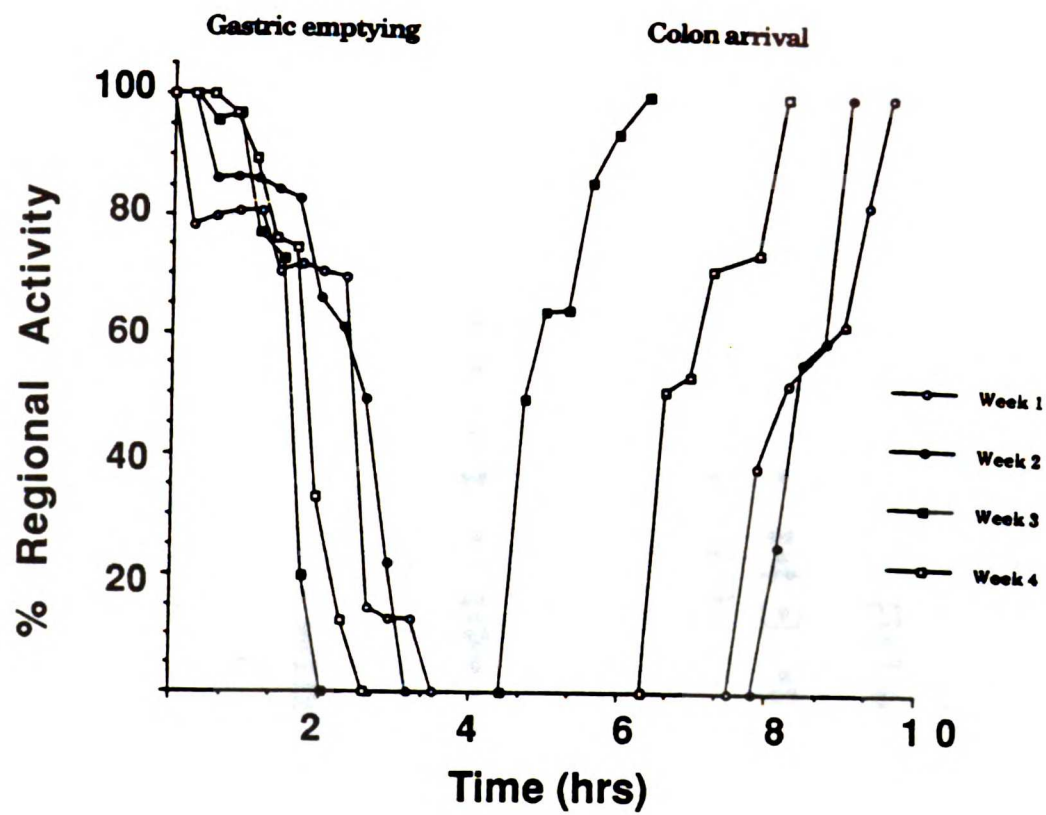
Fig. 2-2   Gastric emptying and colonic filling of ^{99m}Tc-radiolabelled pellets (diameter 0.8 - 1.1 mm) in the same volunteer measured weekly. The pellets empty from the stomach, after an initial lag phase, with apparent zero-order kinetics. From Coupe et al., 1991
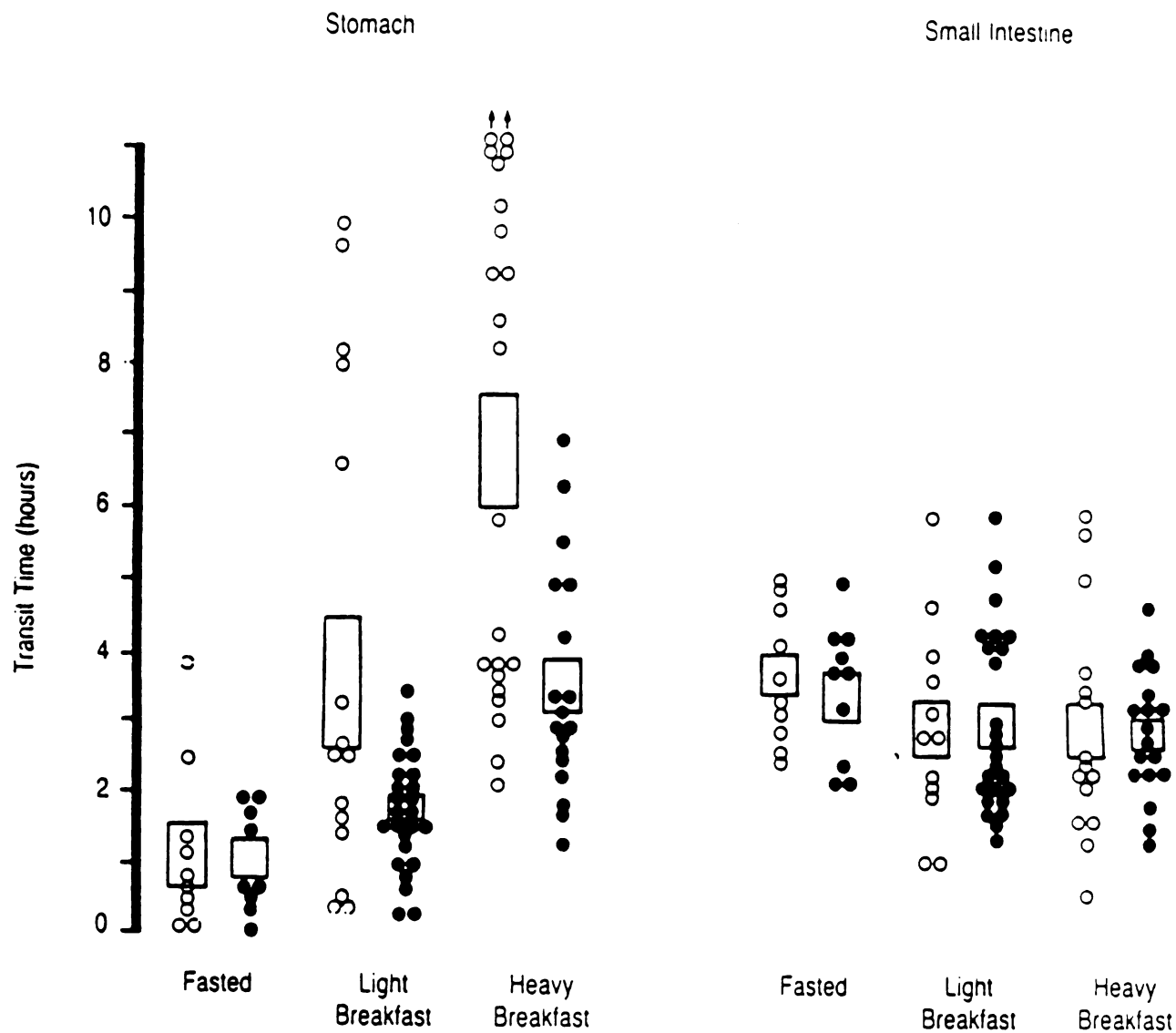
Fig. 2-3 Food, particularly a heavy meal, increases the gastric transit time of small
pellets (●) and, even more markedly, of large single units (○). In contrast,
neither food nor the physical size of the solid affects the small intestine transit
time. The data (individual points,● or ○, and their mean ± s.e., indicated by
the rectangles) were obtained in healthy young adults using drug-free
nondisintegrating materials. The points with an arrow indicate that solid was
still in the stomach at the time of the last observation, the time indicated. From
Rowland and Tozer, 1989

solids, and small particles are emptied at a faster rate than large (Hinder and Kelly, 1977). This concept, together with the effect of food, is shown in Figure 2-3.

The specific gravity of an ingested dosage form affects its gastric emptying rate, with both very heavy (specific gravity 1.85) and very light pellets (specific gravity 0.9) taking longer to be emptied than pellets of specific gravity 1.25 (Mori et al., 1989)

The position of the patient also affects gastric emptying. This was demonstrated by a floating antacid formulation that emptied from the stomach at a faster rate when patients lay on their left side (Bennett et al., 1984) The total contents of the stomach, however, emptied faster when patients lay on their right side.

Viscosity of the stomach contents is another important variable; however, it may be an indirect consequence of slowed nutrient absorption from the intestine which has a negative feedback on gastric emptying rate (Meyer et al., 1988; Reppas, 1991).

Gastric emptying rate is altered according to the caloric value of the stomach contents (Moore et al., 1981). It is well known that fatty meals empty at a much slower rate than carbohydrate meals (Kelly, 1981). The emptying rate is adjusted physiologically so that the rate of presentation of calories to the intestine tends to remain constant (McHugh and Moran, 1979). The salt content of the stomach is another important factor, as hyperosmotic liquid meals are emptied at a slower rate than iso-osmotic meals (McHugh and Moran, 1979).

The gastric emptying rate depends on the animal species studied (Ings, 1984). Although gastric emptying in man has been well studied, ethical considerations often prevent preclinical testing of controlled-release dosage forms in humans. For this reason, it is necessary to find an animal model with gastrointestinal motility similar to that of humans for preclinical testing of oral controlled-release dosage forms (Hinder and Kelly, 1977). The rat (Kaniwa et al., 1988) and the dog (Dressman, 1986) have been discussed as possibly useful models.

The intrasubject and intersubject variabilities in gastric emptying rate have been studied (Brophy et al., 1986; Petring and Flasch, 1990; Coupe et al., 1991) using various techniques. Coupe *et al*. (1991) measured gastric emptying rate in a number of subjects under standardized conditions. They reported that the variability in gastric emptying of single- and multilple-unit dosage forms was large; the intrasubject variation was less than the intersubject variation.

Gastric Emptying in the Fasting State

It was previously mentioned that the distal stomach selectively and gradually releases liquids and small-sized particles until the stomach is empty of food. Any large, enteric-coated dosage form will remain in the stomach until all the food is emptied, a period that may last more than 12 hours if the meal is large and contains large amounts of fat (Davis et al., 1984).

The stomach behaves quite differently when it is empty of nutrients. The emptying pattern during fasting follows a cycle comprised of four phases.(Sarna, 1985). The entire cycle lasts approximately 90-120 minutes. Phase I occupying 40-60% of the cycle, is the quiescent phase when the stomach undergoes no muscular contractions. Phase II, typified by muscular contractions of increasing frequency, lasts 20-30% of the cycle. Phase III, characterized by forceful contractions that sweep down the entire antrum and virtually obliterate the stomach lumen, lasts 5-10% of the cycle. Such contractions are known as *house-keeping* waves. The stomach is swept clean so that large particles or pharmaceutical dosage forms, not previously emptied, are now cleared from the stomach. The final phase, phase IV, a period of diminishing contractility, is of short duration (0-5% of the cycle) (Dressman 1986).

The overall effect of this pattern is that it can delay colon delivery, when a tablet or capsule formulation is taken with food. Such formulations should ideally be administered to fasting patients (Wilson, 1989). A prodrug colonic-delivery system, however, should

not be greatly affected by food, if the prodrug rapidly disperses into solution or suspension. The prodrug would leave the stomach continuously with the liquid phase. Prodrug therefore could be taken by a patient at any time.

The Small Intestine

In contrast to the stomach, the small intestine has a remarkably consistent motility, and is largely unaffected by many potential variables (Davis et al., 1986). The entry of material into the duodenum is determined by the gastric emptying rate (Lagerlof et al., 1974). However, the majority of the small intestine is unaffected by gastric motility (Read et al., 1982). The flow of chyme through the terminal ileum has been examined and it has been reported that chyme is intermittently delivered to the colon as discrete boluses (Camileri et al., 1989). This 'hold-up' at the ileocecal junction has also been reported for radiolabelled nondisintegrating tablets (Davis et al., 1988a). Other researchers report that pooling at the ileocecal valve is infrequent (Spiller et al., 1987).

Small intestine transit time is generally 3 to 4 hours and is unaffected by particle size, density or meal composition (Davis et al., 1986).

Both the degradation and absorption rates in the small intestine are critical to successful colonic drug-delivery. For systemic drug delivery, using the colon as the site of absorption, degradation in the small intestine should be minimal, otherwise low drug bioavailability results. If, on the other hand, the drug is intended for local action in the colon, e.g., antiinflammatory drugs, then absorption in the small intestine must be small to avoid systemic exposure to the drug and possible side-effects.

A recent study was performed to determine the effect of two drugs, loperamide (which slows intestinal motility) and metoclopramide (which speeds up intestinal motility) on the absorption of theophylline from a sustained-release tablet (Bryson et al., 1989). It was hypothesized before the study that speeding up tablet transit through the small intestine would result in reduced bioavailability and that slowing tablet transit would give increased

bioavailability. In fact, the complete opposite occurred. The authors concluded that decreased motility resulted in poorer mixing and decreased dissolution as a consequence of an increased thickness of the unstirred layer in the vicinity of the tablet. Thus, theophylline dissolved and reached the epithelium more slowly, resulting in poor absorption (Bryson et al., 1989).

The observation with theophylline has important consequences for colon-specific delivery. A long residence time in the colon does not necessarily mean that the drug will be readily and completely absorbed. The conditions for drug diffusion from the dosage form to the site of absorption are also important.

The Colon

The colon historically has been divided into a number of sections, largely on the basis of gross anatomical features. The physiology of the colon, however, dictates that it should be regarded as an organ with three different physical areas (Elliott and Barclay-Smith, 1904).

The first portion, including the cecum, ascending colon, and part of the transverse colon has a unique pattern of motility compared to the subsequent sections. This area has a motility first described by Cannon, as antiperistaltic (Cannon, 1902). Peristalsis, a common pattern of motility, in the small intestine, results in an aboral movement of contents (Christensen, 1981). In the proximal colon, antiperistalsis causes the chyme to be pushed back towards the ileocecal junction. This results in thorough mixing and increased efficiency of absorption of water and electrolytes from the chyme (Christensen, 1981). Antiperistalsis leads to a prolonged residence time in the proximal colon. This effect, together with the fact that the contents are less viscous at this site, (Cummings et al., 1990) make the proximal colon an ideal site for drug release.

The second area of the colon, the remainder of the transverse colon and the descending colon, has a different pattern of motility compared to the proximal colon. The

chyme undergoes coordinated peristalsis and tonic contraction rings moving caudad are observed (Christensen, 1981). Contents are progressively dehydrated and segmented into discrete units.

The sigmoid colon and rectum store the feces and are involved in the defecation reflex. Strong muscular contractions are characteristic of the motility in this third region of the colon (Christensen, 1981).

Scintigraphic analysis of the colonic transit of a small infusion pump (Osmet[®]) has been correlated with the absorption of drug released from the pump (Davis et al., 1988b). It was found that the absorption rate was lower when the pump was held up in the hepatic and splenic flexures of the colon. This behavior may have been due to local changes in osmolarity, stagnant diffusion layers, poorer absorption, or the existence of muscular sphincters at these sites. Whatever the mechanism, it is important to be aware of differences in absorption rate at different sites in the colon.

The whole gut transit time is a combination of the individual organ transit times (Hinton et al., 1969). Transit rate through the colon is different in the proximal, middle and distal regions (Proano et al., 1990) A study of the effect of polysaccharides on the transit through the colonic segments in the rat showed that although the total transit time is similar in certain instances, transit times through the various segments differ (Lupton and Meacher, 1988).

Colonic transit time varies markedly among individuals. In one study it ranged from 13 to 68 hours (Hardy et al., 1985). This transit time through the colon constitutes, on average, approximately 80% of the whole gut transit time. The time spent within the proximal colon ranged from 10.7 to >28.4 hours (Hardy et al., 1985).

Factors that influence colonic motility include diet, stress, diurnal variations and pharmacologic agents (Christensen, 1981). The existence of colonic disease, e.g., ulcerative colitis (Rao and Read, 1990) and irritable bowel syndrome, (Cann et al., 1983) also markedly affect colonic motility.

Kinetics of Delivery

Colonic delivery can be advantageous for two reasons. First, direct delivery to the active site can produce greater drug exposure than would be attained by systemic administration. A second major reason for an advantage is first-pass metabolism in the liver. These aspects are, in turn, examined from a pharmacokinetic point of view.

For site-specific delivery to the colon to be advantageous over oral or intravenous administration, one would expect the extent of drug delivery to the colon to be greater following the delivery system than after the other modes of administration. The movement of a delivery system down the gastrointestinal tract and the release of drug in the contents is shown in Figure 2-4. The competing processes of elimination and drug absorption in the upper gastrointestinal tract are also shown. A delivery system is maximally effective, relative to giving drug orally if processes **a, b, e,** and **g** do not occur. In this situation, no drug reaches the colon after oral administration, but the entire dose does after delivery system administration. The objective then of a delivery system is to not release any drug in the upper GIT and to provide complete and reproducible release within the colon. In the subsequent argument, drug reaching the colon from the general circulation after its absorption from the upper gastrointestinal tract is not considered.

From Figure 2-4, it is apparent that delivery of drug to the colon depends on what fraction escapes loss at each of several sites. Defining these fractions as follows:

$$f_1 = c/(a + b + c) \tag{1}$$

$$f_2 = d/(d + e) \tag{2}$$

$$f_3 = b/(a + b + c) \tag{3}$$

$$f_4 = g/(f + g) \tag{4}$$

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Drug-Delivery System   **Drug**

```
            ↓                                  ↓

     a ←  ┌──────────┐   b    ┌──────────┐   f →
          │ Upper GI │ ────→  │ Upper GI │
          │  Tract   │        │  Tract   │
          └──────────┘        └──────────┘
                │ c                 │ g
                ↓                   ↓
          ┌──────────┐   d    ┌──────────┐
          │ Colon /  │ ────→  │ Colon /  │
          │ Rectum   │        │ Rectum   │
          └──────────┘        └──────────┘
                │ e                 │ h
                ↓                   ↓
```
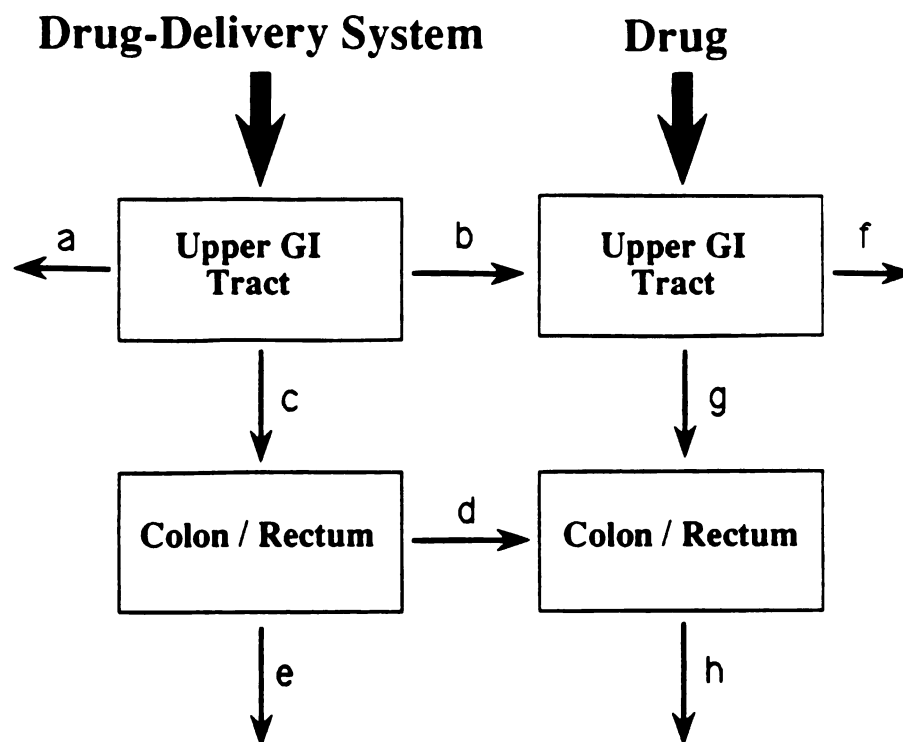
Fig. 2-4 Schematic drawing showing how more drug can be delivered to the colon after oral administration of a drug delivery system (prodrug or formulation), than after oral administration of drug alone. The processes denoted by arrows are as follows;

Release

b = premature release of drug   d = release of drug in the
    in the upper GI tract   colon/rectum

Transport

c = movement of drug-delivery system   g = movement of drug into the
    into the colon/rectum   colon/rectum

Elimination

a = elimination of drug-delivery system e = elimination of drug-delivery
    system due to absorption   from the colon/rectum,
    or degradation   either due to absorption,
                                             degradation or defecation

f = elimination of drug from the   h = elimination of drug from the
    upper GI tract, either due to   colon/rectum, either due to
    absorption or degradation   absorption, degradation or
                                             defecation

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the overall fraction of a dose reaching the colon after administration of a delivery system becomes:

$$\text{Fraction reaching} = f_1 \cdot f_2 + f_3 \cdot f_4 \qquad (5)$$
$$\text{colon}$$

After oral administration of drug in a conventional formulation, the fraction reaching the colon is f_4. The advantage of the delivery system is the ratio of these two fractions. Therefore,

$$\text{Advantage} = \frac{f_1 \cdot f_2}{f_4} + f_3 \qquad (6)$$

The optimum condition is then one in which f_1 and f_2 approach one (the entire dose from the delivery system reaches the colon) and f_4 approaches zero (drug is completely degraded or absorbed before reaching the colon when orally administered). Although it would appear that a high value of f_3 is desirable, one must keep in mind that a high value of f_3 means that the value of f_1 must be small (see Eqs 1 and 3).

The approach used by Sandberg-Gertzén et al..(1983) exemplifies the specificity of colonic delivery. Olsalazine, a prodrug of 5-aminosalicylic acid, reaches the colon in patients with ileostomies. Indeed, 100.2 ± 14.4 % of the administered dose of prodrug is recovered in the ileostomy bags. Although others have indicated that movement down the gastrointestinal tract may be different in these patients (Dew at al, 1983), this quantitative delivery indicates that colonic delivery is probably complete in typical ulcerative colitis patients.

Drug Delivery Index

Steady-State

The administration of a delivery system has a potential advantage relative to oral or intravenous (IV) administration. How selective the delivery system is can be assessed pharmacokinetically by determining the concentration (C) of drug in colonic tissue (ct) relative to the blood (b) at steady state following the two routes of administration. The *drug delivery index (DDI)* is then:

$$\text{Drug Delivery Index (DDI)} \quad [\text{Colonic delivery (CD) vs. oral (PO)}] = \frac{\dfrac{C_{ct}(CD)}{C_{ct}(PO)}}{\dfrac{C_{b}(CD)}{C_{b}(PO)}} \tag{7}$$

while for colonic delivery vs. intravenous administration

$$\text{Drug Delivery Index (DDI)} \quad [\text{Colonic delivery (CD) vs. IV}] = \frac{\dfrac{C_{ct}(CD)}{C_{ct}(IV)}}{\dfrac{C_{b}(CD)}{C_{b}(IV)}} \tag{8}$$

The numerators of Eqs. 7 or 8 are the relative colonic exposures following administration by the two routes. The denominators are the relative systemic exposures and, if corrected for dose differences, are also by definition the relative bioavailability of drug from the colonic delivery system.

Single Dose

Selective advantage can also be assessed following single doses. Equations 7 and 8 then become:

$$\text{Drug Delivery Index (DDI)} \quad [\text{Colonic delivery (CD) vs. oral (PO)}] = \frac{\dfrac{AUC_{ct}(CD)}{AUC_{ct}(PO)}}{\dfrac{AUC_{b}(CD)}{AUC_{b}(PO)}} \tag{9}$$

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$$\text{Drug Delivery Index (DDI)} \quad = \frac{\dfrac{AUCct(CD)}{AUCct(IV)}}{\dfrac{AUCb(CD)}{AUCb(IV)}} \qquad (10)$$

[Colonic delivery (CD) vs. IV]

where AUCct and AUCb refer to the areas under the concentration-time curves of drug in the colonic tissue and blood, respectively.

The DDI, as expressed in Eqs. 9 and 10, is particularly difficult to obtain. Measurement of drug in colonic tissue means that only one time point can be obtained from each animal. Such studies require large numbers of animals to generate an AUC. Furthermore, only a single DDI value is generated, thus preventing the use of statistics to compare different dosage forms. This is in contrast to the steady-state approach which, in theory, estimates the DDI using fewer animals (See Eqs. 8 and 9).

Significance of the Drug Delivery Index

In theory, the drug delivery index is the factor by which the dose of drug in the colonic delivery system may be decreased relative to that required by systemic administration of drug. There are many factors that may modify this conclusion. For example, the drug may elicit some or all of its effects via systemic mechanisms. A second example could be in the treatment of colon cancer, where the blood supply to the tumor may be such that drug absorbed from the lumen does not reach the tumor cells as well as when delivered via the systemic circulation.

Chapter 3

Approaches Towards Achieving Colon-Specific Drug Delivery

All colonic-delivery systems require that the drug be reproducibly released. The rate of release in the colon may be tailored according to specific objectives. For example, peptide delivery may require release in the liquid contents of the proximal colon (Cummings et al., 1990) to optimize absorption into the systemic circulation. On the other hand, local antiinflammatory drug therapy may require gradual, sustained drug release throughout the colon if inflammation is widespread.

The following sections describe methods for colon-specific drug delivery, e.g., dextran prodrugs, small molecular-weight prodrugs and pharmaceutical formulations.

Prodrugs

This method of altering drug distribution has been widely used in both local and systemic drug delivery (Stella and Himmelstein, 1980; Friend and Pangburn, 1987). Prodrugs are pharmacologically inactive and have different physicochemical properties compared to the active molecule. There are two requirements for prodrug-mediated colonic drug delivery. First, the prodrug should not be absorbed in the small intestine. A high molecular weight, or hydrophilic nature can be used to achieve this goal. Second, the rate of conversion to the active drug should be faster in the colon than in the stomach and small intestine. These two factors ensure a slow rate of drug absorption in the upper gastrointestinal tract and a complete conversion to drug in the colon.

Dextran Prodrugs

In a pioneering paper written by Völker in 1962, the first dextran-prodrug colon-specific delivery system was described. Völker synthesized a dextran-dye conjugate, which gave delayed and prolonged urinary excretion of the dye after oral administration of the dextran-prodrug to humans. This prodrug was proposed as a means of sustaining the release of drugs throughout the gastrointestinal tract. Subsequent work by Larsen *et al.*.(1989a) indicates that dextran-prodrugs are degraded by the action of dextranases and esterases, and subsequently release the drug in the colon. The proposed scheme of the release is shown in Figure 3-1.

Harboe *et al.* (1988a,b) synthesized a variety of dextran naproxen conjugates and demonstrated colon-specific delivery in rabbits. The oral administration of dextran-naproxen conjugates to pigs gave complete bioavailability, with an average T_{max} of 15 h which was longer than that following the administration of naproxen solution (2 hours). Representative blood/time profiles obtained in rabbits are shown in Figure 3-2. The long lag time indicated that naproxen was released and absorbed in the colon after administration of the dextran conjugate: 2-3 hours is consistent with the time required to reach the colon after oral administration to the rabbit (Harboe *et al.* 1988). Subsequent experiments *in vitro* using pig and rabbit GI tract content and tissue homogenates confirmed this hypothesis, Table 3-1. The molecular weight of the dextran used in dextran-naproxen conjugates had little effect on the extent of absorption of naproxen in pigs.

Dextran conjugates incubated with GI tract homogenates showed a rapid decrease in the molecular weight (Larsen et al.,1989a). Hydrolysis was inhibited by coincubation of the drug conjugates with plain dextran and also by coincubation with glucose. These results indicate that the breakdown of the conjugates is enzymatically mediated, perhaps by a mixture of endo- and exodextranases.

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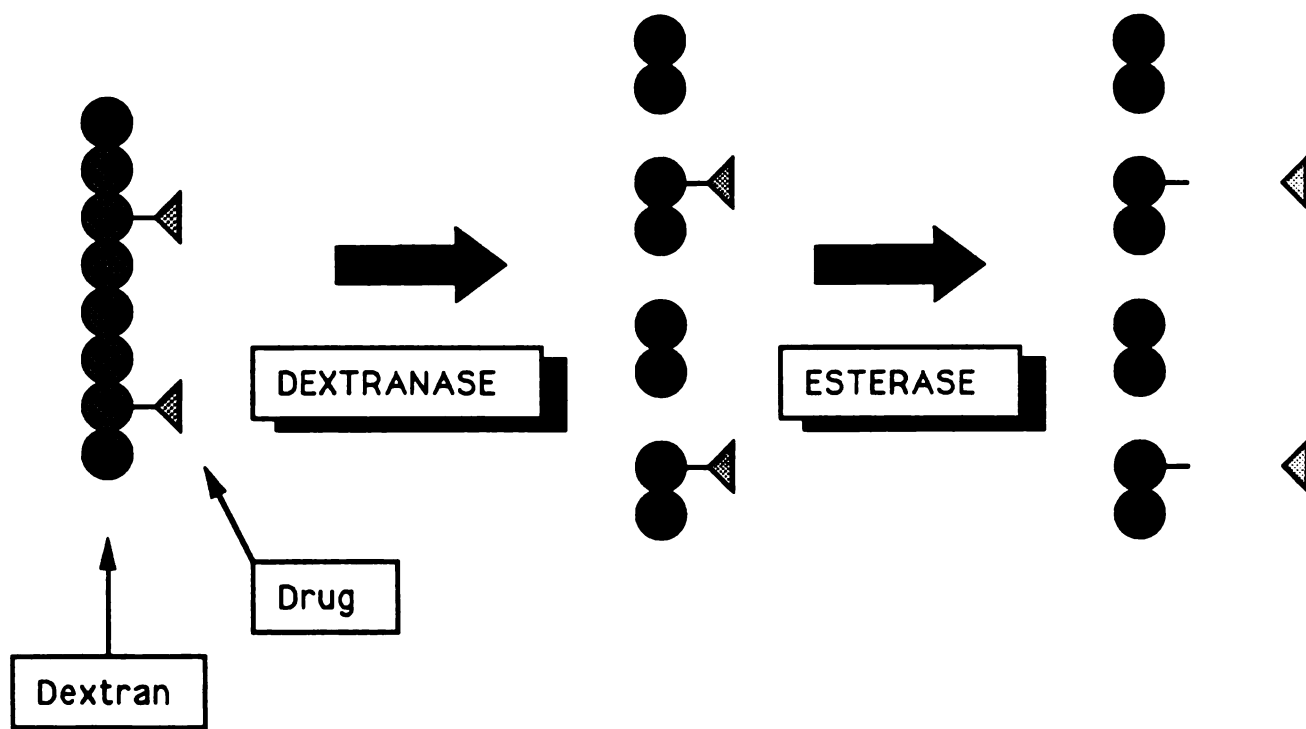
Fig. 3-1   Scheme of proposed hydrolysis of dextran-drug conjugates in the
gastrointestinal tract. For esterases to release drug, the dextran conjugate
must be broken down by dextranases into smaller fragments.

Table 3-1

Naproxen Released fron Naproxen-Dextran[a] during Incubation with Pig and Rabbit GI Tract Homogenates[b]

Homogenate/ Buffer[c]	Initial Rate, μg/ml-hr	
	Rabbit	Pig
Stomach	-	11.2
Duodenum	7.1	7.4
Jejunum	6.0	7.1
Ileum	6.3	8.1
Cecum	65.1	86.5
Colon (1st half)	32.6	107.0
Colon (2nd half)	-	111.2
0.1M Phosphate pH 7.40	6.6	6.6

[a]Mol. wt. = 70,000 daltons, 8.3 mg naproxen per 100mg dextran-conjugate.
[b]Data published by Larsen et al. (1989a).
[c]The reaction solutions: 33% homogenate-0.2 M phosphate buffer, pH 7.4 (1:1, v/v).
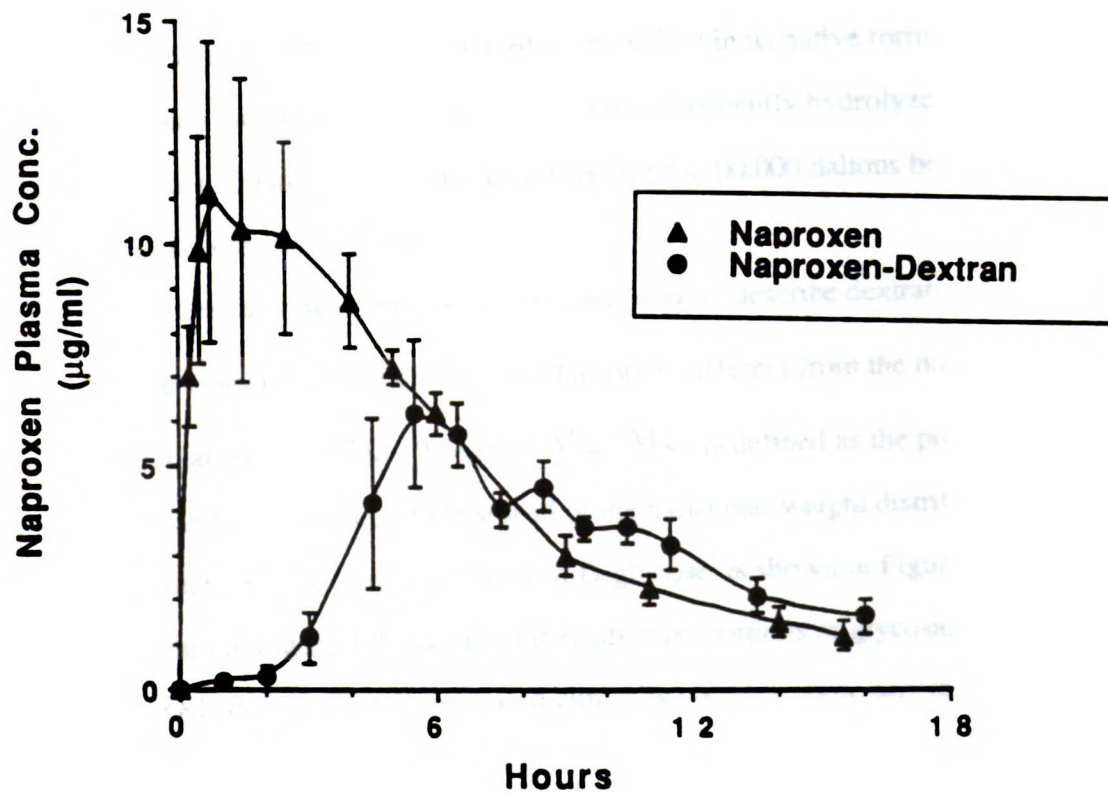
Fig. 3-2   Average naproxen plasma concentration-time curves from 2 rabbits after oral
            administration (10 mg) of a solution of naproxen (triangle) and equivalent
            dose of a dextran T-70 conjugate (circle). From Harboe et al, 1988.

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Source and Chemical Structure of Dextran

Many bacteria have been found to synthesize the extracellular polysaccharides known as dextrans (Walker, 1978). Dextran, produced by the bacterium Leuconostoc mesenteroides NRRL B-512, has a high molecular weight in its native form ($M_{\overline{W}}$ = 50-100 x 10^6 daltons) (Walker, 1978). This dextran is subsequently hydrolyzed under acidic conditions to yield average molecular weights of <100,000 daltons before it is used clinically (Klotz and Kroemer, 1987).

The molecular weight term most commonly used to describe dextran is the weight-average molecular weight, $M_{\overline{W}}$.which is often different from the number-average molecular weight, $M_{\overline{N}}$. The ratio $M_{\overline{W}}/M_{\overline{N}}$ is defined as the polydispersity, (Schott, 1983) and is a measure of the spread of the molecular weight distribution.

The structure of dextran, a polymer of D-glucose, is shown in Figure 3-3. The symbolic diagram in Figure 3-4 indicates the relative proportions of glycosidic linkages in Dextran NRRL B-512. The dextran used clinically contains approximately 95% α-1,6 and 5% α-1,3 glucosidic linkages (Walker, 1978) This high content of α-1,6 linkages makes dextran essentially a linear molecule.

Physicochemical Properties of Dextran

Dextran has a high degree of stereoregularity, as evidenced by high optical rotation. The specific rotation ($\left[\alpha\right]_D^{25°C}$) of NRRL B-512 dextran in aqueous solution is +199° (Walker, 1978) The intrinsic viscosity of native dextrans is low; [η] = 0.95 dl/g, considering their high molecular weights. Dextrans are flexible about the α-1,6 linkages and are free to take many configurations. The relative proportion of the α-1,3 to α-1,6 linkages determines the water solubility of the dextran molecule (Walker, 1978). This may be accounted for by the inflexibility of the α-1,3 linkage.
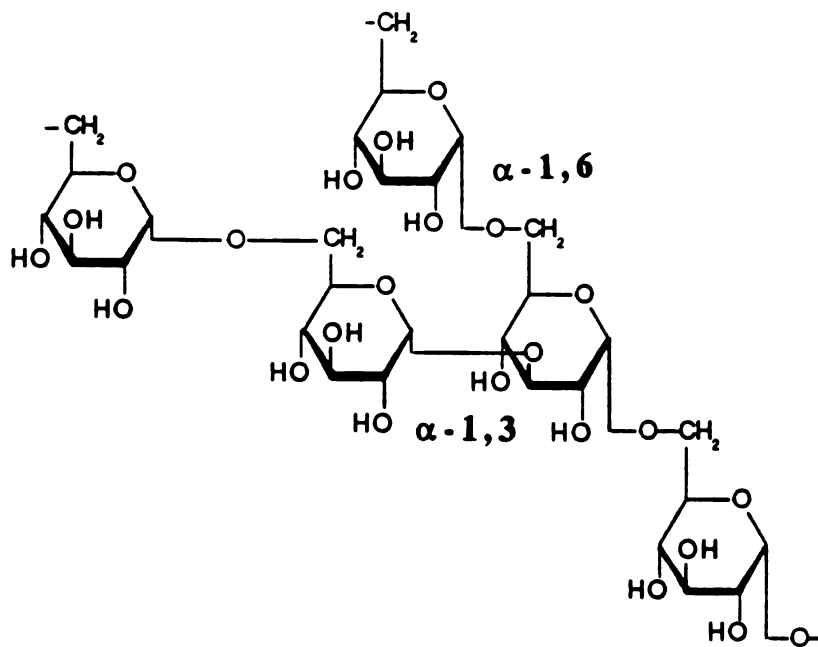
Fig. 3-3   The chemical structure of clinical dextran, showing the repeating subunits of

glucose linked by α-1,6 and α-1,3-glucosidic linkages.

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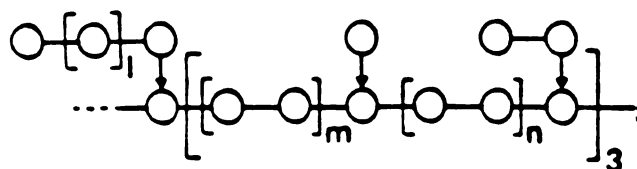
Fig. 3-4   Symbolic representation of clinical dextran (NRRL B-512). D-glucose

residues are drawn as circles (O). The arrows represent α-1,3-linkages, and

horizontal bars are α-1,6-linkages. The sum (m+n) averages 17. This figure

shows the linearity of clinical dextran. From Walker, 1978.

Dextran NRRL B-512 dissolves in water at 4°C to concentrations of 30-40%(

Walker, 1978). Dextran is soluble in dimethylsulfoxide and formamide, but insoluble in

alcohols.

Dextranases

Dextranases are produced by bacteria of the large intestine (Åberg, 1953; Sery,

1956; Sery and Hehre, 1956). In addition, molds (Tsuchiya et al., 1952; Hultin and

Nordström, 1949) and mammalian cells (Bloom and Wilhelmi, 1952; Dalqvist, 1963)

also produce dextranases. They are classified into two groups, depending on the

products formed by enzymatic hydrolysis of dextran. Endodextranases randomly cleave

α-1,6 or α-1,3-glucosidic bonds along the dextran chain producing a mixture of

polysaccharides. The viscosity of a dextran solution decreases sharply after the addition

of endodextranase as a result of the rapid decrease in the average molecular weight of the

dextran (Hultin and Nordström, 1949). Exodextranases cleave the terminal linkages

releasing mono- and disaccharides. The molecular weight of the parent chain, and

consequently the viscosity, are relatively unaffected by exodextranase cleavage

(Zevenhuizen, 1968; Wheatley and Moo-Young, 1975). Theoretical models of

dextranase activity have been devised, (Suga et al., 1949) and experiments confirm that

there is accelerated breakdown of dextran when both enzymes are present (Wheatley and

Moo-Young, 1975). A synergistic effect between dextranases and glucosidases has been

postulated to occur in the mammalian gut (Larsen et al.,1989a).

Dextranase activity has been demonstrated in the intestinal mucosa of the rat

(Dalqvist, 1963) and pig (Dalqvist, 1961) in human fecal samples, (Åberg, 1953) and in

anaerobic bacteria cultured from human feces (Hehre and Sery, 1952). An indirect

means of assessing dextranase activity is the measurement of drug release from a

dextran-drug conjugate during incubation *in vitro* with GI tract tissue homogenates and

luminal contents. Harboe et al. (1988b) and Larsen et al. (1989a) have measured the

release of naproxen from its dextran conjugate in the rabbit and pig and have demonstrated (Table 3-1) that drug release is fastest in the large intestine.

Esterases

The rationale for colon-specific delivery from dextran conjugates is that dextranases in the lower gastrointestinal tract degrade the conjugate into smaller fragments. These smaller oligosaccharides are postulated to be better substrates for esterases (Larsen et al. 1989a), due to either altered polarity or smaller molecular size. Esterases are found throughout the GI tract (Malhotra and Philip, 1965). Sloughed intestinal mucosal cells are most probably the major source of intraluminal esterase (Hänninen et al., 1987). The luminal levels, shown in Figure 3-5, are one third of the mucosal levels, whether studied in normal or germ-free rats.

Most angiotensin converting enzyme (ACE) inhibitors are orally administered as ethyl ester prodrugs because the parent acids are poorly absorbed. Pharmacokinetic studies in normal and germ-free rats showed no difference in the bioavailability of the ACE inhibitor enalapril between the two groups (Pelkonen and Ylitalo, 1989). This result, together with direct measurements of similar enzyme levels in germ-free rats, indicates that the contribution of bacterial esterase is small.

Aldridge proposed a system for the classification of esterases, based upon susceptibility to inhibitors (Aldridge, 1954). An A-type esterase is inhibited by a thiol group inhibitor, e.g., p-chloromercuribenzoate, and a B-type esterase is inhibited by a serine group inhibitor, e.g., diethyl-p-nitrophenol phosphate (Inoue et al., 1979). Esterases have been demonstrated to have broad substrate specificities, so that the use of terms, such as carboxylesterase, lipase, amidase and phosphatase, may be misleading (Walker and Mackness, 1983).

Small Molecular-Weight Prodrugs

Small molecular-weight prodrugs for colonic drug delivery have been used since antiquity. The laxative action of senna is due to glycosides (sennosides), which are largely unabsorbed in the small intestine, and converted to the active moiety in the colon (Hardcastle and Wilkins, 1979). Colon-specific delivery of antiinflammatory steroids by means of various ß-glycosides has been studied in both rat (Friend and Chang, 1984)and guinea pig (Tozer et al., 1991). Glucuronides of naloxone and nalmefene have been synthesized as possible means of treating opiate-induced constipation (Simpkins et al., 1988) Sulfasalazine was the first colon-specific treatment for ulcerative colitis (Truelove et al., 1962) This compound is an azo prodrug in which 5-aminosalicylic acid is attached to a polar sulfonamide moiety. Again, prodrug absorption, azo cleavage and drug absorption are minimal in the upper GI tract, but the prodrug is rapidly cleaved in the colon (Schröder and Johansson, 1973). Sulfasalazine has dose-dependent systemic side effects, largely attributed to the sulfonamide carrier co-absorbed (Azad Khan et al., 1977). When devising a colon-specific delivery system using a small molecular weight carrier, the toxicity and mutagenicity of the carrier must be fully investigated (Selby et al., 1985). Olsalazine, a dimer of 5-aminosalicylic acid (Selby et al., 1985), is sufficiently polar to slow absorption in the intestine; it splits in the colon to release two molecules of drug (Van Hogezand, 1988).

Overall, the method requires that the compounds be very polar, e.g., possess many hydroxyl groups and that there be rapid conversion to drug in the colon relative to the upper GI tract (Friend and Chang, 1984).

Pharmaceutical Formulation

Formulation approaches do not require specific functional groups on the drug, which is vital in prodrug synthesis. The formulation method may take advantage of the following characteristics of the GI tract:

1. Large pH difference between stomach and small intestine.

2. Reproducible transit time through the small intestine.

3. Long residence time in the colon.

4. Large numbers of anaerobic bacteria in colon.

Polymeric Coating

This method requires coating the dosage form with an acrylic polymer, which acts as an enteric coating. The dosage form remains intact in the acidic stomach, even for prolonged times after ingestion of a fatty meal (Davis et al., 1984) Once the dosage form is ejected from the stomach into the neutral conditions of the duodenum, it begins to dissolve and, by carefully controlling the thickness of this coating, the end of dissolution coincides with entry into the colon (Chacko et al., 1990). Thus, by taking advantage of the reproducible small-intestinal transit time, the dose can be released in the proximal colon (Hardy et al., 1985). The gastric hold-up varies with the nature and volume of food consumed; therefore, the arrival time at the colon can be delayed and variable (Sjögren and Bogentoft, 1982).

Slow Release

There are already 5-aminosalicylic acid products on the market that take advantage of the fact that colonic transit time is approximately 80% of total gut transit time (Robinson, 1989). They release 5-AS at a slow rate throughout the GI tract. Most of the dose is released in the colon due to the long residence time at this site.
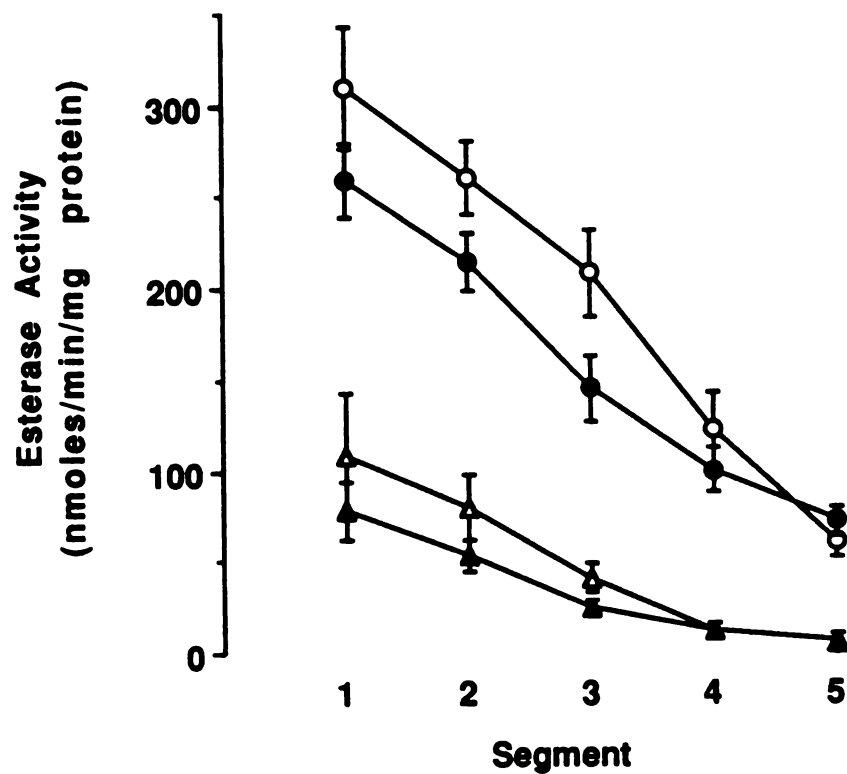
Fig. 3-5   The distribution of carboxylesterase in the small intestinal mucosa (circles) and

luminal contents (triangles) in germ-free (open symbols) and specified pathogen-

free (closed symbols) male rats measured from the 10,000 x g supernatant

fraction (1-5 = five equally-long segments, numbered from the gastric end. Data

are from five animals, and expressed as mean ± s.d.).

From Hänninen et al., 1987.

Bacterially-Degradable Polymers

Instead of using pH-sensitive enteric coatings, these dosage forms use polymers that are degradable by colonic bacteria. The polymers may either disintegrate at crosslinking sites (Saffran et al., 1986) or the polymer itself may degrade (Sintov et al., 1990). Bacterial enzymes such as azoreductase (Schröder and Johansson, 1973) or chondroitin sulfatase (Sintov et al., 1990) are found at higher concentrations in the colon; thus, polymer degradation is enhanced and drug release is faster at this site.

Chapter 4

Synthesis and Chemical Stability
of Dextran-Conjugates

Antiinflammatory glucocorticoids do not possess carboxylic acid groups and first must be chemically transformed in order to form prodrugs with dextran. Various linker molecules have been previously used in the synthesis of drug-polysaccharide conjugates, namely glucocorticoid-carbonates (Khue and Galin, 1985; Khue et al., 1986) benzyl alcohol-carbonate (Weibel et al., 1991) and metronidazole-hemiesters (Larsen, 1987, Larsen et al., 1988b).

In this study, dexamethasone and methylprednisolone were attached to dextran using succinic acid. Dexamethasone-glutarate conjugate also was made to determine whether a longer linker has an effect on drug release. Succinic and glutaric acids were chosen because they have been previously used in the synthesis of metronidazole-dextran conjugates. Dicarboxylic acid linkers have not, however, been tested in colon-specific dextran prodrug mediated drug delivery. Structures of the conjugates are shown in Figure 4-1.

The chemical stability of these conjugates was studied as a function of pH at 60°C. Methylprednisolone-21-hemisuccinate has been previously demonstrated to undergo an acyl migration reaction, forming the 17-hemisuccinate (Anderson and Taphouse, 1981; Anderson et al., 1984). This phenomenon was studied for the dexamethasone hemiesters. The glucocorticoid-hemiesters and dextran conjugates were incubated at 37°C in isotonic buffer at pH 6.8 to measure the chemical degradation expected to occur *in vivo*.
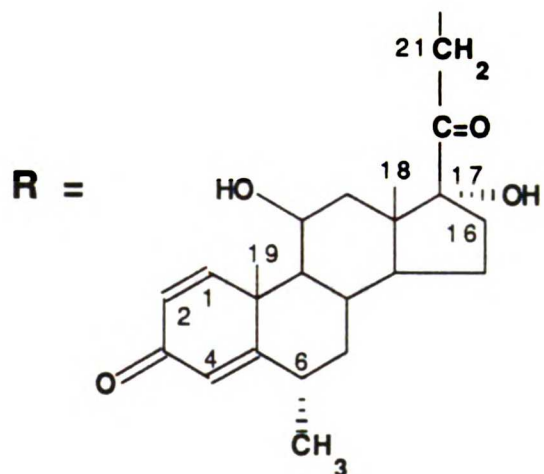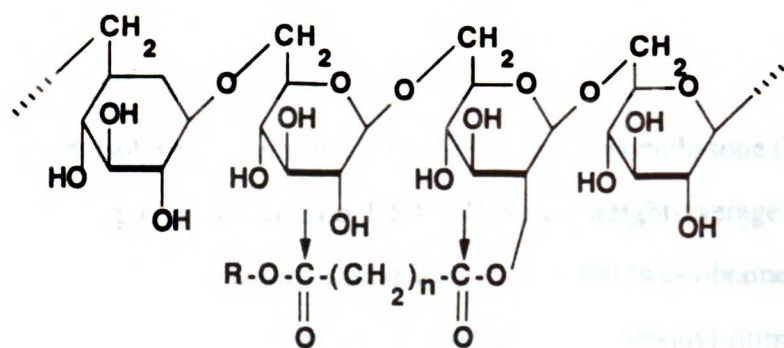
Methylprednisolone
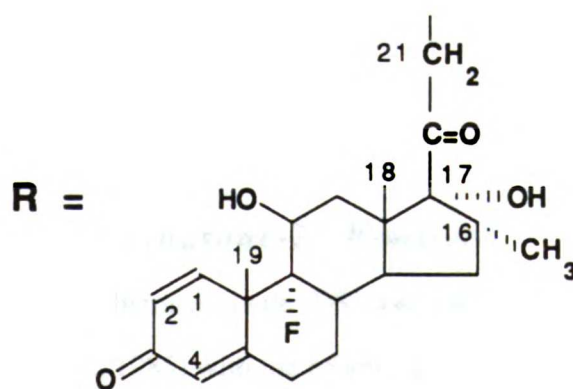
Dexamethasone

Fig. 4-1   Chemical structures of the dextran conjugates. Arrows indicate possible sites of ester hydrolysis.

Materials and Methods

Methylprednisolone-21-hemisuccinate (MPS) and dexamethasone (D) were generous gifts from Upjohn (Kalamazoo, USA). Dextran (weight-average molecular weight = 72,600; number-average molecular weight = 43,400) was obtained from Sigma (St. Louis, USA). 4-Dimethylaminopyridine (DMAP), 1,1'-carbonyl-diimidazole, succinic anhydride and glutaric anhydride were all obtained from Aldrich (Milwaukee, WI). Acetonitrile, acetone, diethyl ether, methanol, methylene chloride and dimethylsulfoxide (DMSO) were of HPLC grade.

Apparatus

HPLC was performed on the following equipment: Shimadzu SCL-6A system controller, LC-6A pumps, SPD-6AV variable wavelength detector (Kyoto, Japan); Waters WISP 710B autoinjector (Milford, MA, USA) and a Hewlett Packard HP3396A integrator (Avondale, PA, USA). Other equipment included: an Orion pH meter (model 231, Cambridge, MA, USA) and a Brinkmann Homogenizer (Sybron Corp., Cantiague, NY, USA) fitted with a Polytron-Aggregate® blade assembly (Kinematica, Switzerland). ^{1}H-NMR spectra of the compounds in d_6-DMSO solutions at room temperature were obtained on a General Electric QE-300 (300 MHz) NMR spectrometer, and referenced to 0.1% w/v tetramethylsilane at 0 ppm.

Synthesis of Dexamethasone-21-hemiesters

Dexamethasone-21-hemisuccinate (DS) was prepared by a previously published method (Vermeersch et al., 1985) with the following modifications. Dexamethasone, succinic anhydride and 4-dimethylaminopyridine (12.7 mmol of each) were dissolved in 400 ml anhydrous acetone. After reacting for 30 minutes at 25°C the acetone was removed by vacuum evaporation in a rotating flask. Dexamethasone-21-hemisuccinate was obtained in the monohydrate form by recrystallization from ethanol/water (29:71). The number of

moles of water of crystallization was determined by weight loss after drying at 115°C for 15 hours. M.P.:209-213°C. ^{1}H-NMR : δ [ppm]: 0.787 (d; 3H; 16-CH$_3$); 0.879 (s; 3H; 18-CH$_3$); 1.487 (s; 3H; 19-CH$_3$); 2.499 (s; 4H; succinate C2 & C3); 6.008 (s; 1H; C4-H); 6.226 (d; 1H; C2-H); 7.287 (d; 1H; C1-H); 12.252 (broad; 1H; succinate COOH). Elemental analysis; Calculated for C$_{26}$H$_{33}$FO$_8$: C, 61.17%; H, 6.91%; N, 0%. Found: C, 61.33%; H, 6.92%; N, 0%.

Dexamethasone-21-hemiglutarate (DG) was also synthesized by the same method (Vermeersch et al., 1985), however, 19 mmol glutaric anhydride was used. After removal of the acetone, the gummy residue was dissolved in 40 ml methanol. After the addition of 15 ml water, the white precipitate obtained was removed by filtration and discarded. The filtrate was diluted to 100 ml with water, and left at 4°C overnight to produce white crystals. Dexamethasone-21-hemiglutarate did not contain water of crystallization. M.P.:224-229°C. ^{1}H-NMR : δ [ppm]: 0.790 (d; 3H; 16-CH$_3$); 0.886 (s; 3H; 18-CH$_3$); 1.489 (s; 3H; 19-CH$_3$); 1.771 (m; 2H; glutarate C3); 2.307 (t; 4H; glutarate C2 & C4); 6.008 (s; 1H; C4-H); 6.227 (d; 1H; C2-H); 7.295 (d; 1H; C1-H); 12.140 (broad; 1H; glutarate COOH).

Elemental analysis; Calculated for C$_{27}$H$_{35}$FO$_8$: C, 64.02%; H, 6.96%; N, 0%. Found: C, 63.93%; H, 7.00%; N, 0%.

Thin Layer Chromatography

The glucocorticoids and hemiesters were analyzed by thin-layer chromatography. Silica gel plates with fluorescent markers were used (250 μm thickness, K5F, Whatman, New Jersey, USA). The mobile phase consisted of methylene chloride: diethyl ether: methanol: water (77:15:8:1.2). Spots were visualized by UV (254 nm). Rf values were as follows: dexamethasone 0.43; methylprednisolone 0.38; dexamethasone-hemisuccinate

0.11; dexamethasone-hemiglutarate 0.21; methylprednisolone-hemisuccinate 0.09. These values are comparable to published data (Pharmaceutical Codex, 1979).

Synthesis of Dextran Conjugates

Dextran conjugates were synthesized using a modification of a previously published procedure (Harboe et al., 1988). All steps were performed in a glovebag under nitrogen. Five millimoles of the hemiester (MPS, DS or DG) was dissolved in 15 ml of anhydrous DMSO. Next, 11 mmol of 1,1'-carbonyldiimidazole was added, and reacted with the hemiester for 30 minutes. Dextran (200 ml of a 5% w/v solution in DMSO), and 17.5 ml of triethylamine were then added. The reaction was continued for 21 hours at 25°C. The dextran conjugate was precipitated by adding 300 ml of ethanol:diethyl ether (50:50) to the DMSO solution while stirring. The liquid was discarded, and the gummy polymer was redissolved in 200 ml DMSO. The precipitation step was repeated, then the polymer was dispersed in ethanol using a Brinkmann homogenizer, fitted with a Kinematica rotor attachment. The polymer powder was washed twice with diethyl ether, and dried under a stream of dry nitrogen. The yield was typically 90-95%.

Drug content of the dextran conjugates was determined by measurement of glucocorticoid released after hydrolysis in 0.1M NaOH. MPS-Dextran, DS-Dextran and DG-Dextran contained 9.5, 7.4 and 4.3 mg glucocorticoid per 100 mg dextran conjugate, respectively. Water solubility of the MPS-Dextran, as expected for all the conjugates, decreased with an increasing amount of attached glucocorticoid. Poor water solubility was observed in MPS-Dextran conjugates containing more than 14 mg glucocorticoid per 100 mg polymeric prodrug.

^{1}H-NMR of the dextran conjugates showed peaks of the glucocorticoid methyl groups and unsaturated carbon protons. There was complete absence of free carboxylic protons downfield at 12 ppm, indicating that the hemiesters were covalently attached to

dextran. In addition, analysis by HPLC showed that the dextran conjugates contained less than 0.1% non-covalently bound drug by weight.

HPLC Analysis

The glucocorticoids and hemiesters were measured by reversed-phase HPLC. The column (15cm x 4.6mm i.d.) contained a polystyrene-vinyl benzene copolymer (5 μm, PRP-1, Hamilton, Reno USA). The mobile phase composition for the separation of MP, D, MPS and DS was 30% acetonitrile and 70% trisodium citrate buffer (20mM) adjusted to pH 5.5 with phosphoric acid. For the analysis of DG, the buffer portion of the mobile phase was pH 5.65. In all cases, the flow rate was 1 ml/min with detection at 242nm. In this HPLC method, dextran conjugates were eluted in the solvent front. The retention times of glucocorticoids and hemiesters were all less than 9.5 minutes.

Chemical Stability at Elevated Temperature

Solutions of the hemiesters, or their respective dextran conjugates, were made in pH 7 buffer. This stock solution was added to preheated $(60 \pm 0.2°C)$ buffers to give a final concentration of 50 μM. All buffers were 0.1M and adjusted to an ionic strength of 0.5 M by the addition of NaCl. This ionic strength was used in many previous studies of dextran conjugate hydrolysis kinetics (Larsen, 1986; Larsen, 1989; Larsen and Johansen, 1987; Larsen et al., 1989b; Larsen et al., 1988b). The buffer systems were as follows: hydrochloric acid (pH 1.4), citrate (pH 3), acetate (pH 5), phosphate (pH 7) and borate (pH 9). Samples were withdrawn at various times, and immediately chilled and adjusted to pH3 to stop the reaction. The samples (200 μL) were then analyzed by HPLC. Rate constants were calculated using the initial rate method (Anderson and Taphouse, 1981). In these experiments a known concentration of reactant is incubated for a short period and the rate of product formation is measured. Generally less than 5% of the reactant is consumed; thus reactant concentration remains essentially constant throughout the incubation. The rate

of product formation divided by the reactant concentration yields the rate constant. The rate constants k_3 and $k_{21 \to 17}$ were measured by incubating 21-hemiester and measuring the production of glucocorticoid and 17-hemiester respectively. The dextran hydrolysis rate constants k_2 and k_1 were measured by incubation of the dextran conjugates and measuring the production of hemiester and glucocorticoid respectively. In dextran conjugates the concentration of glucocorticoid was calculated from the drug content of the conjugate multiplied by the conjugate concentration (e.g., for DS-Dextran which contains 7.4 mg dexamethasone per 100 mg conjugate, a 0.265 mg/ml solution is equivalent to 50 μM dexamethasone).

Hydrolysis in Buffer at Physiologic Conditions

In contrast to the elevated temperature studies the initial reactant concentration was 500 μM. This concentration was necessary to obtain measurable rates of product formation in all tissues during a 3-hour incubation period. Samples (200 μL) were withdrawn at predetermined intervals using a wide-bore pipet tip. Samples were added to chilled tubes containing 200 μL of saturated aqueous sodium chloride and 60 μL of 6 % phosphoric acid. After addition of internal standard, the samples were extracted with methyl-t-butyl-ether/pentane (60:40) and the organic phase removed and evaporated. The residue was dissolved in 100 μL of methanol, and 50 μL was analyzed by HPLC. The mobile phase consisted of 35 % acetonitrile and 65 % of 50 mM trisodium citrate adjusted to pH 4.1 with phosphoric acid. HPLC instrumentation was the same as previously described. These chromatographic conditions were necessary to move the 21-hemiester and glucocorticoid away from interfering peaks. Under these conditions, however, interfering peaks obscured the 17-hemiesters, therefore acyl migration was not measured.

As a direct measure of the chemical stability of the hemiesters and dextran conjugates at physiologic conditions, control incubations at pH 6.8, 37°C were also performed.

Results and Discussion

Synthesis

Vermeersch *et al.* (1985) described the use of 4-dimethylaminopyridine as an acyl transfer catalyst in the synthesis of metronidazole-hemisuccinate. These authors reported high yields (95 %) using 0.05 moles of 4-dimethylaminopyridine per mole of metronidazole. When this method was used for the synthesis of dexamethasone hemiesters we obtained low yields (<10 %) even after 20 hours at room temperature. Increasing the molar ratio of 4-dimethylaminopyridine to one gave higher yields. After recrystallization the final yields were 94 % for dexamethasone-21-hemisuccinate and 56 % for dexamethasone-21-hemiglutarate.

The dextran conjugates were readily purified in high yields (>90 %). The purification method enabled rapid production of large batches (10 g) of the conjugates. HPLC analysis of the conjugates indicated that the amount of free drug (non-covalently bound) was less than 1 % of the total drug content.

Hydrolysis and Acyl Migration at 60°C

HPLC analysis of 21-hemiester incubation products showed, in each case, the formation of an early eluting peak, at approximately three minutes. Figure 4-2 demonstrates that this peak increases with time. Based on previously published data this peak was hypothesized to be a 17-hemiester (Anderson and Taphouse, 1981). The mobile phase containing this peak was collected and subjected to further alkaline hydrolysis. The unknown peak was found to produce 21-hemiester and subsequently to yield glucocorticoid. This sequence of events is in agreement with observations using methylprednisolone-21-hemisuccinate (Anderson and Taphouse, 1981), and supports the model proposed in Figure 4-3. Due to the unavailability of authentic 17-hemiesters, a single standard curve using 21-hemiester peak area was used to measure both 17- and 21-hemiesters. Molar absorptivity was assumed to be identical for the two hemiesters.
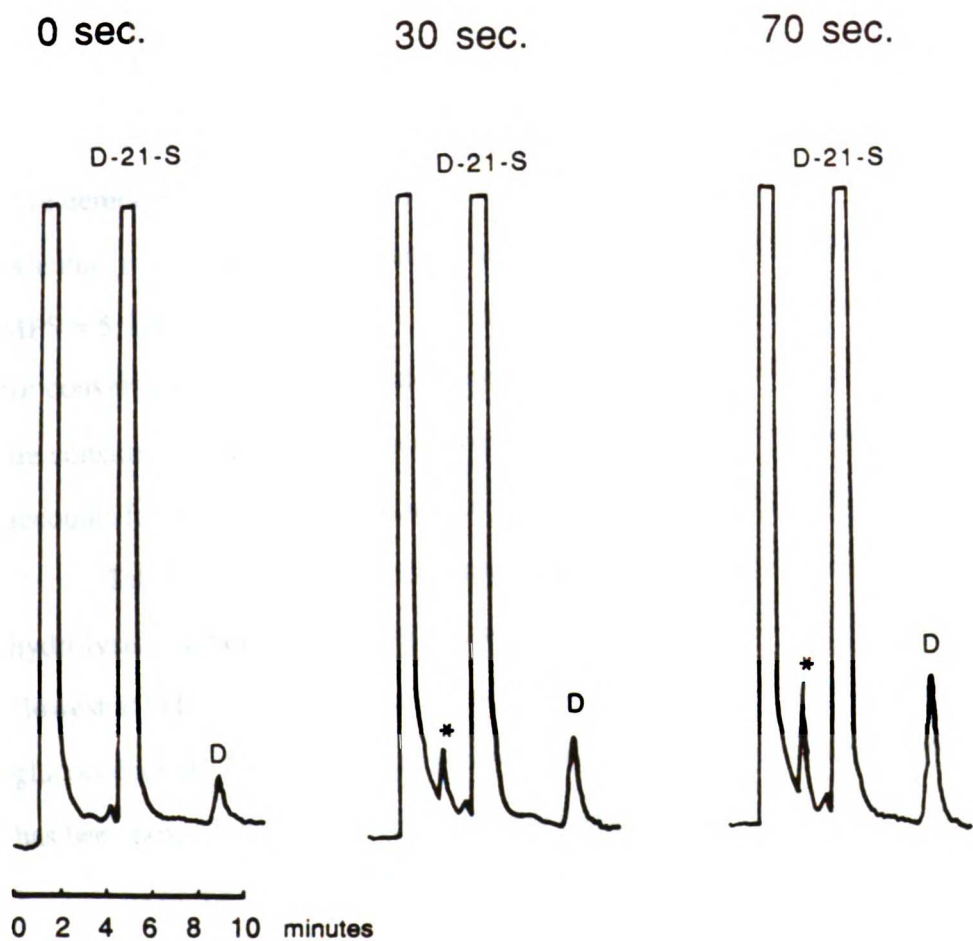
Fig. 4-2 HPLC chromatograms showing the hydrolysis products of dexamethasone-21-hemisuccinate (D-21-S initial concentration 50 μM, pH9, 60°C), after 0, 30 and 70 seconds of incubation. The unknown peak (*) is postulated to be dexamethasone-17-hemisuccinate, and the peak labeled (D) is dexamethasone.

As shown in Figure 4-1, both the dextran-linker and glucocorticoid-linker ester bonds are susceptible to hydrolysis. Therefore, during incubation of the dextran conjugates both drug and hemiester are released.

The model shown in Figure 4-3 includes both ester hydrolysis and acyl migration reactions. As shown by the model, the ratio of $k_{17\rightarrow21}$ and $k_{21\rightarrow17}$ is identical to the hemiester concentration ratio occurring at the time the 17-hemiester concentration peaks. At this time, the rates of formation and degradation are equal. Anderson and Taphouse (1981) have demonstrated using methylprednisolone-hemisuccinates that this ratio remains essentially constant in the pH range from 7-9. The hemiester ratios at pH 9 are as follows; MPS = 5, DS = 61 and DG = 67. These ratios indicate that acyl migration rate constants for conversion of glucocorticoid-17-hemiesters to the respective 21-hemiesters ($k_{17\rightarrow21}$) are considerably larger than the opposing acyl migration rate constants ($k_{21\rightarrow17}$), which accounts for the very small amount of 17-hemiester observed during incubation.

Table 4-1 shows the rate constants at 60°C as a function of pH. In the ester hydrolysis reactions, i.e., k_1, k_2 and k_3 the hydrolysis is faster at extreme pH values, and slowest at pH 3. In all cases, the release rate of hemiester is faster than the release of glucocorticoid. This difference is most pronounced for the dexamethasone conjugates. It has been postulated that adjacent hydroxyl groups on the dextran molecule catalyze the hydrolysis of the dextran-linker bond, thus making k_2 greater than k_1 (Larsen, 1986).

Ester hydrolysis rates are quite similar for DS and DG, which is in accordance with work on hydrocortisone-hemiesters, (Garrett, 1962a; Garrett, 1962b) and metronidazole-hemiesters (Larsen et al., 1988a). Acyl migration ($k_{21\rightarrow17}$) increases with pH, in accordance with published work on methylprednisolone-21-hemisuccinate (Anderson and Taphouse, 1981). Anderson et al. (1984) have postulated that a tetrahedral intermediate species is formed during 21→17 succinate migration in methylprednsiolone-hemisuccinate. Dexamethasone possesses a 16α-methyl group, not present in methlyprednisolone, which may theoretically

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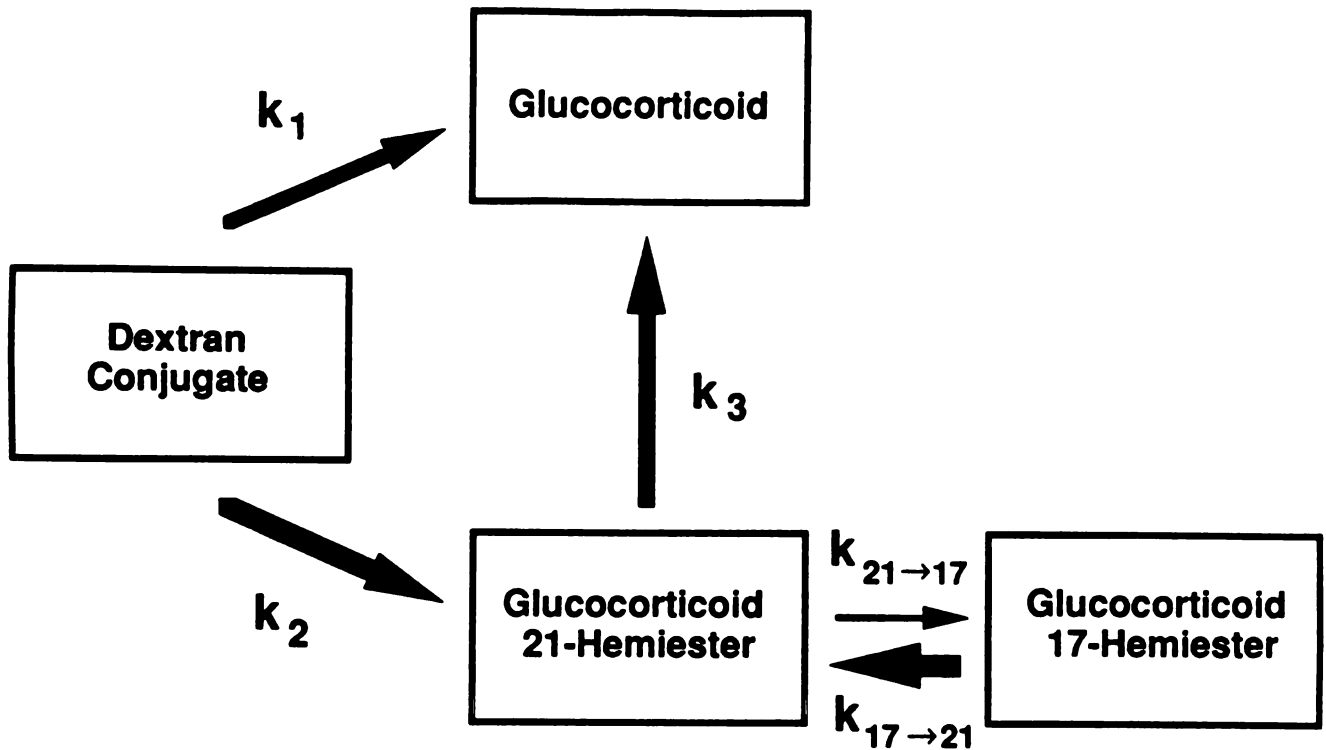
Fig. 4-3   Schematic model showing ester hydrolysis and acyl migration pathways. Rate constants: k_2 and k_1 measured after the incubation of dextran conjugates describe the production of hemiester and glucocorticoid, respectively. The rate constant k_3 measured after the incubation of glucocorticoid hemiesters, describes the production of glucocorticoid. The rate constants $k_{21 \to 17}$ and $k_{17 \to 21}$ quantify acyl migration to and from the 17-hemiester (see text for methods used to measure these rate constants). The thicknesses of the arrows indicate the relative magnitude of the rate constants.
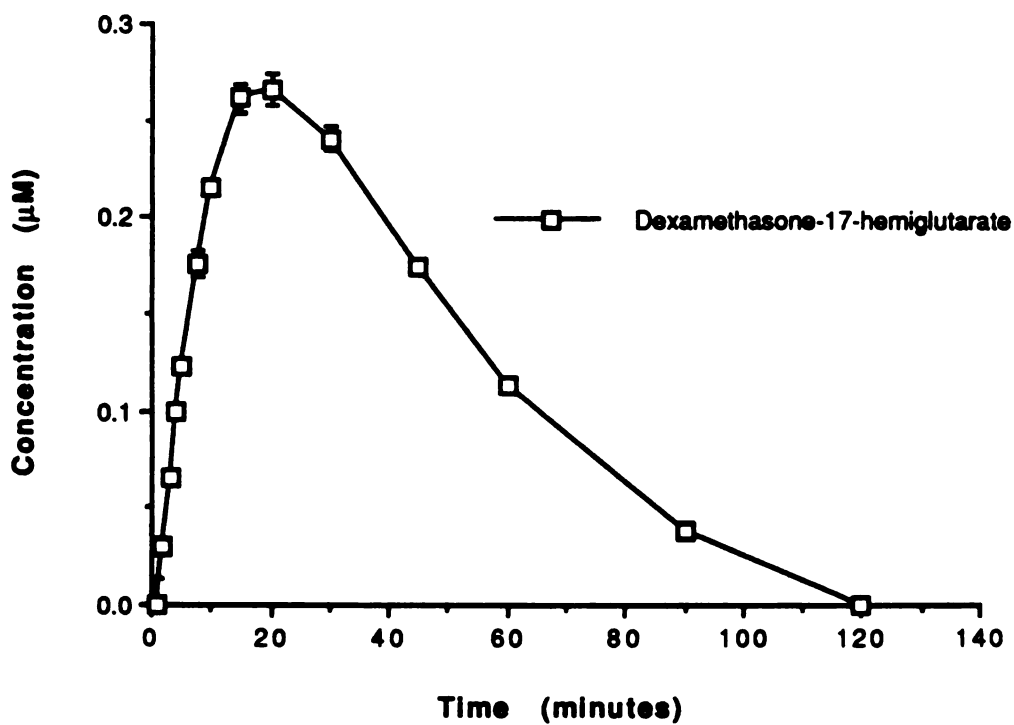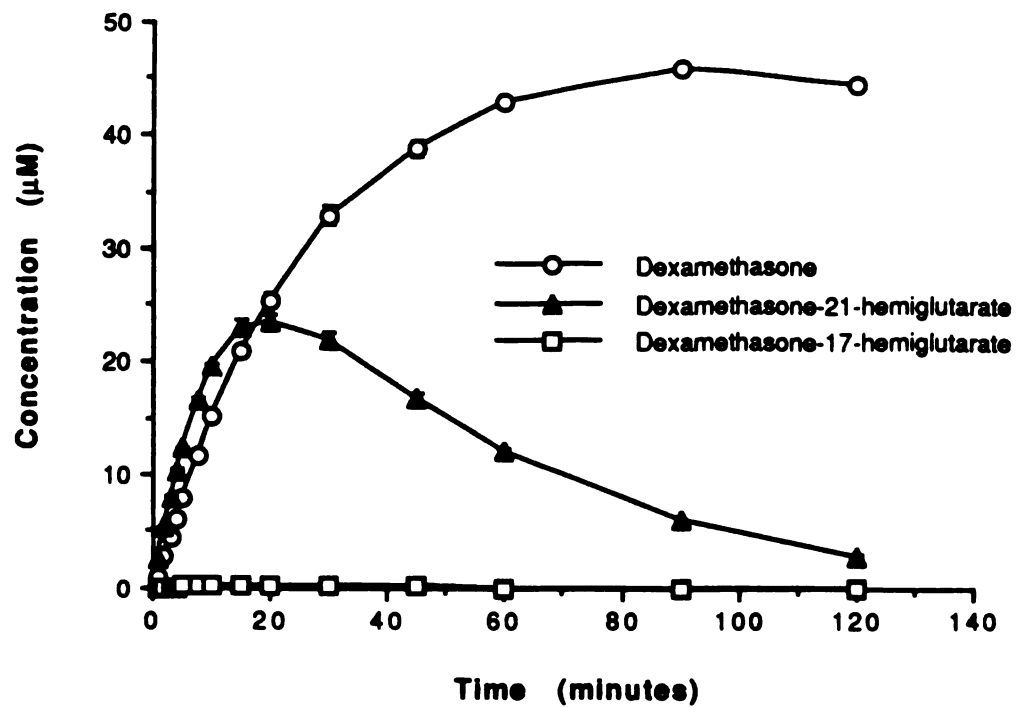
Fig. 4-4   Kinetics of the reaction products after incubation of DG-Dextran

(containing the equivalent of 50 μM dexamethasone), at pH 9 and 60°C.

Note the very low levels of dexamethasone-17-hemiglutarate (shown in

more detail in the lower graph) compared to dexamethasone-21-

hemiglutarate concentrations during the reaction. Data points are the means

of three replicates, error bars indicate standard deviation.

lessen the formation of the tetrahedral intermediate in dexamethasone-hemiesters. This may be the reason for diminished acyl migration in dexamethasone compared to methylprednisolone.

Figure 4-4 shows the time-course of the reaction products formed during the incubation of DG-Dextran at pH 9, 60°C. Note the rise and fall of the two hemiglutarates accompanied by the accumulation of dexamethasone. Dexamethasone-17-hemiglutarate was present in low concentrations (< 0.3 μM) throughout the incubation.

Hydrolysis in Buffer at Physiologic Conditions

The chemical hydrolysis of glucocorticoid-hemiesters and dextran conjugates proceeds slowly at pH 6.8, 37°C (Table 4-2). In agreement with the elevated temperature data k_2 is always larger than k_1. Furthermore, k_3 is larger for methylprednisolone-21-hemisuccinate (0.203 day^{-1}) than for the two dexamethasone-hemiesters (DS = 0.063 day^{-1} and DG = 0.025 day^{-1}.

To determine the overall chemical stability of the dextran conjugates, a degradation rate constant (k_{DEGRAD}) was defined;

$$k_{DEGRAD} = k_1 + k_2$$

This parameter was used to calculate the chemical stability half-life ($t_{1/2}$) at pH 6.8, 37°C. The values were as follows: MPS-Dextran, 82 hours; DS-Dextran, 75 hours and DG-Dextran, 103 hours. These values indicate that the dextran-conjugates should only undergo minor chemical hydrolysis during passage down the gastrointestinal tract.

Table 4-1

Rate Constants for Ester Hydrolysis and Acyl Migration at 60°C as a Function of pH

pH	MPS $k_{21\rightarrow17}$ (day^{-1})	MPS k_3 (day^{-1})	DS $k_{21\rightarrow17}$ (day^{-1})	DS k_3 (day^{-1})	DG $k_{21\rightarrow17}$ (day^{-1})	DG k_3 (day^{-1})
1.4	n.d.[a]	0.72[b](19)[c]	n.d.	0.65 (6)	n.d.	1.09 (5)
3	0.04 (6)	0.07 (37)	0.02 (12)	0.07 (6)	0.01 (19)	0.03 (25)
5	0.49 (4)	0.12 (15)	0.14 (15)	0.12 (4)	0.02 (9)	0.03[d](16)
7	1.71 (16)	0.81[d] (5)	0.34 (8)	0.45 (15)	0.20 (3)	0.30 (8)
9	38.02 (5)	33.26 (7)	7.20 (11)	19.50 (10)	8.29 (23)	17.11 (27)

pH	MPS-Dextran k_2 (day^{-1})	MPS-Dextran k_1 (day^{-1})	DS-Dextran k_2 (day^{-1})	DS-Dextran k_1 (day^{-1})	DG-Dextran k_2 (day^{-1})	DG-Dextran k_1 (day^{-1})
1.4	0.32 (2)	0.38[d] (2)	0.46 (4)	0.43 (7)	1.09 (10)	1.25 (9)
3	0.01 (37)	0.01 (45)	0.13 (16)	0.01 (20)	0.03 (11)	0.02 (20)
5	0.06 (22)	0.05 (17)	0.07 (6)	0.03 (3)	0.03[d](10)	0.01 (21)
7	1.60 (7)	1.00 (8)	2.20 (2)	0.94 (3)	1.80 (6)	0.77 (11)
9	67.39 (11)	50.98 (4)	94.61 (7)	46.80 (6)	74.45 (10)	38.86 (14)

[a] not detectable.
[b] mean of three and [d] mean of two replicates.
[c] numbers in parentheses are coefficients of variation (percent).

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Table 4-2

Rate Constants for Ester Hydrolysis in Buffer at Physiologic Conditions (pH 6.8, 37°C)

	k_2 (day^{-1})	k_1 (day^{-1})	k_3 (day^{-1})
MPS-Dextran	0.113 ± 0.009[a]	0.091 ± 0.007	0.204 ± 0.012
DS-Dextran	0.152 ± 0.012	0.069 ± 0.006	0.063 ± 0.007
DG-Dextran	0.124 ± 0.015	0.037 ± 0.002	0.025 ± 0.002

[a] mean ± standard deviation, n=5.

Chapter 5

Stability in Gastrointestinal Tract Contents

The bacterial count in the colon is higher than that of preceding sections of the gastrointestinal (GI) tract by many orders of magnitude in humans and other animals, Figure 5-1 (Hawksworth et al., 1971; Williams-Smith, 1965). Enzymes associated with the colonic bacteria have been used to achieve colonic delivery via prodrugs, e.g., sulfasalazine and senna glycosides. The ideal prodrug is both stable and unabsorbed from the upper GI tract, yet completely releases drug in the colon where the drug is fully absorbed (McLeod and Tozer, 1992). Larsen et al. (1989a) directly esterified naproxen to dextran for use in colon-specific delivery. Drug remained attached in the upper GI tract but was released by bacterial enzymes in the colon. Conjugates using dicarboxylic acid linkers have not, however, been tested for colon-specific drug delivery.

Chapter 4 described the attachment of dexamethasone and methylprednisolone to dextran using succinic acid. In addition, dexamethasone-glutarate conjugate was made to determine whether a longer linker has an effect on drug release. In this chapter all three hemiesters and conjugates were incubated in the contents of the stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum and colon of the rat. These results were used to estimate the extent of drug release expected after dosing *in vivo*. In addition, the nature of the hydrolytic enzymes present in rat cecal contents was investigated. These experiments included determination of the effects of pH, centrifugation, homogenization, anaerobic conditions and esterase inhibitors on hydrolysis kinetics. In a further group of experiments, dextran and methylprednisolone-succinate-dextran were incubated with
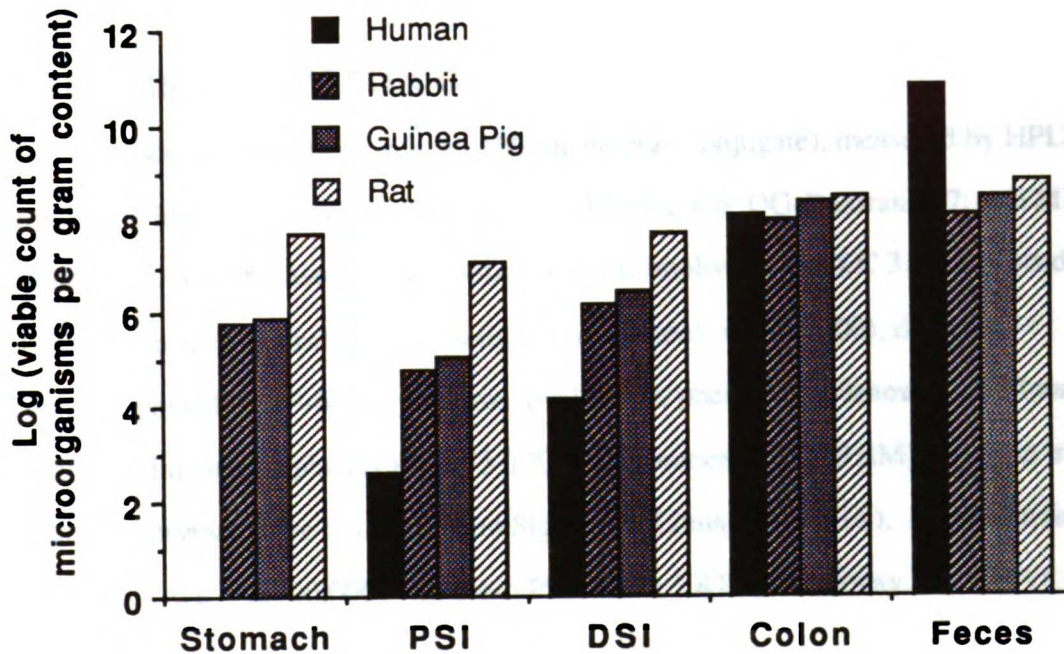
Fig. 5-1   Total microorganism count in the gastrointestinal tract in humans
(mean, n = 30) and laboratory animals (median, n = 7). Microorganisms
studied were Enterobacteria, Enterococci, Lactobacilli, Veillonelae, Yeasts,
Clostridia, Bacteroides and Bifidobacteria. From Hawksworth et al. (1971)

purified dextranase. Degradation was monitored by size-exclusion chromatography to determine if the attachment of glucocorticoid to dextran alters substrate specificity for dextranase.

Materials and Methods

Glucocorticoid content (mg per 100 mg dextran conjugate), measured by HPLC after alkaline hydrolysis, were as follows: DS-Dextran, 8.6; DG-Dextran, 5.7; and MPS-Dextran, 10.5. Dextranase (1,6-α-D-Glucan-6-glucanohydrolase, EC 3.2.1.11, produced by Penicillium sp.), dextran sulfate (weight-average mol. wt. = 5,000), diethyl-*p*-nitrophenyl phosphate (E-600), lysozyme, ovalbumin, phenylmethylenesulfonyl fluoride (PMSF), prednisone, Sephadex® (G-75-120, bead diameter 40-120 μM), soybean trypsin inhibitor and trypsin were obtained from Sigma (St. Louis, MO, USA). Blue dextran (approx. mol. wt. = 2,000,000) was from Pharmacia LKB (Piscataway, NJ, USA). Acetonitrile, methanol, methyl-t-butyl ether and pentane were HPLC grade (Fisher Scientific, Pittsburgh, PA, USA).

Analysis

Samples (200 μL) were withdrawn at predetermined intervals using a wide-bore pipet tip and added to chilled tubes containing 200 μL saturated aqueous sodium chloride and 60 μL of 6% phosphoric acid or 200 μL of chilled 10% trichloroacetic acid. These conditions were found in Chapter 4 to prevent further ester hydrolysis. After addition of internal standard (either dexamethasone, methylprednisolone or prednisone) the samples were extracted by vortexing with either a mixture of methyl-t-butyl ether: pentane (6:4) or methyl-t-butyl ether for 20 seconds. After centrifuging for 2 min at 1,000 x g, the organic phase was removed and evaporated at 50°C in a stream of nitrogen. The residue was dissolved in 100 μL of methanol and 50 μL was analyzed by HPLC. An octadecylsilane

column, (250 mm x 4.5 mm, Fisher, Pittsburgh, PA), with a PRP-1 precolumn, (Hamilton, Reno, NV), was used in contrast to the HPLC described in Chapter 4. The mobile phase consisted of 35% acetonitrile and 65% buffer (50 mM trisodium citrate adjusted to pH 4.1 with phosphoric acid). A flow rate of 1.5 mL/min and a detection wavelength of 242 nm were used.

Incubations with Rat GI Tract Contents

Male Sprague-Dawley rats (180-220 g) were used throughout these experiments and were fed a standard diet (Purina Rodent Chow, # 50-01, Ralston-Purina, Richmond, IN, USA). Rats were decapitated and the GI tract was removed and chilled within 10 min. The contents of each tissue (stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum and colon) were removed and diluted to 15% w/v with chilled isotonic buffer. Stomach contents were diluted with acetate buffer (prepared by mixing 0.15 M sodium acetate with 0.3 M acetic acid to achieve pH 4.4, a typical value for rat stomach contents (Williams-Smith, 1965)). Contents further down the GI tract were diluted with phosphate buffer (prepared by mixing 0.1 M Na_2HPO_4 and 0.15 M NaH_2PO_4 to achieve pH 6.8, a typical value for rat intestinal contents (Williams-Smith, 1965)). The latter buffer is subsequently referred to as 'pH 6.8 buffer'.

The diluted contents (2 mL) were placed in glass culture tubes and warmed to 37°C in a shaking water bath. Stock solutions of glucocorticoid-hemiesters and dextran conjugates (all containing 1.5 mM glucocorticoid) were prepared in pH 6.8 buffer and warmed to 37°C. The reactions were initiated by adding 1 mL of the stock solutions to the diluted contents. Thus, the final concentration of glucocorticoid during the incubations was 500 μM. The pH of the diluted stomach contents was increased to 4.7 by addition of the stock solution, however, this pH is within the range of values normally measured in the rat stomach (Williams-Smith, 1965).

Hydrolysis of Glucocorticoid-Hemiesters in Rat Cecal Content
Effect of pH

Cecal content, obtained from rats as previously described, was diluted to 20% w/v with 0.9% sodium chloride solution. Large particulate matter was removed from the dispersion by centrifuging at 500g for 0.5 min. The supernate (250 µL) was added to tubes containing 200 µL of 0.25 M citrate/phosphate buffer at the following pH's: 4.3, 5.1, 5.9, 6.9 and 7.9. The tubes were warmed to 37°C and 50 µL of 500 µM glucocorticoid-hemiester solution was added. Due to faster kinetics the substrate concentration was smaller (50 µM) than in the dextran-conjugate experiments (500 µM).

Centrifugation and Homogenization

A 15% suspension of rat cecal content was prepared using chilled pH 6.8 isotonic phosphate buffer. The suspension was centrifuged at 15,000 x g for 2 min and after aspirating the supernate the pellet was resuspended with buffer. The process was repeated twice. The final pellet was reconstituted with buffer to the same volume as the pooled supernates (3.4 mL). In additional experiments, the cecal content suspension was homogenized (setting 7) for 1 min and compared to a nonhomogenized control.

One milliliter of DS-Dextran solution (containing 1.5 mM dexamethasone in pH 6.8 buffer) was added to 2 mL of each of the suspensions obtained in the experiments described above.

Anaerobic/Aerobic Incubation

In these experiments cecal contents were removed anaerobically in a nitrogen-filled glove bag. All solutions had been degassed by stirring under reduced pressure for 12 hr and subsequently purged with nitrogen. The cecal contents were dispersed to 15% w/v using chilled pH 6.8 isotonic phosphate buffer. Two milliliters were placed in each of six vials. After warming to 37°C, 1 mL of DS-Dextran solution (containing 1.5 mM

dexamethasone in pH 6.8 buffer) was added. During sampling, three vials were exposed to the atmosphere while those remaining were kept under anaerobic conditions.

Enzyme Inhibitors

The effect of esterase inhibitors on the hydrolysis of dexamethasone-hemisuccinate in cecal contents was studied. The following ranges of carboxylesterase inhibitor concentrations were used: diethyl-p-nitrophenyl phosphate (0.001 - 100 μM); phenylmethylenesulfonyl fluoride (0.01 μM - 800 μM); physostigmine sulfate (0.01 - 100 μM) and p-hydroxymercuribenzoate (1 μM - 4000 μM). To examine the effect of metal ions (Nègre et al., 1988), EDTA (0.6 - 50 mM) and $CaCl_2$ (2.5 - 250 mM) were added. To determine whether the enzyme was alkaline phosphatase (Fleisher et al., 1986; Kim et al., 1986), incubations were performed with the addition of phosphate (5 - 20 mM) and L-phenylalanine (10 - 40 mM). The alkaline phosphatase experiments were performed at pH 7.4 in tris buffer (20 mM). To determine whether the esterase was membrane-bound, the effect of Triton X-100 (0.8 - 25 g/L) was also studied (Nègre et al., 1988).

Cecal content was diluted to 20% w/v with isotonic pH 6.8 phosphate buffer. Next, the test compounds were dissolved in buffer and 200 μL was added to 250 μL of the diluted cecal content. The tubes were incubated at 37°C for 5 min before the addition of 50 μL of 500 μM dexamethasone-hemisuccinate solution.

## *Incubation of MPS-Dextran with Dextranase*

Size-exclusion chromatography was used to monitor the breakdown of dextran and MPS-dextran during incubation with a purified endodextranase (Wheatley & Moo-Young, 1977). Solutions of dextran or MPS-Dextran (both 5% w/v) were prepared in 0.2 M pH 6 citrate/phosphate buffer. Dextranase was added to give a final activity of 5 units/mL. The solution was incubated at 37°C and 1 mL samples were withdrawn at 0.5, 6, 15, 25 and 40 min. The reaction was stopped by immersing the samples in boiling water for 1 min,

followed by rapid cooling on ice. One hundred microliters was injected onto the column (30 mL bed volume packed with Sephadex®) and eluted with 0.2 M citrate/phosphate buffer at pH 6. Two milliliter fractions were collected and diluted twofold with buffer. Methylprednisolone content of each fraction was measured by absorbance at 242 nm. Polysaccharide content was measured by adding 2 mL of anthrone reagent (35 mg anthrone in 100 mL of concentrated sulfuric acid) to1 mL of each fraction. The solution was allowed to cool for 20 - 30 min before measuring the absorbance at 630 nm. A standard curve of log molecular weight vs. elution volume was constructed using methylprednisolone-hemisuccinate, dextran sulfate, lysozyme, soybean trypsin inhibitor, trypsin and ovalbumin. Void volume was 7 mL calculated using blue dextran.

Data Fitting and Statistical Analysis

Initial rate kinetics were used to calculate k_3, the rate constant describing the hydrolysis of glucocorticoid-hemiesters. This method required a high substrate concentration (500 μM) because product formation was measured in the initial stages (generally < 5% conversion). The slope of the product concentration vs. time curve divided by the initial substrate concentration was k_3. Hydrolysis of the glucocorticoid-hemiesters was rapid in the PSI and DSI, therefore incubations typically were over 6 - 12 minutes. It was shown in Chapter 4 that acyl migration was a minor hydrolytic pathway, especially for the dexamethasone-hemiesters. For this reason, the hydrolysis model in Figure 4-3 was modified to exclude this pathway and is shown in Figure 5-2.

The revised model was fitted to the data using Minim, a nonlinear regression program for the Apple Macintosh (1.8a, from Dr. D.R. Purves). Analysis of variance and t-tests were used to determine statistical differences.
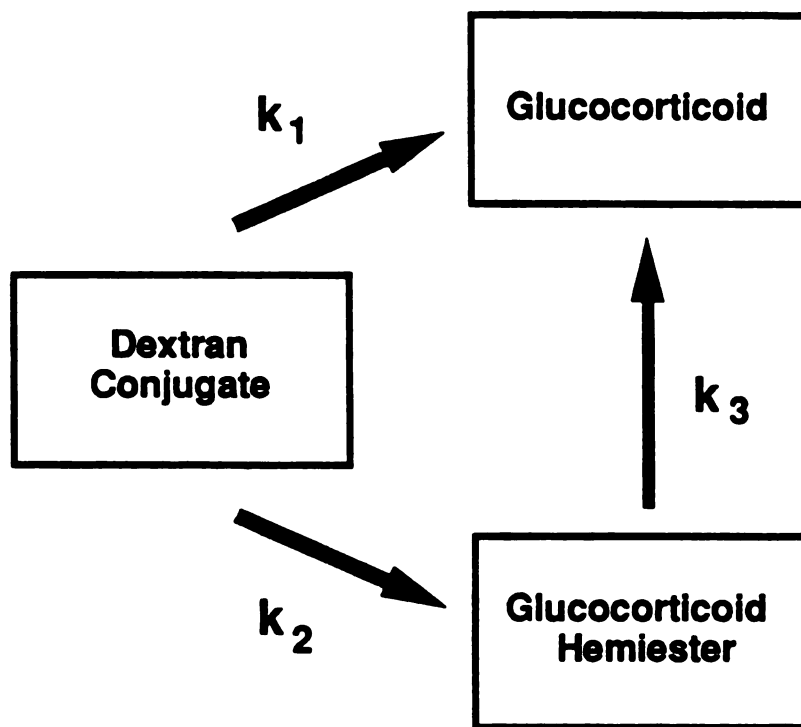
Fig. 5-2   Glucocorticoid may be released directly from the dextran conjugate by

hydrolysis of the glucocorticoid-linker ester bond (k_1) or by the sequential

hydrolysis of the linker-dextran (k_2) and glucocorticoid-hemiester

(k_3) bonds. See Fig. 4-1 for chemical structures of dextran-conjugates

and glucocorticoids.

Results and Discussion

Figure 5-3 shows that the hemiesters are rapidly hydrolyzed in the proximal small intestine (PSI) and that the rate declines progressively further down the GI tract. Taking the data from dexamethasone-hemiglutarate, the corresponding half-lives in the contents of the PSI, DSI, cecum and colon were 0.5, 4.8, 54, and 68 hours, respectively. A similar enzyme distribution has been reported for glucocorticoid-hemisuccinate esterase in the rat GI tract (Hattori et al., 1981). The enzyme distribution suggests that the esterase is either secreted in bile or released from the small intestinal mucosa. The esterase activity in PSI contents is lower after preincubation for 120 minutes at 37°C. Degradation of the esterase was almost completely prevented by adding trypsin inhibitor, Figure 5-4

Release of glucocorticoid from the three conjugates is shown in Fig. 5-5. Figure 5-6 shows the release of glucocorticoid-hemiester. Most hydrolysis occurs in the cecum and colon. It appears that dextran protects both bonds with the linker from hydrolysis by esterases in the PSI and DSI. Curvature of the graphs suggests that hemiester production slows slightly with time, even though less than 10% of the total glucocorticoid has been released. This may be a result of some glucocorticoid sites on the dextran being less accessible to enzymatic attack.

The hydrolysis model shown in Figure 5-2 assumes that all rate constants remain unaltered with time. However, hydrolysis of glucocorticoid-hemiesters in PSI contents slows as a function of time due to destruction of esterase by trypsin. For this reason, it was not possible to fit the model to the upper GI tract data. The rate constants, k_1 and k_2, for the three conjugates in cecal and colonic content, however, are shown in Fig. 5-6. Most drug is released as glucocorticoid-hemiester (via k_2) and virtually no drug is hydrolyzed directly from the conjugates (via k_1). These results were surprising as one would expect that the dextran-linker ester bond would be more sterically hindered. One explanation is hydrolysis of the glucose-linker bond by a glucosidase after previous disintegration of the dextran backbone by dextranases (Wheatley & Moo-Young, 1977).
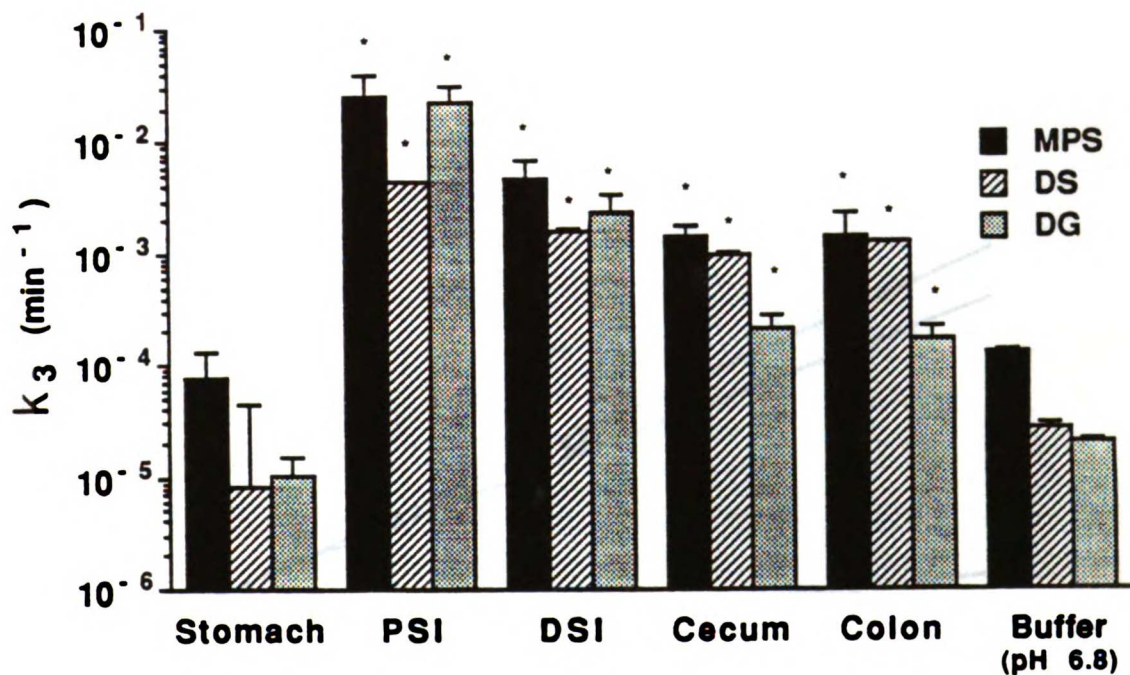
73

Fig. 5-3   Hydrolysis rate constant (k_3) of glucocorticoid-hemiesters

MPS = methylprednisolone-hemisuccinate, DS = dexamethasone-

hemisuccinate and DG = dexamethasone-hemiglutarate.

Data are mean + S.D. (n=5 animals) on a semilogarithmic scale.

The asterisk (*) indicates significant ($p<0.05$) difference

compared to buffer hydrolysis rate constants.

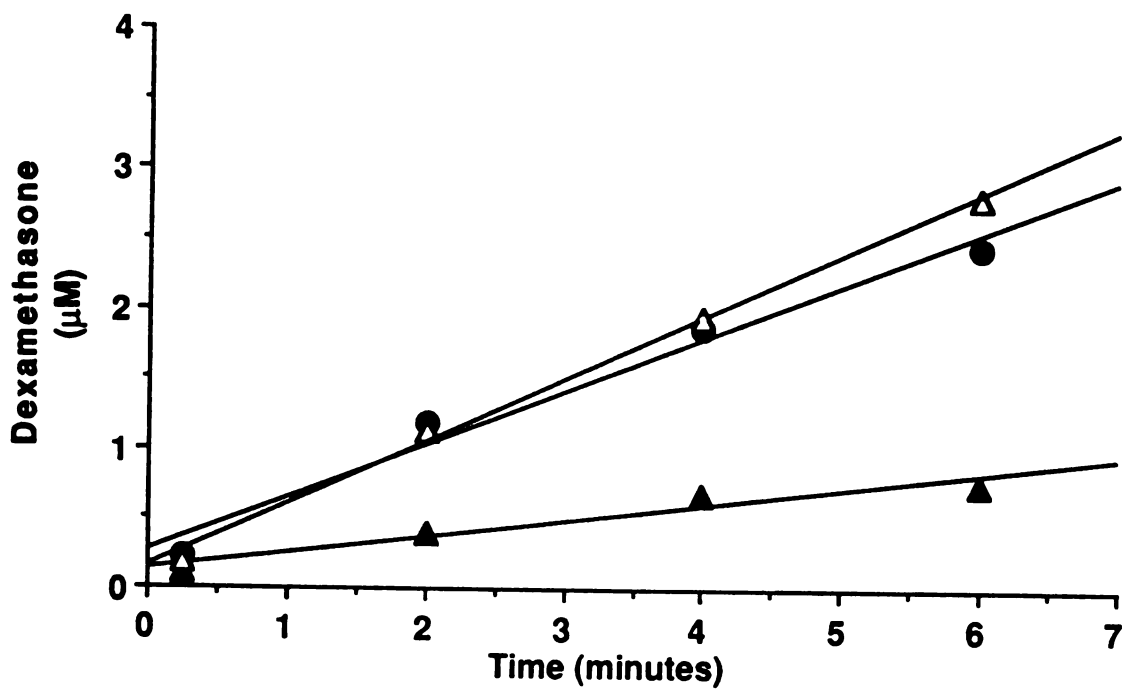Fig. 5-4   Hydrolysis of dexamethasone-hemiglutarate in PSI contents measured at

t=0 (●) and again after 120 min at 37°C in the absence (▲) and

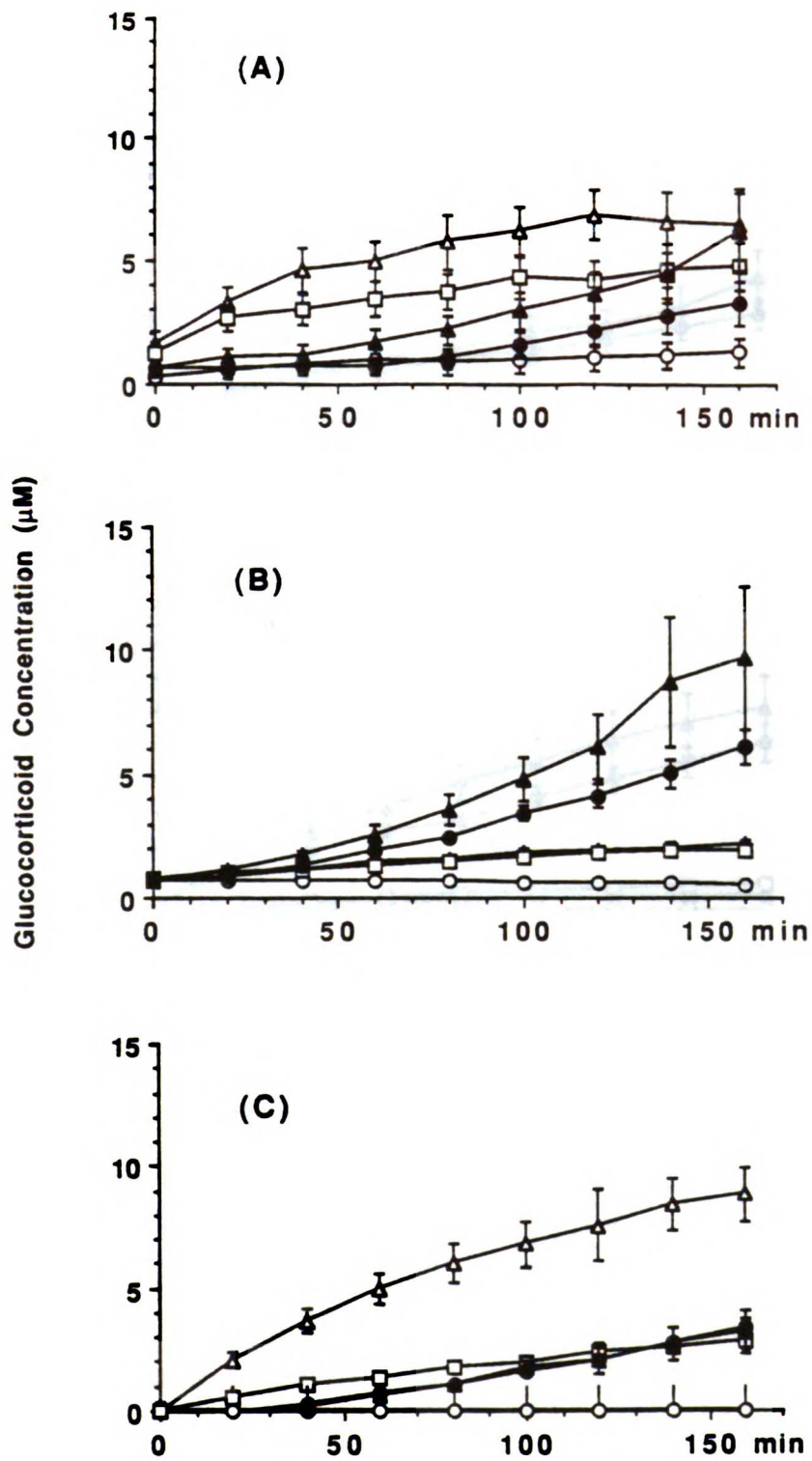presence of 7.5 mg/mL trypsin inhibitor (Δ).

Fig. 5-5   Release of glucocorticoid in contents of the GI tract during

incubation of 500 μM (equivalent concentration of glucocorticoid)

solutions of (A) MPS-Dextran, (B) DS-Dextran and (C) DG-Dextran.

Stomach (O), PSI (Δ), DSI (□), Cecum (●), Colon (▲).

Data are mean ± S.E.M. (n=5 animals)

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Fig. 5-6   Release of glucocorticoid-hemiester in contents of the GI tract during

incubation of 500 μM (equivalent concentration of glucocorticoid)

solutions of (A) MPS-Dextran, (B) DS-Dextran and (C) DG-Dextran.

Stomach (O), PSI (Δ), DSI (□), Cecum (●), Colon (▲).
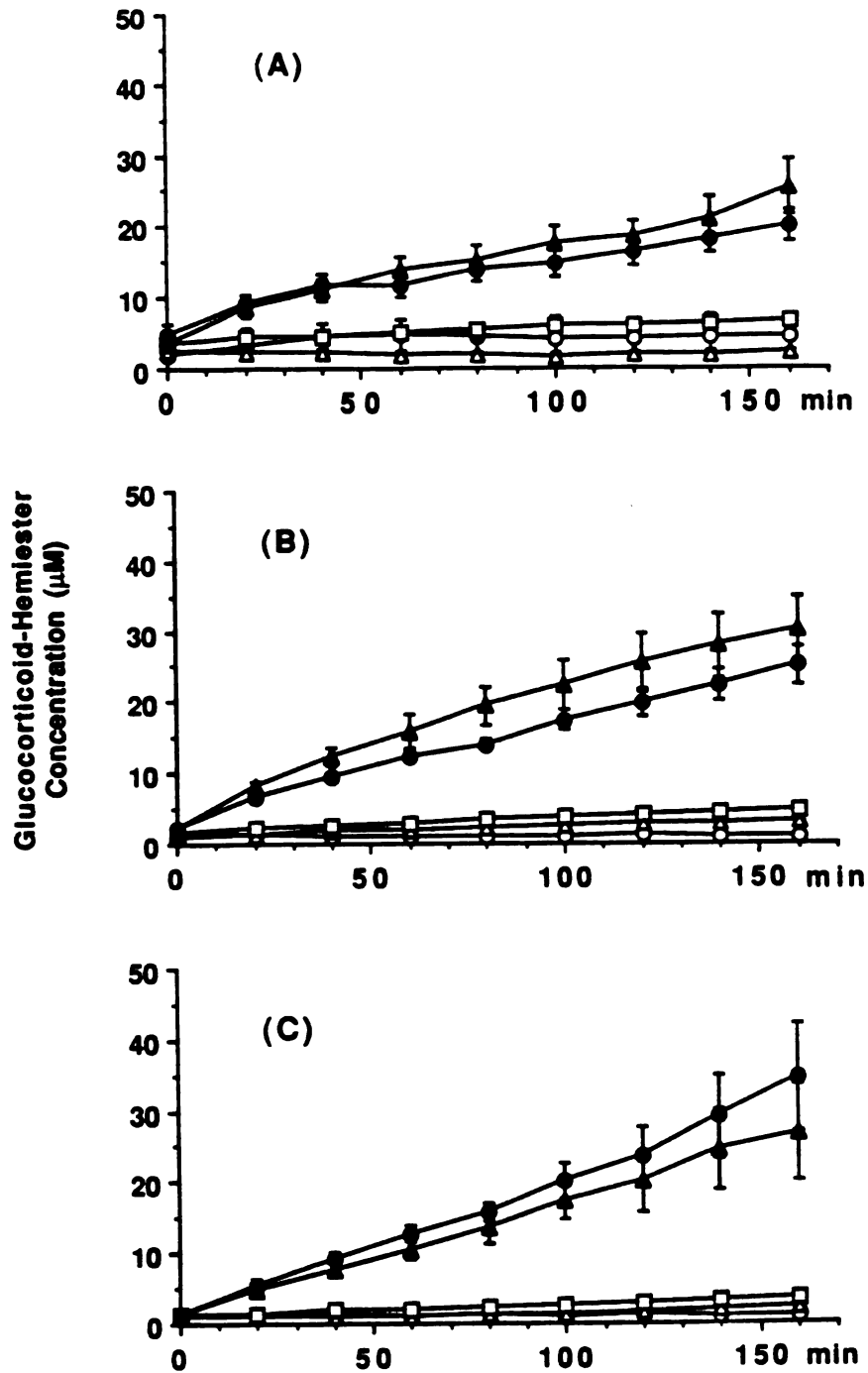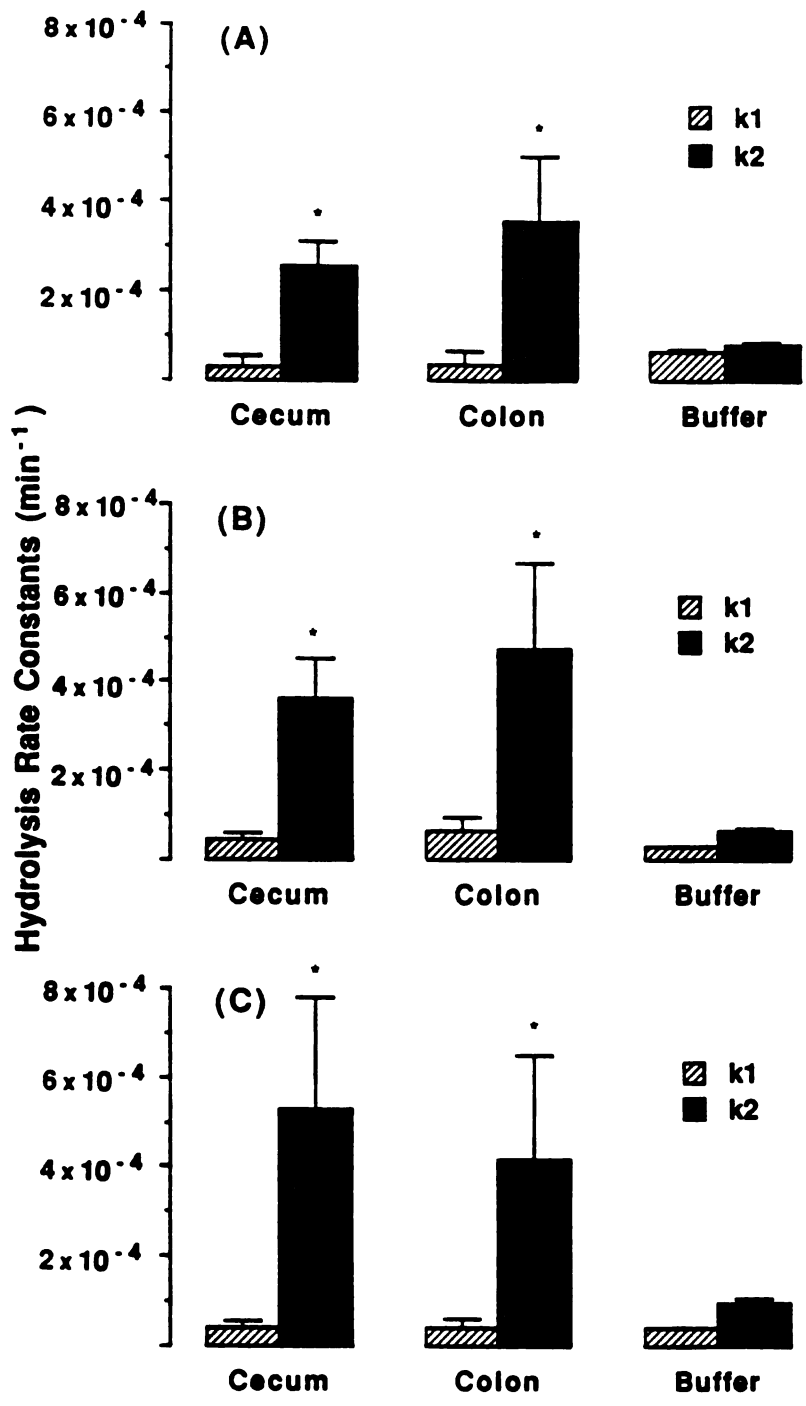
Data are mean ± S.E.M. (n=5 animals)

Fig. 5-7   Rate constants for the diluted contents obtained after fitting the model

shown in Fig. 5-2 to (A) MPS-Dextran, (B) DS-Dextran and

(C) DG-Dextran hydrolysis data. Data are mean ± S.D. (n=5 animals)

on a semilogarithmic scale. The asterisk (*) indicates significant

(p < 0.05) difference compared to hydrolysis rate constants in buffer.

Maximal hydrolysis of all hemiesters occurred at pH 6.9 as shown in Fig. 5-8. This pH maximum indicated that the cecal enzyme was an alkaline esterase (Nègre et al., 1988). This pH profile is quite different from hydrolysis of glucocorticoid-hemiesters in hamster liver microsomes (Hattori et al., 1981). These authors found that glucocorticoid-hemiesters were hydrolyzed fastest at pH 5.5 whereas glucocorticoid-acetates were hydrolyzed fastest at pH8.

The pellet obtained by centrifugation of cecal contents rapidly hydrolyzed DS-Dextran but the supernate had little activity. This observation, together with the lack of activity in PSI and DSI contents where the bacterial count is much lower (Hawksworth et al., 1971; Williams-Smith, 1965), suggests that bacterial enzymes in the large intestine are responsible for hydrolysis of the dextran conjugates. Anaerobic conditions did not affect the hydrolysis of DS-Dextran in cecal contents; however, homogenization increased the release rate of dexamethasone-hemisuccinate. Similar results were observed during incubations of dexamethasone-β-D-glucoside in guinea pig cecal contents (Tozer et al., 1991).

Hepatic (Ali et al., 1985; Hattori et al., 1981) and intestinal mucosa (Campbell et al., 1987; Inoue et al., 1979) carboxylesterases have been described; however, there is a lack of information on esterases in the large intestine (Larsen et al., 1991b). In most cases the enzyme isolated has been classified as carboxylesterase-B, due to inhibition by organophosphates. In the present study, hydrolysis of dexamethasone-hemisuccinate was unaffected by the organophosphates diethyl-p-nitrophenyl phosphate and phenylmethylenesulfonyl fluoride or the cholinesterase inhibitor physostigmine, at all concentrations studied. Hydrolysis was, however, reduced to 33% of control by the addition of 4 mM p-hydroxymercuribenzoate. The addition of EDTA and Ca^{2+} had no effect at all concentrations indicating that metal ions are not required. Glucocorticoid-hemiester hydrolysis was unaffected by phosphate and L-phenylalanine, eliminating the possibility that the enzyme is an alkaline phosphatase.
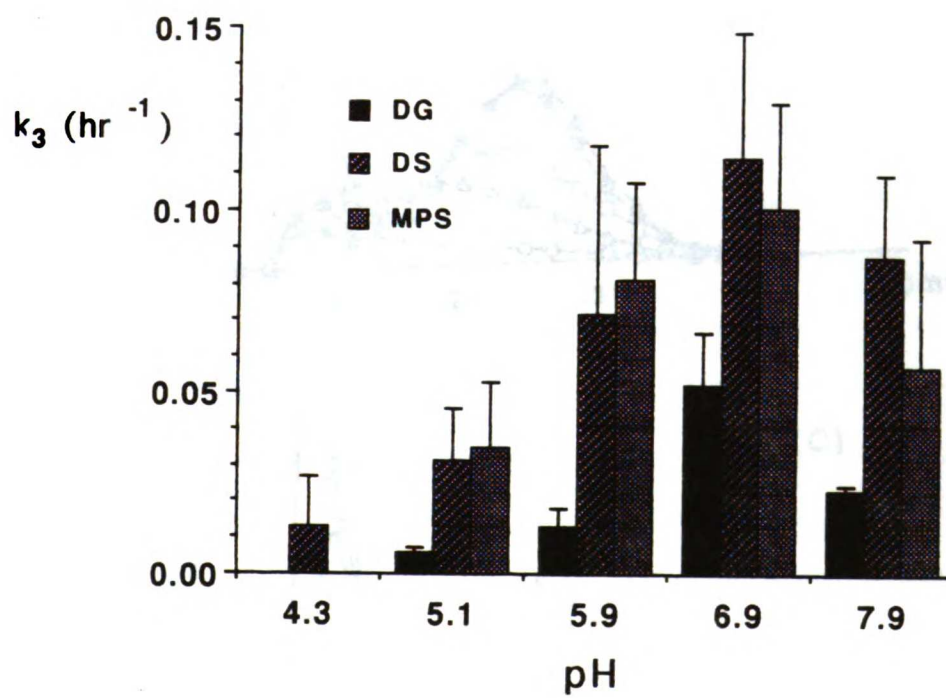
Fig. 5-8   Effect of pH on hydrolysis of the glucocorticoid-hemiesters during incubation with rat cecal content.
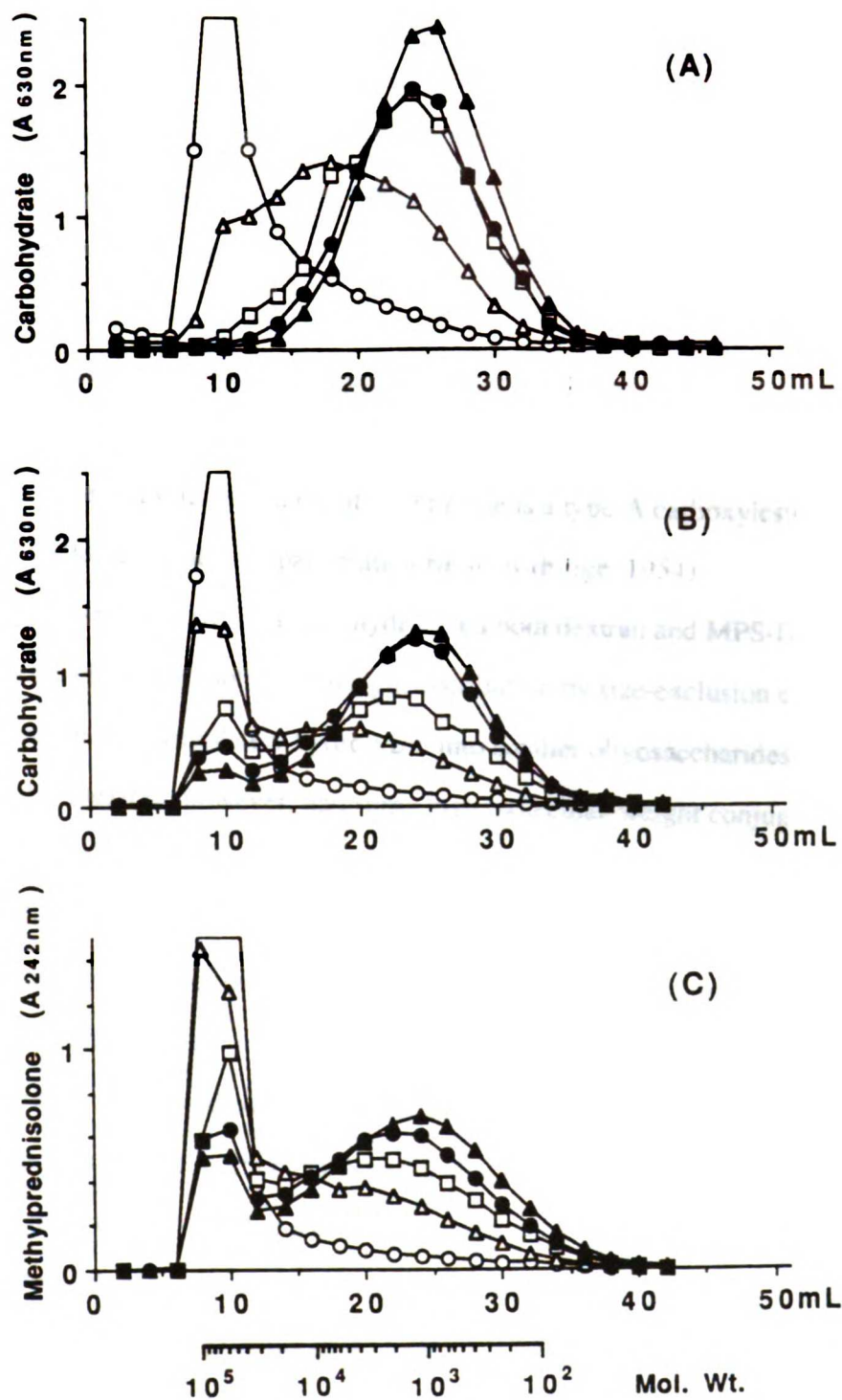
Fig. 5-9   Size-exclusion chromatography of dextran (A) and
MPS-Dextran (B,C) after incubation with dextranase for
0.5 (○), 6 (△), 15 (❑), 25 (●) and 40 (▲) min. Graphs (A) and (B)
show carbohydrate content and graph (C) shows
methylprednisolone content. The molecular weight scale is
indicated under the x-axis.

Triton X-100 increased the hydrolysis rate by 31% at 0.8 g/L but then decreased the hydrolysis rate by 50% at 25 g/L. Hydrolysis rate enhancement and inhibition by increasing concentration of Triton X-100 has been observed with other esterases (Nègre et al., 1988).

In summary, the enzyme has cysteine rather than serine at the reactive site, is not dependent on heavy metal ions and is membrane associated. The enzyme is not an alkaline phosphatase. These results indicate that the enzyme is a type-A carboxylesterase (Walker & Mackness, 1983), possibly of pancreatic origin (Aldridge, 1954).

Dextranase (5 units/ml) rapidly hydrolyzed both dextran and MPS-Dextran as indicated by the change in molecular weight distribution by size-exclusion chromatography, Fig. 5-9. Dextran was completely hydrolyzed into smaller oligosaccharides within 40 min, Graph A. MPS-Dextran, however, had some high molecular-weight conjugate remaining after 40 min, Graphs B and C. This fraction (up to 12 mL elution volume) was 7% of the total area in the carbohydrate chromatogram (Graph B) whereas it was 18% of the total area of the methylprednisolone-content chromatogram (Graph C). Thus, this high molecular-weight fraction containing a higher proportion of methylprednisolone appears to be less susceptible to hydrolysis by dextranase. This hydrolysis-resistant fraction may explain why the production of glucocorticoid-hemiester slows slightly during incubation of the dextran conjugates with rat cecal and colonic contents.

This study shows that these glucocorticoid-dextran conjugates resist breakdown by high levels of esterase in the contents of the small intestine but are readily degraded in the contents of the cecum and colon where the bacterial count is high. These conjugates, therefore, may be a useful means of selectively delivering glucocorticoid to the large intestine for the treatment of colitis.

Chapter 6

Colonic Permeability

In Chapter 5 incubations of dextran-conjugates with luminal contents of the large intestine were shown to release more glucocorticoid-hemiester than glucocorticoid. Glucocorticoids are well absorbed from the GI tract (Rohdewald et al., 1987), but little is known about the absorption of glucocorticoid-hemiesters (Fleisher et al., 1986). Currently Caco-2 cell monolayers are used extensively as model systems to simulate the intestinal absorption of drugs (Artursson and Karlsson, 1991). Thus, the permeabilities of Caco-2 cell monolayers to glucocorticoids and their derivatives are determined in this chapter to gain insight into absorption from the GI tract (Artursson et al., 1991). In addition, the permeability of rabbit colon mucosa to methylprednisolone-hemisuccinate is measured.

The Caco-2 cell line is derived from a moderately well differentiated colon carcinoma of a 72-year old patient (Pinto et al., 1983). The cells are grown in standard media on collagen-coated polycarbonate membranes (Hidalgo et al., 1989). The collagen coating is believed to regulate cell attachment, growth and polarity (Hidalgo et al., 1989). Microporous polycarbonate membranes allow the presentation of nutrients to the cells' basolateral surface, as occurs in the GI tract (Hidalgo et al., 1989). The cells have apical microvilli, a histological feature commonly found in intestinal epithelial cells (Pinto et al., 1983). Caco-2 cells form well-polarized monolayers joined by tight junctions and resemble colonic-crypt cells with regard to electrical resistance and permeability properties (Grasset et al., 1984). Caco-2 cells further resemble intestinal epithelial cells by expressing aminopeptidase N, sucrase-isomaltase, lactase, alkaline phosphatase and phenol sulfotransferase, but not uridine-diphosphate-glucuronyl-transferase (Neutra and Louvard, 1989, Baranczyk-Kuzma et al., 1991).
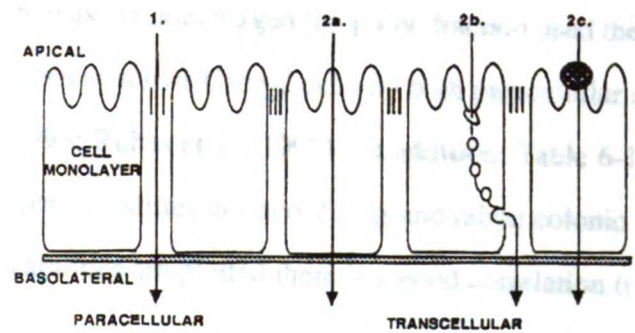
Fig. 6-1   Diagram of routes and mechanisms for the transport of substances

across a cell monolayer. 1, paracellular; 2, transcellular (2a, diffusion;

2b, transcytosis and 2c, carrier-mediated).

Figure from Wilson, (1990).

Drugs permeate cell monolayers by paracellular (between cells) or transcellular (through cells) routes (Wilson, 1990) Figure 6-1. Hidalgo et al. (1989) studied paracellular transport of polar compounds across Caco-2 cell monolayers. When a solution of dextran (mol. wt. 70,000) was applied to the apical surface of a 10 day-old Caco-2 monolayer, only 0.045 % permeated across the cells in an hour (Hidalgo et al., 1989). The permeability of a number compounds was measured in Caco-2 cell monolayers and rabbit colonic mucosa, Table 6-1. The authors hypothesized that the negatively-charged fraction of compounds moved across the monolayer by the paracellular route, whereas the noncharged lipophilic fraction used the transcellular route, e.g., naproxen moves across Caco-2 cells by both paracellular and transcellular routes (Jezyk et al., 1993; Rubas et al., 1992). In addition, Table 6-1 shows a relationship between permeabilities in Caco-2 cells and rabbit colonic mucosa. When the six pairs of permeabilities are plotted there is a good correlation (r = 0.97). Thus, permeability measurements through Caco-2 cells can be used to estimate colonic mucosal permeability.

Materials and Methods

Cell Culture

Caco-2 cells were grown as previously described (Rubas et al., 1992). Briefly, the cells were maintained at 37°C in Dulbecco's modified Eagle's medium with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate), supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were trypsinized and seeded at a density of 63,000 cells/cm^2 on prewetted collagen-coated polycarbonate membranes (Snapwells®). The monolayers were used between

days 20 and 30.

Table 6-1

Log (Partition Coefficients) and Permeability Values of Selected Compounds in Caco-2 Cells and Rabbit Colonic Mucosa[a]

Compound	Mol. Wt.	Log (Partition coefficient)[b]	Caco-2 Permeability Coefficient, (x 10^{-7} cm/sec)	Colonic Mucosa Permeability Coefficient, (x 10^{-7} cm/sec)
Methanol	32	-0.74	1318 ± 72[c]	690 ± 71
Mannitol	182	-3.10	32 ± 10	55 ± 5
Ganciclovir	255	-1.65	27 ± 7	38 ± 6
PEG 900	900	n.d.[d]	121 ± 3	18 ± 3
PEG 4000	4000	-5.10	10 ± 3	n.d.[d]
Naproxen	250	0.42	742 ± 89	394 ± 50
Hydrocortisone	362	1.20	354 ± 10	39 ± 7
Progesterone	315	3.89	789 ± 57	348 ± 52

[a] Table composed from data published by Rubas et al. (1993) and Jezyk et al. (1992).
[b] measured in n-octanol/Kreb's Ringer bicarbonate buffer (pH 7.4), at 37°C.
[c] Mean ± S.E.M., with 7 to 15 replicates in each group.
[d] not determined.

Rabbit Colon Mucosa Preparation

A male albino New Zealand rabbit (Hazelton), weighing between 2.5 and 3.5 kg was sacrificed by rapid injection of sodium pentobarbital (80 mg/kg) through a marginal ear vein. Following a midline incision, the intestinal tract was removed and colon was isolated. Colon tissue was placed immediately into chilled Kreb's Ringer bicarbonate buffer, (pH 7.4). The entire procedure lasted less than 10 minutes.

The colon was opened along the mesenteric border, and stripped of the muscle layers to prepare the musosa for permeability experiments described below.

Permeability Measurements

Experiments were performed using the Sweetana/Grass diffusion cell (Figure 6-2) for both the Caco-2 and colonic mucosa preparations as previously described (Grass and Sweetana, 1988; Rubas et al., 1992). Caco-2 monolayers were washed free of media with Kreb's ringer bicarbonate buffer just prior to use. The monolayer, with attached polycarbonate membrane, was placed between the two halves of the diffusion cell. The rabbit colon mucosa was placed into the diffusion cell in an similar manner.

Saturated solutions of: dexamethasone, (D); methylprednisolone, (MP); dexamethasone-hemisuccinate (DS); methylprednisolone-hemisuccinate, (MPS) and dexamethasone-hemiglutarate, (DG) were prepared at 37°C in oxygenated Kreb's Ringer bicarbonate buffer. A 1 mL sample of the saturated solution was immediately taken and quick-frozen for later analysis. The saturated solutions are used as 'donor' solutions while plain Kreb's Ringer bicarbonate buffer was the 'receiver' solution.

Mucosal (donor) and serosal (receiver) solutions, 1 mL each, were placed into the respective chambers of the diffusion cell. Carbogen (95%O_2/5% CO_2) was bubbled into each chamber from a gas port, Figure 6-2. The bubbling gas fulfilled two purposes: constant aeration maintained tissue viability during the experiment, and fluid convection
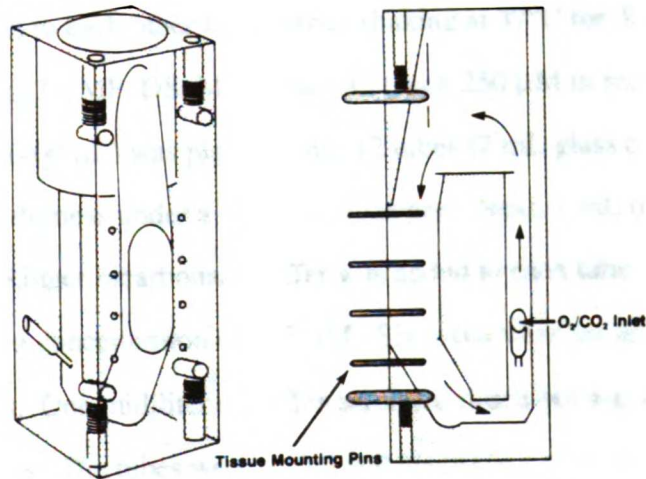
Fig. 6-2   Sweetana/Grass diffusion cell. Caco-2 cell monolayer or colonic mucosa is

mounted between acrylic half-cells. Buffer is circulated by gas lift

(O_2/CO_2) at inlet and flows in the direction of arrows,

parallel to the tissue surface. Figure from Grass et al. (1988).

ensured that the solutions were always well-mixed. The whole apparatus was maintained at 37°C throughout the experiment. The entire receiver phase was withdrawn and replaced with fresh, prewarmed Kreb's ringer bicarbonate buffer approximately every 30 minutes over 2 -3 hours. The samples were quick-frozen on dry ice. At the end of the experiment the entire donor phase was removed, and also quick-frozen for later analysis.

Partition Coefficients

n-Octanol (Aldrich, Milwaukee, WI) and Kreb's Ringer bicarbonate buffer were saturated with respect to each other by vigorous shaking at 37°C for 30 minutes. A stock solution containing D, MP, DS, MPS, and DG (each 250 μM in methanol) was prepared. Stock solution (400 μL) was pipetted into 12 tubes (7 mL glass culture tubes), and evaporated to dryness under a stream of nitrogen. Next, 1 mL of the octanol-saturated Kreb's Ringer bicarbonate buffer was added to each tube. Thus, each compound was present at a concentration of 100 μM. Six tubes were set aside to serve as prepartitioning controls. One milliliter of buffer-saturated n-octanol was added to each of the remaining tubes. The tubes were sealed and placed in a shaking water bath at 37°C in a horizontal position to ensure that the phases had maximal contact. After 2 hours the tubes were centrifuged at 1,000 x g for 30 seconds to separate the phases. The buffer phase was removed for analysis.

Sample Analysis

The 1 mL samples from the permeability and partitioning experiments were adjusted to pH 5 by the addition of 50 μL of 6% phosphoric acid. Next, 100 μL of prednisone solution (5 μM in methanol) was added as an internal standard. The donor phase solutions from the permeability experiments were diluted by the addition of 1 mL of methanol. This step ensured the complete dissolution of drug which was

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precipitated during freezing. The buffer/methanol solution was then diluted 100-fold in Kreb's Ringer bicarbonate buffer. A 1 mL aliquot was taken and treated as described above for the samples. Stock solution, (10, 20, 40, 80, 160 and 320 μL) was pipetted into glass culture tubes and evaporated under a stream of nitrogen. Next, 1 mL of Kreb's Ringer bicarbonate buffer was added to each tube, and treated as the experimental samples. One hundred microliters of each sample and standard was injected into the HPLC for the simultaneous analysis of glucocorticoids and their hemiesters. HPLC conditions were the same as described in Chapter 5. Permeability was calculated using the following relationship:

$$\text{Permeability (cm/sec)} = \frac{\frac{dC}{dt}}{C_o} \cdot \frac{V}{A}$$

where:

dC/dt = initial slope of receiver concentration vs time plot

C_o = initial concentration of drug in the donor phase

V = volume of the receiver phase (5 cm^3 in Caco-2 experiments and 1 cm^3 in rabbit colon experiments).

A = area of monolayer/tissue sample (1.13 cm^2 in Caco-2 experiments and 0.64 cm^2 in rabbit colon experiments).

Statistical Analysis

Results are presented as means ± standard error of the means (S.E.M.). Overall statistical significance was determined with one-way analysis of variance. Post-tests were individual pairwise comparisons with the Student's t-test.

Results and Discussion

The Caco-2 cell monolayer permeability results are given in Table 6-2 and plotted on a semilogarithmic scale in Figure 6-3. The cells were highly permeable to both dexamethasone and methylprednisolone; however, dexamethasone crossed significantly faster than methylprednisolone ($p < 0.01$). Dexamethasone and methylprednisolone permeated the cells faster than all of their respective derivatives ($p < 0.0001$). The cells were significantly more permeable to dexamethasone-hemiglutarate than dexamethasone-hemisuccinate and methylprednisolone-hemisuccinate ($p < 0.05$).

The permeation of methylprednisolone-hemisuccinate through rabbit colon mucosa was $3.3 \pm 1.1 \times 10^{-7}$ cm/sec (n=3). In addition, a permeability measurement was made with rectal tissue, with permeability of 10.2×10^{-7} cm/sec (n=1). These colonic permeability measurements are an order of magnitude lower than hydrocortisone ($38.5 \pm 6.5 \times 10^{-7}$ cm/sec) (Rubas et al., 1992).

Partition coefficients are shown in Table 6-2. Dexamethasone and methylprednisolone had good lipid solubility, evidenced by their relatively high log partition coefficients (1.55 and 1.75, respectively). In contrast, the hemiesters had low partition coefficients at these conditions (-0.57, -0.91 and -0.26, respectively). The hemiesters were most probably ionized at pH 7.4 and thus had poor solubility in the octanol phase. The partition coefficient results indicate that the glucocorticoids are sufficiently lipophilic to pass through the cells by the transcellular route.
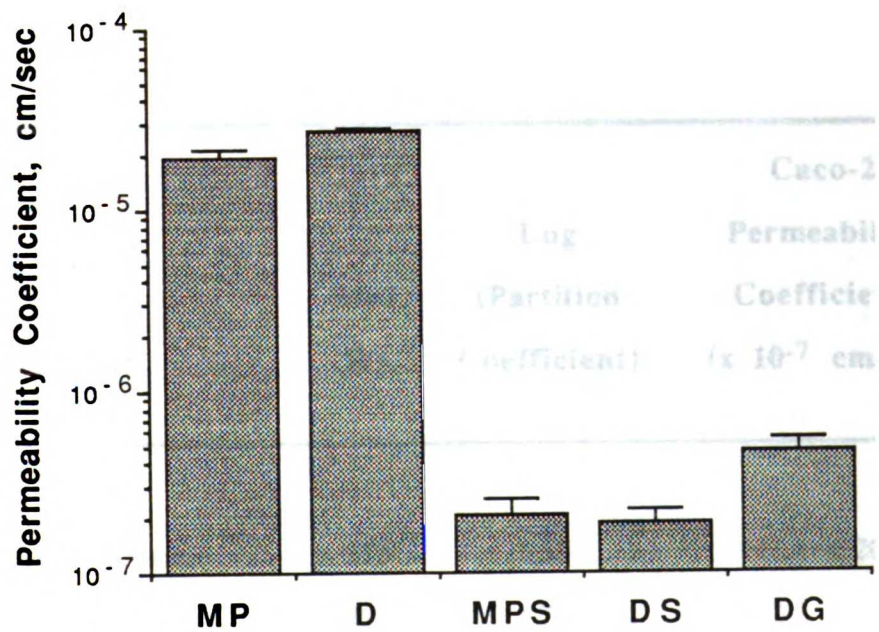
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Fig. 6-3   Semilogarithmic plot of permeability coefficients of glucocorticoids and

hemiesters through Caco-2 cells. Abbreviations: MP, methylprednisolone;

D, dexamethasone; MPS, methylprednisolone-hemisuccinate;

DS, dexamethasone-hemisuccinate and DG, dexamethasone-hemiglutarate.

Means + SEM. See Table 6-2 for number of replicates in each group.

Table 6-2

Log (Partition Coefficients) and Permeability Values of Glucocorticoids and Glucocorticoid-Hemiesters in Caco-2 Cells

Compound	Mol. Wt.	Log (Partition Coefficient)	Caco-2 Permeability Coefficient, (x 10^{-7} cm/sec)	n
Methylprednisolone	375	1.55	197.8 ± 20.6	5
Dexamethasone	393	1.73	272.3 ± 11.5	7
Methylprednisolone-Hemisuccinate	475	-0.57	2.0 ± 0.5	3
Dexamethasone-Hemisuccinate	493	-0.91	1.9 ± 0.3	3
Dexamethasone-Hemiglutarate	507	-0.26	4.7 ± 0.7	4

[a] Mean ± S.E.M.

When solutes are charged, as in the case of the hemiesters, the transcellular route is not favored, so paracellular transport is the predominant pathway. The Caco-2 cell monolayer has been shown to possess numerous tight junctions, restricting the paracellular route to small molecules (Hidalgo et al., 1989). As previously shown in Chapter 5, incubation in vitro of dextran-conjugates with contents of the large intestine resulted mainly in the production of glucocorticoid-hemiester. Based on Caco-2 and colonic mucosal permeabilities, we propose that glucocorticoid would be absorbed more readily than hemiester (Artursson et al., 1991). This may limit the usefulness of dextran-hemiester-conjugates for colon-specific drug delivery.

Caco-2 cells, however, are a model system. They should be used as a predictive tool and not, however, as a replacement for experiments in vivo. Certain enzymes, e.g., esterases, may be produced in vivo to a higher degree than in Caco-2 cell monolayers (Baranczyk-Kuzma et al., 1991). The ultimate test is to administer the dextran-conjugates in vivo. This, therefore, is the subject of Chapters 7 and 8.

Chapter 7

Pharmacokinetic Assessment of Colonic Delivery

In this chapter dexamethasone-succinate-dextran (DS-Dextran) and dexamethasone-glutarate-dextran (DG-Dextran) were administered to two groups of rats by intragastric infusion. In two additional groups dexamethasone disodium phosphate and dexamethasone-hemisuccinate were each administered by subcutaneous infusion. In a fifth group, dexamethasone was administered by intragastric infusion. All five groups of rats were infused for sufficient time to achieve steady-state in both blood and GI tract tissues. The steady-state concentrations at these sites were used to calculate a drug delivery index (DDI). The DDI is identical to the previously described selective advantage (Eriksson and Tozer, 1987) and drug targeting index (Hunt et al., 1986). The pharmacokinetics of methylprednisolone was investigated in a final group of rats after subcutaneous infusion. These results were used to determine the feasibility of using methylprednisolone-succinate-dextran (MPS-Dextran) as a colon-specific prodrug for oral administration.

Materials and Methods

Methylprednisolone, methylprednisolone disodium phosphate and dexamethasone were generous gifts from Upjohn (Kalamazoo, MI, USA). Dexamethasone disodium phosphate was a gift from Merck Sharp & Dohme Research Lab. (Rahway, NJ, USA). Acetonitrile, methanol, methyl-t-butyl ether and pentane were of HPLC grade (Fisher

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Scientific, Pittsburgh, PA, USA). Propylene glycol (USP Grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA).

Glucocorticoid-hemiesters (dexamethasone-hemisuccinate (monohydrate), dexamethasone-hemiglutarate and methylprednisolone-hemisuccinate) were reacted with dextran to form covalently-linked conjugates: DS-Dextran, DG-Dextran and MPS-Dextran as described in Chapter 4. Glucocorticoid content (mg per 100 mg dextran conjugate), measured by HPLC after alkaline hydrolysis, were as follows: DS-Dextran, 7.4; DG-Dextran, 5.7 and MPS-Dextran, 10.5.

Apparatus

The glucocorticoids were measured by HPLC using the following equipment: Shimadzu SCL-6A system controller, LC-6A pumps, and a SPD-6AV variable wavelength detector (Kyoto, Japan); an octadecylsilane column, (250 mm x 4.5 mm, Fisher, Pittsburgh, PA), with a PRP-1 precolumn, (Hamilton, Reno, NV); Waters WISP 710B autoinjector (Milford, MA, USA); and a Hewlett Packard HP3396A integrator (Avondale, PA, USA). The mobile phase consisted of 35% acetonitrile and 65% buffer (50 mM trisodium citrate adjusted to pH 4.1 with phosphoric acid). For the analysis of methylprednisolone in cecal and colonic tissues, 30% acetonitrile was used. A flow rate of 1.5 mL/min and a detection wavelength of 242 nm were used. A Brinkmann Homogenizer (Sybron Corp., Cantiague, NY, USA) fitted with a Polytron-Aggregate® blade assembly (Kinematica, Littau,Switzerland) was used to homogenize tissue samples. Implanted osmotic pumps (ALZET®, Lot # 047101, Model 2001D, 9 ± 0.3 µL/hr for 24 hours, Alza Corporation, Palo Alto, CA) were used for all infusions. Pumps were filled with the drug solutions and equilibrated at 37°C for 2 hours in isotonic saline. Catheters were attached to the pumps for intragastric infusions, Figure 7-1.
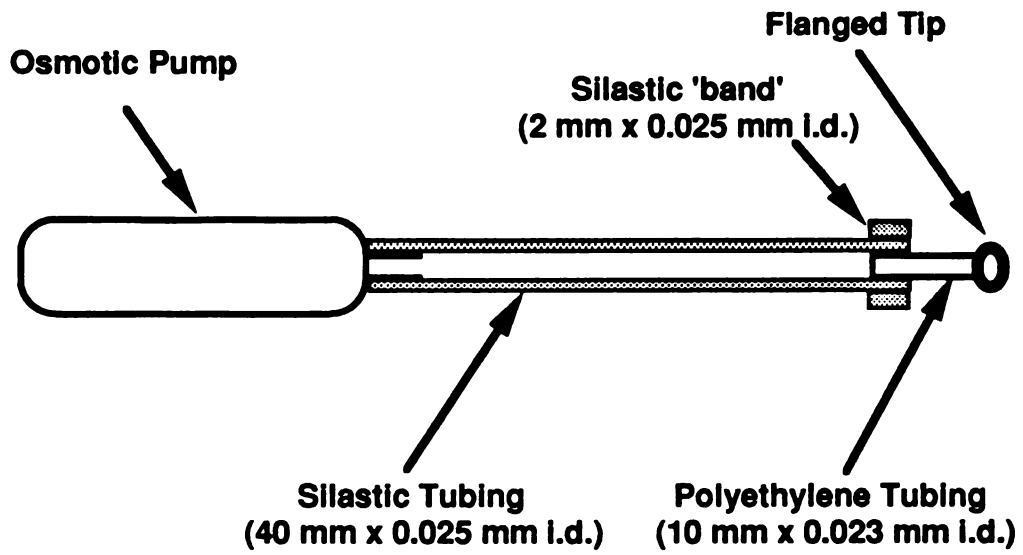
Fig. 7-1   Specifications of the cannula used for intragastric infusions.

Animals

Male Sprague-Dawley rats (260-320 g) were fed a standard diet (Purina Rodent Chow, # 50-01, Ralston-Purina, Richmond, IN, USA). Food and water were provided *ad libitum*. The rats were housed in a room with 12-hour light/dark cycle (9:00 am - 9:00 pm). Because glucocorticoids undergo circadian fluctuations (Fisher et al., 1992), surgery was performed at a fixed time, 10:00 am.

Surgery

After lightly anesthetizing by inhalation of methoxyflurane, rats were given ketamine (80 mg/kg) and acepromazine (3 mg/kg) by injection into the thigh muscle. The abdomen was shaved and swabbed with povidone-iodine. For subcutaneous infusions, a 1-cm midline incision was made through the skin and a subcutaneous pocket (approx. 15-20 cm^2) was created. After implanting the pump, the skin was sutured with four discontinuous stitches using 3-0 silk. For intragastric infusions, a 5-cm midline incision was made through the skin, and a subcutaneous pocket was created. Next, the abdominal muscle was cut along the midline to create a 4-cm incision. The pump and attached catheter was placed in the subcutaneous pocket and the catheter was passed into the peritoneal cavity through a small hole (1-2 mm) made in the muscle. A square comprising four purse stitches was made in the muscular layer of the hind stomach (approximately 10 mm from the antrum, and midway between the lesser and greater curvatures). A hole was made at the center of this ring of stitches and the tip of the catheter was passed through into the stomach lumen. The purse stitches were pulled tight around the catheter. The muscle and skin layers were closed with 3-0 silk sutures. Animals regained consciousness within 2 hours. Throughout the remainder of the experiment, they were fully ambulatory and were given food and water *ad libitum*.

Dosing

The delivery of drug to the cecum and colon is expected to be delayed by about 5 hours, the transit time in the upper GI tract (Johansson and Nylander, 1968; Mori et al., 1989). In addition, the time for accumulation in the body is expected to be about 7 - 10 hours, as the elimination half life of dexamethasone in the blood has been reported to be 2 -3 hours (Moldenhauer et al., 1991; Varma and Yue, 1984). Overall, a 22-hour infusion was expected to be sufficient to attain steady-state in both the large intestine and blood (Rowland and Tozer, 1989). The same conclusion applies to methylprednisolone for which an elimination half-life of 0.5 hour has been reported in rats (Haughey and Jusko, 1992).

Due to pump volume and drug solubility constraints, concentrations of the solutions placed in the pump were: dexamethasone, 1.17 mg/mL; dexamethasone disodium phosphate, 1.54 mg/mL; dexamethasone-hemisucinate (monohydrate), 1.52 mg/ml; DS-Dextran, 15.81 mg/mL and DG-Dextran, 20.53 mg/ml. All solutions were prepared in deionized distilled water, except dexamethasone and dexamethasone-hemisuccinate monohydrate for which 60% propylene glycol: 40% deionized distilled water was needed.

Dexamethasone plasma clearance (0.26 L/hr/kg) was calculated from data reported in rats (Moldenhauer et al., 1991). In preliminary experiments we found the ratio of blood/plasma concentrations to be 0.74, thus blood clearance was estimated to be approximately 0.35 L/hr/kg. In all cases the infusion rate of glucocorticoid was approximately 35 µg/hr/kg. In a 300-g rat, assuming complete bioavailability, this infusion rate would be expected to give a steady-state blood concentration of 100 ng/mL.

With a plasma clearance of 5.44 L/hr (Haughey et al., 1992), methylprednisolone had to be infused at a rate considerably greater than that for dexamethasone to attain a steady-state blood concentration of approximately 100 ng/mL. In these experiments methylprednisolone disodium phosphate was infused at 900 µg/hr/kg, which is the equivalent of 683 µg methylprednisolone/hr/kg

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In the present experiments, dexamethasone and methylprednisolone were infused at 35 and 683 μg/hr/kg, respectively. The recommended doses of dexamethasone and methylprednisolone for the treatment of colitis in humans are 10 and 60 μg/hr/kg respectively (Haynes and Murad, 1980).

Sample Preparation and Analysis

After 22 hours (8:00 am) the animals were anesthetized with methoxyflurane and the thorax was opened. Blood was withdrawn from the right ventricle using a heparinized 10 mL syringe (containing 0.1 mL of 5,000 u/mL heparin solution). The GI tract was removed and chilled on ice within 10 min. The luminal contents of each tissue (stomach, proximal (PSI) and distal (DSI) small intestine, cecum, and colon) were manually removed and weighed. The tissues were washed in isotonic saline, gently blotted with laboratory wipes and weighed. Isotonic saline was added to give a 20 %w/v dilution and the mixture was homogenized at speed 5 for 30 seconds. Two milliliters of either blood or tissue homogenate was placed in 10 mL culture tubes in duplicate. Sodium bicarbonate (200 μL of 10% w/v solution) and internal standard (100 μL of 5 μM methylprednisolone or dexamethasone in methanol) were added to each tube. Next, n-pentane (5 mL) was added and the tubes were rotated for 5 min. After a 2-min centrifugation at 1,000 g the pentane phase was removed and discarded. Methyl-t-butyl ether (5 mL) was added and the tubes were again rotated and centrifuged. The organic phase was removed and evaporated at 50 °C under a stream of nitrogen. After reconstitution with 100 μL of methanol, 50 μL was injected onto the HPLC. Preliminary experiments showed there to be no significant difference (p > 0.05) between extraction from isotonic saline and the tissue samples, therefore saline was used to construct all standard curves. Standard curves covered concentrations from 10 - 60 ng/mL, samples containing concentrations above this range were diluted prior to assay. Standard curves were linear ($r^2 > 0.98$) throughout this range.

The minimum quantifiable level, (the concentration which had a 20 % coefficient of variation using 5 replicates), of the glucocorticoids was 6 ng/mL.

Calculations and Statistical Analysis

The drug delivery index (DDI) was calculated as follows:

$$
\textbf{Drug Delivery Index (DDI)} \atop \text{[Dextran Conjugate (IG) vs. Glucocorticoid (IG or SQ)]} = \frac{\dfrac{Css_{Tissue}(\text{Dextran Conjugate, IG})}{Css_{Tissue}(\text{Drug, IG or SQ})}}{\dfrac{Css_{Blood}(\text{Dextran Conjugate, IG})}{Css_{Blood}(\text{Drug, IG or SQ})}} \qquad (1)
$$

$$
= \frac{\dfrac{Css_{Tissue}(\text{Dextran Conjugate, IG})}{Css_{Blood}(\text{Dextran Conjugate, IG})}}{\dfrac{Css_{Tissue}(\text{Drug, IG or SQ})}{Css_{Blood}(\text{Drug, IG or SQ})}} \qquad (2)
$$

where the parentheses refer to the compound administered and the route of adminisistration. The abbreviations are: IG, intragastric infusion; SQ, subcutaneous infusion; Css, steady-state concentration in tissue or blood. The numerator of equation 1 is the factor by which concentrations are increased in the target tissue after dextran-conjugate compared to drug administration. This is the factor by which the dextran-conjugate dose could be reduced to maintain the same efficacy. The denominator of equation 1 is the relative bioavailability of the drug following the two administrations. This ratio is an estimate of the reduction in the systemic exposure, and therefore the systemic toxicity. The combination, the DDI, is an estimate of the advantage gained by decreasing both the dose and the systemic exposure. Equation 1 is rearranged to give equation 2. This equation uses tissue to blood concentration ratios after dextran conjugate and drug administration. These ratios were measured in each set of experiments and used to calculate the DDI.

One-way analysis of variance, unpaired t-tests and Student-Newman-Keuls tests were used to measure the magnitude of statistical significance.

Results and Discussion

Table 7-1 shows the steady-state blood and tissue concentrations of dexamethasone after dosing with dexamethasone, DS-Dextran and DG-Dextran by intragastric infusion. In addition, Table 7-1 shows steady-state concentrations of dexamethasone obtained after administration of dexamethasone-phosphate and dexamethasone-hemisuccinate by subcutaneous infusion. In all cases, the rate of dexamethasone infusion was 43 μg/hr/kg.

Steady-state blood concentrations of dexamethasone were significantly lower after intragastric administration of the dextran conjugates (DS-Dextran and DG-Dextran) compared to the other modes of administration. There was no significant difference between blood levels of dexamethasone after the administration of dexamethasone (IG), dexamethasone-phosphate (SQ) or dexamethasone-hemisuccinate (SQ).

It has been shown that adrenosuppression is correlated with exogenous glucocorticoid blood concentrations (English et al., 1975; Loew et al., 1986). Both DS-Dextran and DG-Dextran gave lower blood concentrations of dexamethasone and therefore should give less adrenosuppression than dexamethasone itself.

Dexamethasone concentrations in all tissues were approximately 100 ng/ml after subcutaneous administration of dexamethasone-phosphate, with no statistically significant difference among the tissues. This indicates that dexamethasone formed from dexamethasone-phosphate tends to be evenly distributed throughout the body. The tissue to blood concentration ratios were 0.64 and 0.66 for cecum and colon, respectively. When dexamethasone-hemisuccinate was administered by subcutaneous infusion, dexamethasone concentrations in the cecum and colon were significantly lower ($p < 0.05$) than blood. The tissue to blood ratios were 0.44 and 0.39 for cecum and colon, respectively.

Table 7-1

Dexamethasone Concentrations (ng/mL) in Blood and Other Tissues after a 22-Hour Constant-Rate Infusion

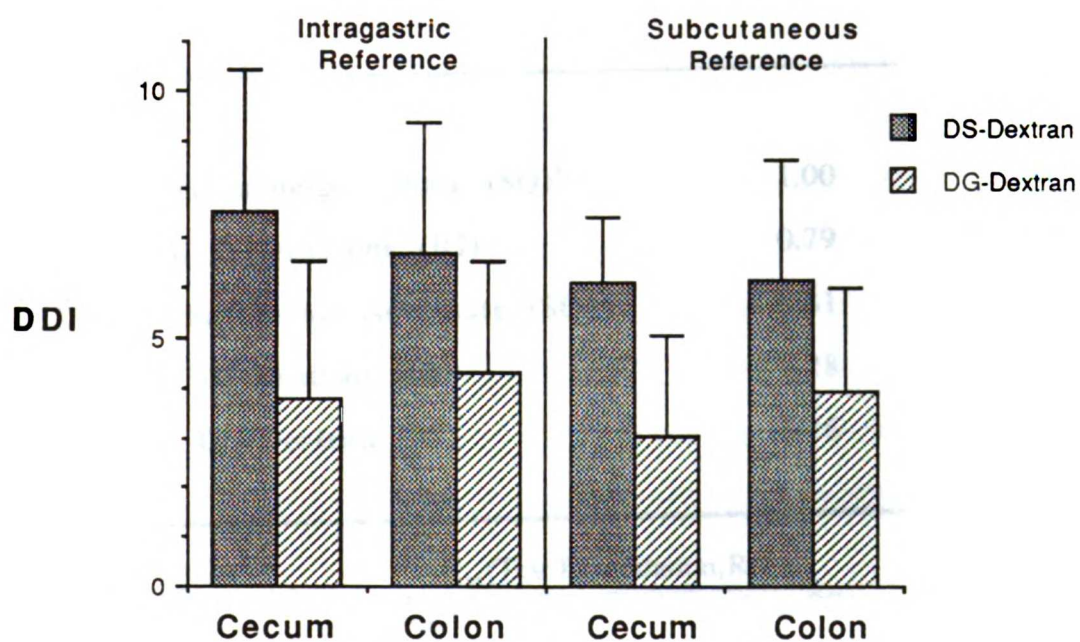|	Dexamethasone-Phosphate SQ[a]	Dexamethasone IG	DS-Dextran IG	DG-Dextran IG	Dexamethasone-hemisuccinate SQ
Blood	107±24[b]	84±17	30±10	17±5	87±11
Stomach	92±42	165±57*	31±11	20±21	61±12
PSI	110±36	110±38	32±9	30±10	67±20
DSI	105±63	90±19	35±10	24±5	64±17
Cecum	69±23	41±11	113±52*	37±32	38±21*
Colon	71±26	50±17	113±20*	44±13	34±18*

[a] SQ = subcutaneous, IG = intragastric.
[b] mean ± standard deviation for n = 5.
* denotes significant difference (p < 0.05) compared to blood concentration within that group.

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Table 7-1 shows that dexamethasone levels were significantly (p < 0.05) higher in the stomach than in blood or further down the GI tract. This confirms that gastric infusion was occurring and that dexamethasone was absorbed in the small intestine. The tissue to blood concentration ratios were 0.48 and 0.60 for cecum and colon, respectively. Oral administration of dexamethasone does not appear to offer any advantage over parenteral administration of a water-soluble prodrug as the cecal tissue to blood ratios were approximately the same: dexamethasone (IG), 0.49; dexamethasone-phosphate (SQ), 0.64 and dexamethasone-hemisuccinate (SQ), 0.44.

DS-Dextran resulted in significantly higher concentrations in the cecum and colon (p < 0.05) compared to the other modes of dexamethasone administration. The tissue to blood ratio was 3.77 in both the cecum and colon. This ratio is due to the combination of higher tissue levels and lower blood levels. DG-Dextran did not give significantly higher tissue levels; however, because of the lower blood levels, the tissue to blood ratios were 2.18 and 2.59 for cecum and colon, respectively. Thus, the dextran conjugates have two major advantages over other modes of administration. First, DS-Dextran gives significantly higher (p < 0.05) levels of dexamethasone in the large intestine which would theoretically lead to enhanced local antiinflammatory effect. Second, the dextran conjugates give significantly lower blood concentrations (p < 0.05) compared to parenteral and oral administration of dexamethasone which may reduce systemic side-effects.

The drug delivery index (DDI, equations 1 and 2) summarizes the advantages of administration of dexamethasone in dextran-conjugates relative to dexamethasone-phosphate SQ and dexamethasone IG. The calculated DDI values are shown in Figure 7-2. The figure shows that DS-Dextran gives higher DDI values than DG-Dextran in both cecum and colon. The dextran conjugates yield larger DDI values when intragastric dexamethasone was used as the reference ratio than when subcutaneous dexamethasone-phosphate was used.

Fig. 7-2   Drug delivery indices for DS-Dextran and DG-Dextran in the
cecum and colon. Blood and tissue reference concentrations were after
intragastric and subcutaneous infusions of dexamethasone and disodium
dexamethasone-phosphate, respectively. Data are obtained from groups of
five rats and error bars are the calculated standard deviations.

Table 7-2

Relative Bioavailability of Dexamethasone

Compound	Relative Bioavailability[a]
Dexamethasone-phosphate (SQ)[b]	1.00
Dexamethasone (IG)	0.79
Dexamethasone-hemisuccinate (SQ)	0.81
DS-Dextran (IG)	0.28
DG-Dextran (IG)	0.16

[a] Relative Bioavailability $= \dfrac{C_{ss}(\text{Test Formulation, Route})}{C_{ss}(\text{Dexamethasone-phosphate, SQ})}$

[b] SQ = subcutaneous, IG = intragastric.

The steady-state blood data were used to calculate the relative bioavailability of dexamethasone in rats (Table 7-2). The reference was dexamethasone levels measured in blood after subcutaneous infusion of dexamethasone-phosphate. Dexamethasone blood clearance after administration of dexamethasone-phosphate was calculated to be 0.411 L/hr/kg, confirming that dexamethasone is a low clearance drug. This result is close to that (0.350 L/hr/kg) calculated from published data (Moldenhauer et al., 1991). Apparent bioavailability was 79% after oral administration of dexamethasone. The apparent bioavailability of dexamethasone after administration of the dextran conjugates was low (28% and 16% for DS-Dextran and DG-Dextran, respectively). It was shown in Chapter 5 that dexamethasone is released from DS-Dextran primarily as dexamethasone-hemisuccinate. If the hemiester is absorbed fom the large intestine it could undergo sequential first-pass metabolism. To test this theory, dexamethasone-hemisuccinate was administered by subcutaneous infusion (Table 7-1, 7-2). The calculated bioavailability was 81%, and blood clearance was 0.461 L/hr/kg indicating that dexamethasone-hemisuccinate is almost completely converted to dexamethasone without further metabolism during the first pass through the liver.

Perhaps the low bioavailabilities of dexamethasone from the dextran conjugates is due to the majority of the dose remaining attached to dextran in the GI tract. After administration of DS-Dextran and DG-Dextran, dexamethasone concentrations were almost identical in both cecum and colon. Thus, the dextran-conjugates release dexamethasone along the whole length of the large intestine. This observation has important clinical consequences for the treatment of both proximal and distal colitis (Hanauer and Kirsner, 1988). It is important to remember that colonic drug delivery does not require complete bioavailability. In fact, low blood concentrations are desirable provided tissue concentrations are high.
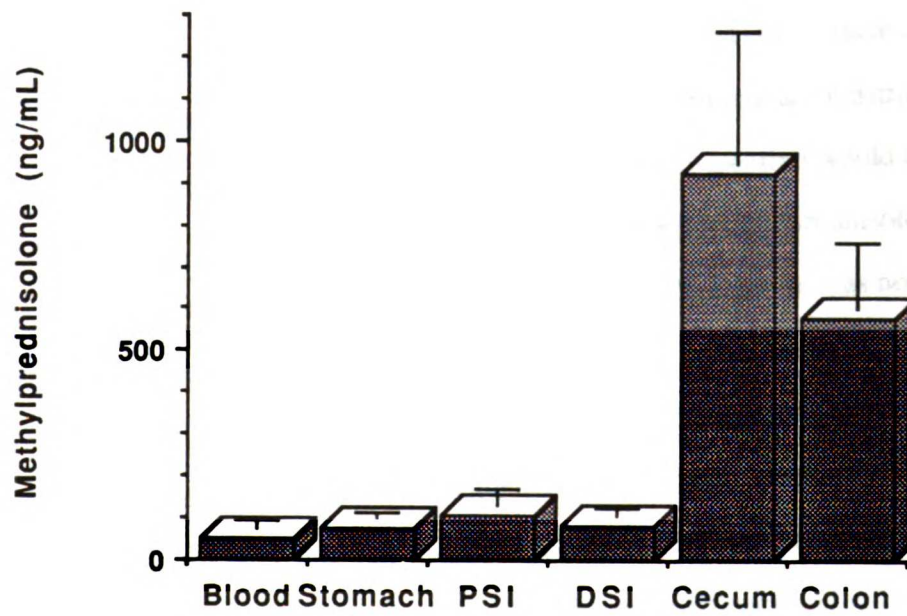
Fig. 7-3   Blood and other tissue methylprednisolone concentrations after subcutaneous infusions of disodium methylprednisolone-phosphate. Mean ± S.D. , n = 5 rats.

Subcutaneous administration of methylprednisolone-phosphate gave very high

levels of methylprednisolone in both cecum and colon, yet blood levels remained low,

Figure 7-3. Blood clearance of methylprednisolone after administration of the phosphate

was 16.4 L/hr/kg, confirming that methylprednisolone is a high clearance drug (hepatic

blood flow in the rat is 4.145 L/hr/kg, (Boxenbaum, 1980)). The tissue to blood

concentration ratios were 19 and 12 for cecum and colon, respectively. One possible

explanation for the high tissue concentrations is enterohepatic cycling, a common

phenomenon with contraceptive steroids (Fotherby and James, 1972). Indeed, the

extremely high concentrations of methylprednisolone in cecum and colon tissue make this

an effective form of colon-specific drug delivery even after parenteral administration. It

was considered unlikely that intragastric administration of MPS-Dextran would deliver

methylprednisolone to the colon as effectively as subcutaneous methylprednisolone-

phosphate. For this reason, intragastric administration of MPS-Dextran was not

investigated in vivo.

These experiments used pharmacokinetic principles to assess the colon-specific

delivery of dexamethasone from dextran-conjugates. DS-Dextran increased concentrations

of dexamethasone in the large intestine threefold and decreased blood concentrations

threefold. Thus, this dextran conjugate should both increase efficacy and reduce toxicity.

DG-Dextran gave a fivefold decrease in blood concentrations while maintaining similar

tissue concentrations. In addition, subcutaneous administration of methylprednisolone as

the disodium phosphate resulted in high concentrations of methylprenisolone in both cecum

and colon, possibly as a result of enterohepatic cycling.

Chapter 8

Pharmacodynamic Assessment of Colonic Delivery

The objectives of the research in this chapter were twofold: the first was to test the efficacy of the glucocorticoid-dextran prodrugs in treating experimental ulcerative colitis. The second was to examine the adrenosuppression following administration of the prodrugs. Colitis was induced in male Sprague-Dawley rats by instillation of acetic acid solution (4%) into the colon (Sharon and Stenson, 1985). This method produces colitis that is macroscopically, histologically and physiologically similar to ulcerative colitis (Fedorak et al. 1990). The colon returns to normal within 12 days. Several measures of drug response were used with major emphasis on the net absorption of fluid and degree of ulceration in the colon. A dose-response model (Gilman et al., 1980) was fitted to both sets of data.

Materials and Methods

Glacial acetic acid, purchased from Fisher Scientific (Nepon, ON, Canada) was used to prepare a 4% solution in water (pH 2.4). All remaining reagent grade chemicals were purchased from Sigma (St. Louis, MO, USA)

Induction of Colitis with 4% Acetic Acid

Colitis was induced as described in detail elsewhere (Sharon et al., 1985). Briefly, non-fasting male Sprague-Dawley rats (250 - 275 g; Biotron, University of Alberta, Edmonton, AB, Canada) were anesthetized via intraperitoneal injection of pentobarbital (55 mg/kg) and atropine (0.5 mg/kg). Through a sterile midline abdominal incision, the colon

was isolated and the junction of the cecum and ascending colon was occluded with a reversible ligature; care was taken to avoid compromising neural or vascular integrity. The colon was cleansed of its luminal contents with 154 mM sodium chloride solution at 37°C, and the residual fluid was manually expressed through the rectum. Acetic acid (4 %, 2 mL) was injected into the lumen of the colon through a 25 gauge needle passed obliquely through the colonic wall just distal to the occluding ligature. Immediately thereafter, 10 mL of air was injected to clear the acetic acid from the colon. The occluding ligature was removed and the midline incision closed. The animals were allowed to recover from the anesthesia in a light-cycled room that provided free access to standard rat chow pellets (5001, Purina Mills Inc., St. Louis, MO, USA) and water. Studies in vivo were carried out 72 hours later. All assays at each drug dosage were performed in the same group of rats.

Administration of Dextran Prodrugs

The dextran conjugate solutions were prepared immediately before their oral administration by mixing the appropriate volume of stock solution with 154 mM sodium chloride to a total volume of 1 mL (pH 7.4). The dextran conjugates were administered by oral gavage 24 and 48 hours after induction of colitis. Dexamethasone 0.22 mmol/kg/day is approximately equivalent to the human dose of prednisone (0.6 mg/kg/day) used to treat idiopathic ulcerative colitis. Rats were administered doses of methylprednisolone and dexamethasone (and equivalent doses of dextran conjugates) ranging from 0.0034 to 0.44 μmole/kg/day.

Colonic Fluid Absorption in Vivo

Colonic absorption of fluid was assessed as described previously (Fedorak et al., 1990). Seventy-two hours after induction of colitis rats were anesthetized with pentobarbital (55 mg/kg) and atropine (0.5 mg/kg) and maintained at 37°C using a

thermostatic heat lamp. The intestinal tract was exposed through a midline abdominal incision. An occluding ligature was placed at the cecal-ascending colon junction. Sodium chloride solution (154 mM, 37°C) was instilled into the proximal colon via a cannula inserted through an incision just distal to the proximal occluding ligature to flush out the luminal contents of the colon. Residual saline was emptied by gentle manual expression. A 12-cm long intestinal loop, beginning 2 cm below the cecal-colonic junction and extending distally to the peritoneal reflection, was created with ligatures. In isolating the loop, care was taken not to compromise mesenteric, vascular or neural integrity. A 27 gauge needle was inserted obliquely through the outer muscle layer along the antimesenteric border, and 2 mL of 37 °C 154 mM sodium chloride was instilled into the empty loop. In all cases, no fluid leakage was detected, and the loop was only mildly distended. The viscera were returned to the abdominal cavity and the incision was closed. Sixty minutes after abdominal closure, the animals were given a pentobarbital overdose (240 mg/kg) and the colonic loop was removed. The length of each loop was recorded. The colonic loops were weighed, this weight (in milligrams) was defined as the 'full weight'. The loops were then opened longitudinally and the tissue was gently patted dry using laboratory wipes. The tissue was weighed again, and this weight (in milligrams) was defined as the 'empty weight.' It was assumed that 2 mL of 154mM sodium chloride solution at 37°C weighed 2000 mg. The net fluid absorption rate was calculated as follows:

$$\text{Net Fluid Absorption Rate } (\mu L/hr/cm) = \frac{2000 \text{ mg} - (\text{Full Weight} - \text{Empty Weight})}{1 \text{ hr} \cdot \text{Loop Length}}$$

The nonlinear regression program *Minim* (Version 1.8a, Dr. R. Purves, Department of Pharmacology, University of Otago, Dunedin, New Zealand) was used to fit the Hill equation (Gilman et al., 1980) to the colonic water-absorption data. To incorporate both positive and negative values, the equation was modified to include the term, Effect_0 ($\mu L/hr/cm$), as follows:

$$\text{Effect} = \text{Effect}_0 + \frac{\text{Effect}_{max} \cdot \text{Dose}^{\gamma}}{(\text{ED}_{50})^{\gamma} + \text{Dose}^{\gamma}}$$

where:

Effect	=	Water flux, $\mu L/hr/$ per cm of intestine
Effect$_0$	=	Water flux in animals pretreated with 4% AAC, $\mu L/hr/$ per cm of intestine
Effect$_{max}$	=	-Effect$_0$ + Control (Control is the value in animals not treated with either 4% acetic acid or drug), $\mu L/hr/$ per cm of intestine
γ	=	Hill coefficient
ED$_{50}$	=	Daily dose which gives half-maximal effect, $\mu mol/kg/day$

Macroscopic Ulceration

The colon was rapidly excised, opened along its mesenteric border, and gently rinsed of its luminal contents with an iced 154 mM solution of sodium chloride. The colon was then placed flat, mucosal surface upwards, on a glass plate chilled to 4°C. A transparent acetate was placed 5 mm above the mucosal surface and the area of ulceration and total surface area were traced by a single observer. Areas in square centimeters were then calculated using a Zeiss computerized videoscope (Videoplan, Carl Zeiss Co., Toronto, ON, Canada). The data were also transformed into a term expressing the improvement in macroscopic ulceration, in which control animals scored 100% and those treated with acetic acid but no drug scored 0%. The Hill equation was then fitted to these data, and the ED$_{50}$ values calculated as described for fluid absorption.

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Myeloperoxidase Activity

An assay of colonic myeloperoxidase activity was used to quantify neutrophil infiltration. The entire length of colon, from the ascending-cecal junction to rectum, was homogenized with a polytron homogenizer (Brinkman Instruments, Rexdale, ON, Canada) three times for 30 seconds each at 4°C in 5 mL of 0.5 % hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6). The homogenate was then sonicated for 10 seconds and assayed for enzymatic activity spectrophotometrically. Supernate (0.1 mL) was combined with 2.9 mL of 50 mM phosphate buffer (pH 6) containing 1.05 mM o-anisidine hydrochloride and 0.15 mM hydrogen peroxide (Bradley et al., 1982). Change in absorbance at 460 nm was measured with a Beckman DU-6 spectrophotometer (Beckman Instruments Inc., Irvine, CA, USA). One unit of myeloperoxidase activity was defined as that degrading 1 μM of peroxide per minute at 25 °C.

Determination of Serum Corticosterone

Blood (2 mL) was collected by intracardiac puncture into an evacuated blood collection tube (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA). The blood was then centrifuged at 4°C for 10 minutes at 4,000 r.p.m. (Centra-7, International Equipment Co., Needham Heights, MA, USA). The serum was removed and stored at -70°C prior to analysis. Stock anti-corticosterone antiserum (B3-163) was obtained from Endocrine Sciences RIA Reagents, (Tarzana, CA, USA). Diluted antiserum was prepared as follows: 10 mL borate buffer (0.05M, pH 8); 400,000 dpm of 1,2-^{3}H-corticosterone; 0.2 mL of 10% of bovine serum albumin in borate buffer; 0.2 mL of 0.25 % bovine gamma globulin in borate buffer; and 100 μL of stock antiserum. The serum sample was placed in a 12 x 75 mm test-tube, diluted with borate buffer and incubated at 60°C for 30 minutes. The solution was transferred to a 2 mL conical tube (Kimax #45150, Fisher Scientific, Nepon, ON, Canada) and incubated with diluted antiserum at 37°C for 45 minutes then at room temperature for 2 hours. Saturated ammonium sulfate solution (0.25

mL) was added to all tubes and the mixture was centrifuged at 3,000 rpm for 10 minutes to separate free and bound steroid. The supernate was decanted into a microvial (Simport Ltd., Quebec City, QB, Canada), 4 mL of scintillation cocktail was added (Scintiverse, bio-HP, Fisher Scientific, Nepon, ON, Canada) and radioactivity was counted by a liquid scintillation counter (LKB Wallace, 1219 Rack Beta, Turka, Finland).

Determination of Plasma ACTH

Blood (3 mL) was collected between 8:00 and 10:00 a.m. by intracardiac puncture following pentobarbital overdose. Blood was drawn into an chilled silanized Vacutainer[®] tube (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA). The blood was then centrifuged at 4°C for 10 minutes at 4,000 r.p.m. (Centra-7, International Equipment Co., Needham Heights, MA, USA). The plasma was transferred to a polypropylene tube and stored at -70°C until analysis. The sample did not come into contact with glass at any time throughout the sample collection procedure. ACTH was measured using a commercial immunoassay kit (Nichols Institute, San Juan, Capistrano, CA, USA). The ACTH immunoassay incorporates a monoclonal antibody and a polyclonal antibody, both with high affinity and specificity for defined amino acid regions of the ACTH molecule. The polyclonal antibody is prepared by affinity chromatography to bind only to the C-terminal region of ACTH. The monoclonal antibody binds only to the N-terminal region of ACTH. Both antibodies bind ACTH without competition or steric interference from each other, and form a soluble sandwich complex. The monoclonal antibody is radiolabeled for detection, while the polyclonal antibody is coupled to biotin. The addition to the reaction mixture of an avidin-coated plastic bead allows for a specific and efficient means of binding the sandwich complex via the high affinity reaction between biotin and avidin (Hodgkinson et al., 1984).

In the assay, standards, controls, and samples were incubated with a solution containing both the radiolabeled antibody and biotin coupled antibody, and an avidin coated plastic bead. At the end of the assay incubation, the bead was washed to remove unbound components and the radioactivity bound to the solid phase was measured in a gamma counter (5000 series Packard Counter, Fisher Scientific, Nepon, ON, Canada). Since the complex occurs only in the presence of an intact ACTH molecule containing both N-terminal and C-terminal regions, the radioactivity of the bead bound complex is directly proportional to the amount of intact ACTH in the sample.

Statistical Analysis

Nonparametric analysis of the dose-response curves was performed using the Kruskall-Wallis test. Statistical analysis of the data was performed by repeated-measure analysis of variance. When the overall analysis showed significance, the Students t-test was used to examine the location and significance of differences.

Results

Colonic Fluid Absorption in Vivo

The colonic fluid absorption data for dexamethasone, DS-Dextran and DG-Dextran are shown in graph A of Figure 8-1 and for methylprednisolone and MPS-Dextran in graph B. Hill plots of the same data are shown in graphs A and B of Figure 8-2. Colonic fluid absorption in vivo in sham operated controls was 102.5 ± 9.9 µL/hr/cm. Colonic fluid absorption in sham operated controls was unaffected by dosing with the following compounds at 0.44 µmol/kg/day: dexamethasone, (118.1 ± 10.2); DS-Dextran, (112 ± 13.3); DG-Dextran, (125.3 ± 20.8); methylprednisolone, (97.2 ± 7.3) and MPS-Dextran (113.0 ± 11.2). Upon induction of colitis with 4 % acetic acid, colonic fluid absorption became negative $(-33.0 \pm 11.2$ µL/hr/cm); i.e., there was net secretion of fluid into the colonic lumen. The curves for the dextran conjugates are shifted to the left, indicating that
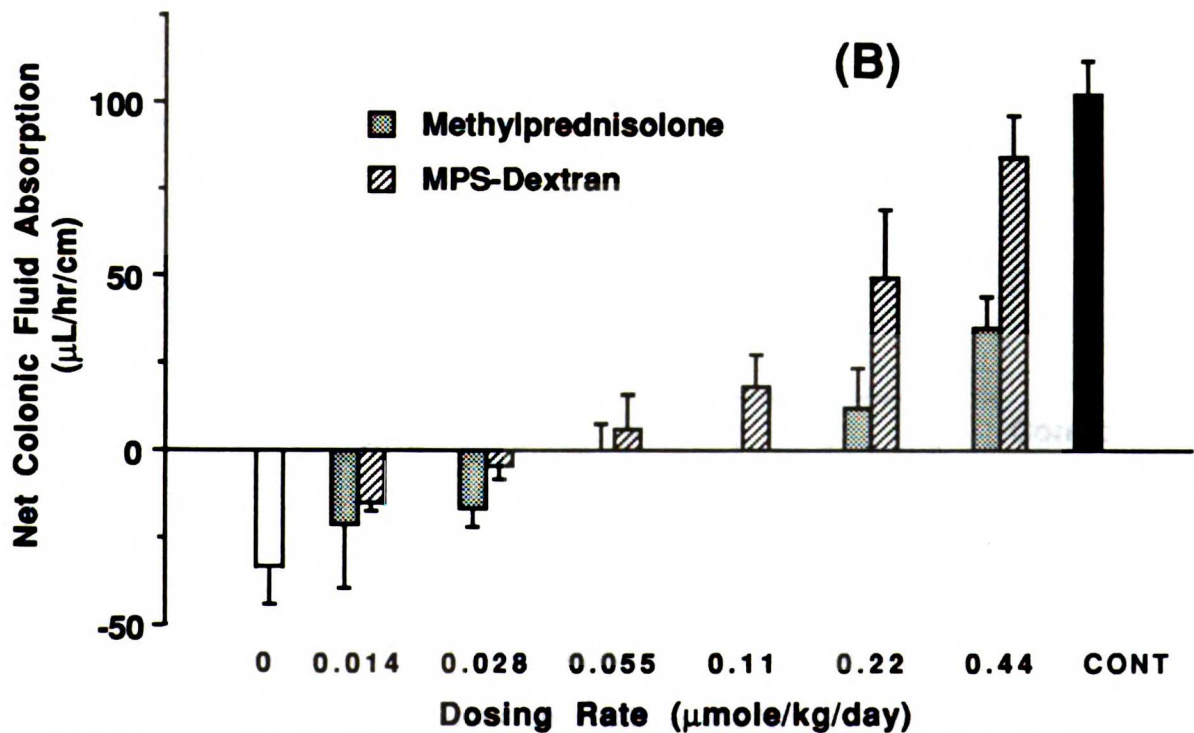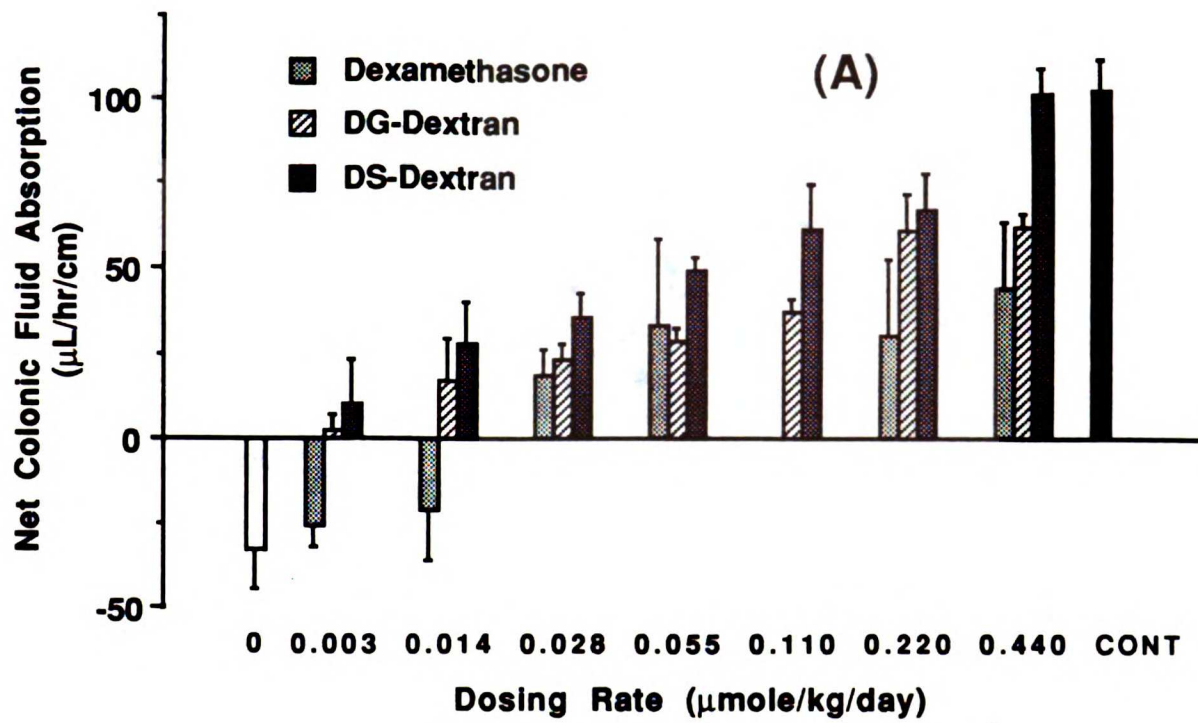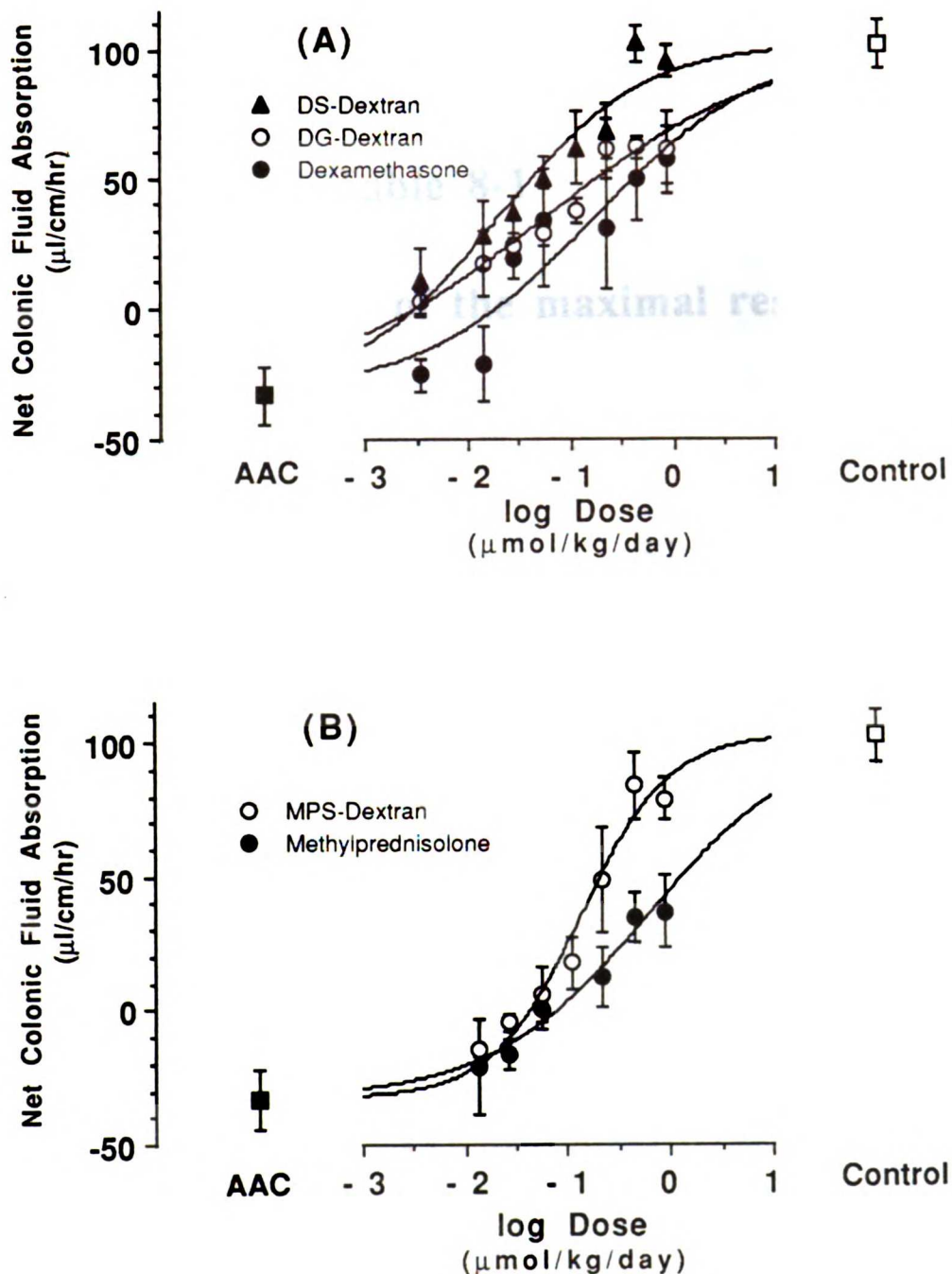
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Fig. 8-1   Colonic fluid absorption measured in sham operated control animals
(CONT) and in those treated with acetic acid. Dexamethasone compounds,
(graph A) and methylprednisolone compounds, (graph B) were
administered by gavage in doses, ranging from 0 to 0.44 μmole/kg/day,
24 and 48 hours after acetic acid treatment.

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Fig. 8-2   Hill plots of colonic fluid absorption data for dexamethasone,

DS-Dextran and DG-Dextran (graph A), and for methylprednisolone

and MPS-Dextran (graph B). AAC (■) indicates the value obtained in

rats treated only with 4% acetic acid. Control (□) is the value measured in

sham operated untreated rats. Points are mean ± S.E.M. (n=5).

Model predictions are superimposed.

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Table 8-1

Doses showing 50% of the maximal response

Compound	Colonic Fluid Absorption ED50 (μmol/kg/day)	Colonic Ulceration ED50 (μmol/kg/day)
Dexamethasone	0.180 ± 0.084[a]	0.049 ± 0.021
DG-Dextran	0.060 ± 0.015	0.011 ± 0.002
DS-Dextran	0.021 ± 0.005	0.005 ± 0.003
Methylprednisolone	0.603 ± 0.227	0.046 ± 0.017
MPS-Dextran	0.136 ± 0.023	0.005 ± 0.004

[a] S.D. of the computer fit of ED50

Fig. 8-3 Ileal fluid absorption measured in control animals and in those treated with acetic acid. Dexamethasone compounds, (graph A) and methylprednisolone compounds, (graph B) were administered by gavage in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours after acetic acid treatment
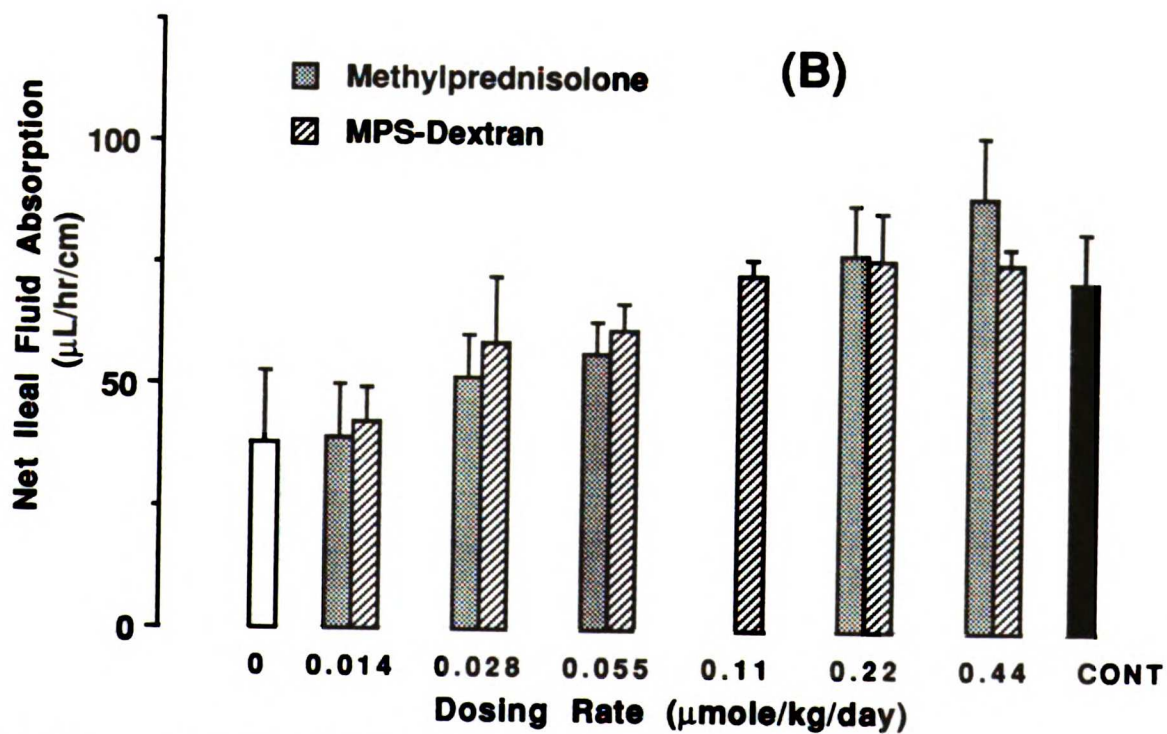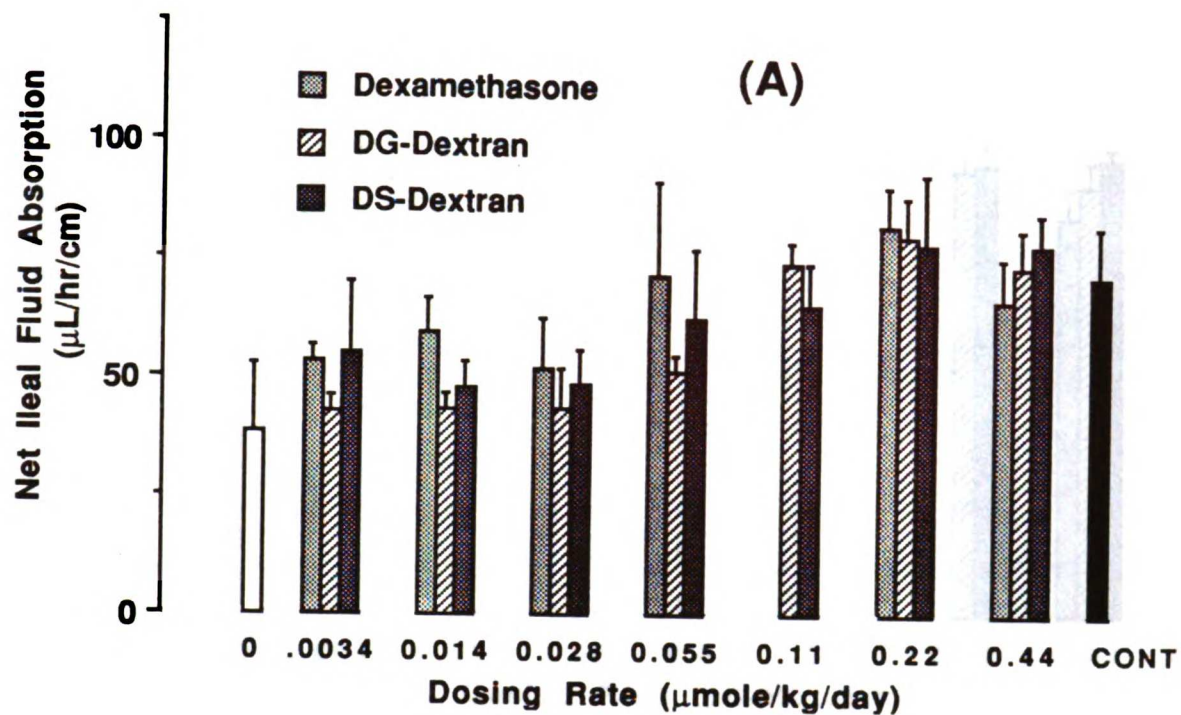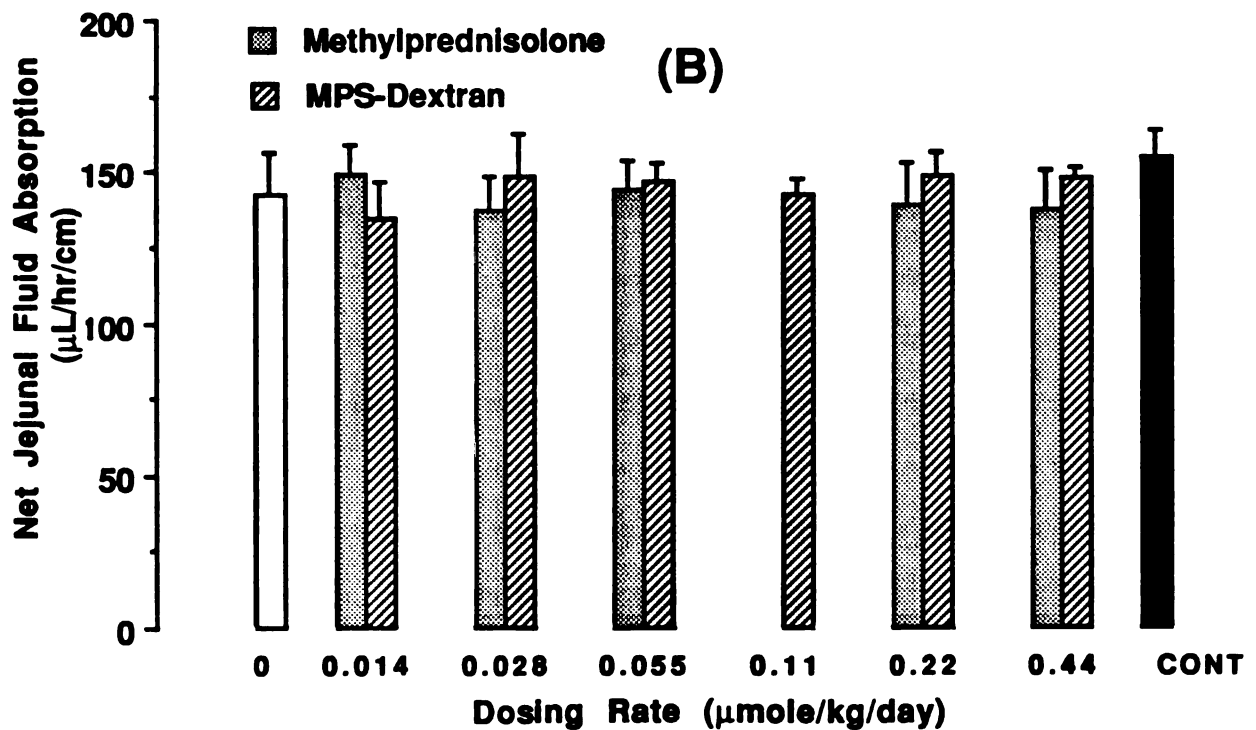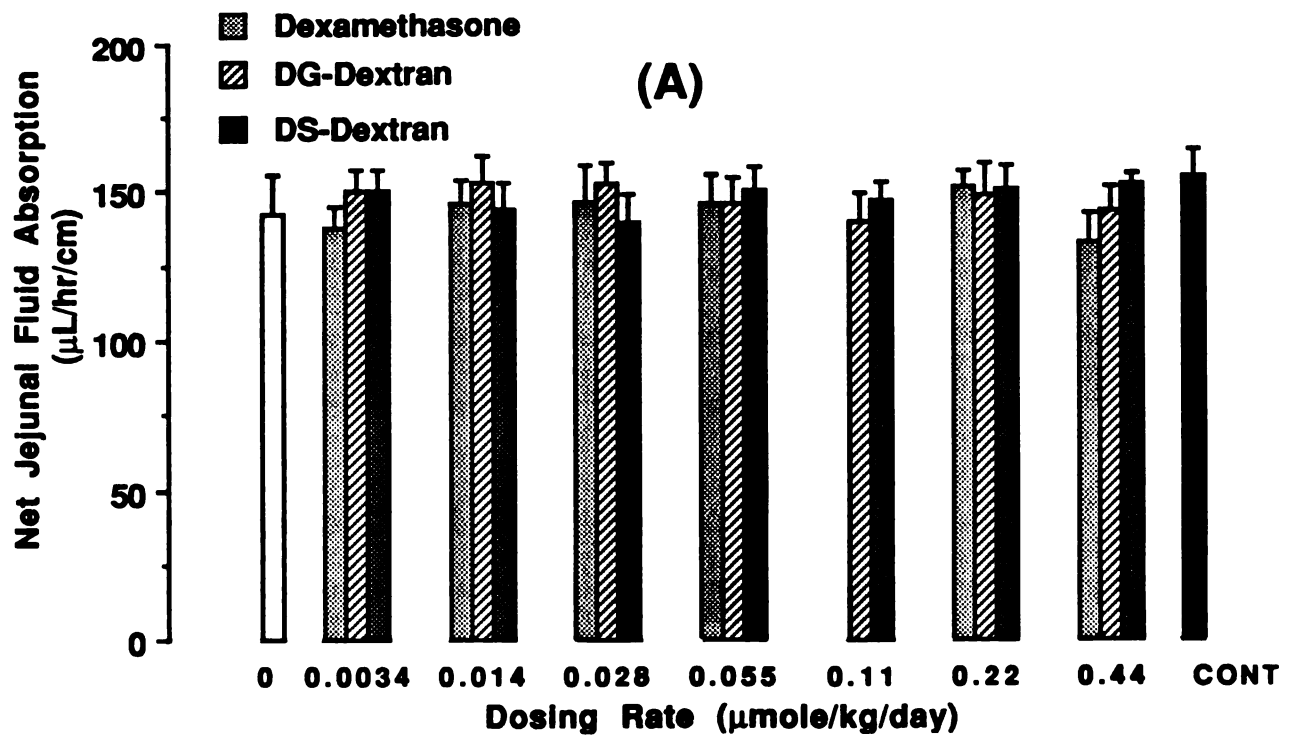
120

Fig. 8-4   Jejunal fluid absorption measured in control animals and in those

treated with acetic acid. Dexamethasone compounds, (graph A) and

methylprednisolone compounds, (graph B) were administered by gavage

in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours

after acetic acid treatment

the conjugates are more potent than the corresponding glucocorticoids. This conclusion was confirmed by nonparametric statistical analysis ($p<0.001$). The ED_{50} values for the compounds are summarized in Table 8-1. Comparing ED_{50} values, DS-Dextran is three times more potent than DG-Dextran and nine times more potent than dexamethasone. MPS-Dextran is 4.3 times more potent than methylprednisolone.

Ileal and Jejunal Fluid Absorption in Vivo

Figure 8-3 demonstrates that colitis induced with 4 % acetic acid also causes a decrease in ileal fluid absorption. Both the dextran conjugates and glucocorticoids increased ileal fluid absorption in a dose-dependent fashion, although the range of data is narrow compared to the colonic fluid absorption experiments. Jejunal fluid absorption (155.0 ± 8.5 $\mu L/hr/cm$) was unaffected by either induction of colitis or treatment with dextran conjugates and glucocorticoids (Figure 8-4).

Colonic Macroscopic Ulceration

Figure 8-5 shows the degree of macroscopic ulceration in each experimental group. Sham operation (control) or treatment of control animals with either dexamethasone or methylprednisolone did not induce macroscopic ulceration of the colon. In contrast, treatment with 4 % acetic acid induced substantial ulceration to approximately 8 % of the colonic surface area. Similar to the results seen with measurement of colonic fluid absorption in vivo, treatment with the dextran-conjugates accelerated healing and improved macroscopic colonic ulceration compared to the respective glucocorticoid. The data were transformed into a term expressing the improvement in macroscopic ulceration, where control animals scored 100% and those treated with acetic acid scored 0%. The Hill equation was then fitted to these data; the ED_{50} values are shown in Table 8-1. The dexamethasone-dextran conjugates had approximately the same relative potency, i.e., DS-Dextran was 10 times and DG-Dextran 4.5 times more potent than dexamethasone.
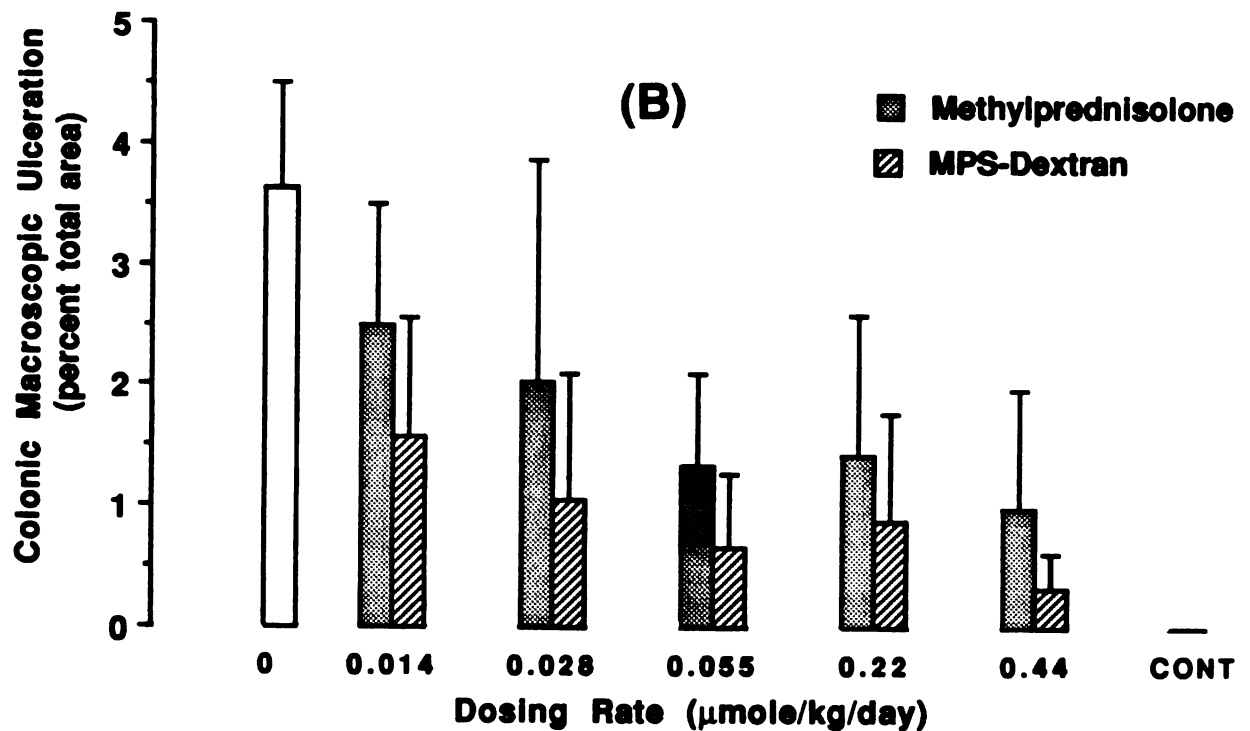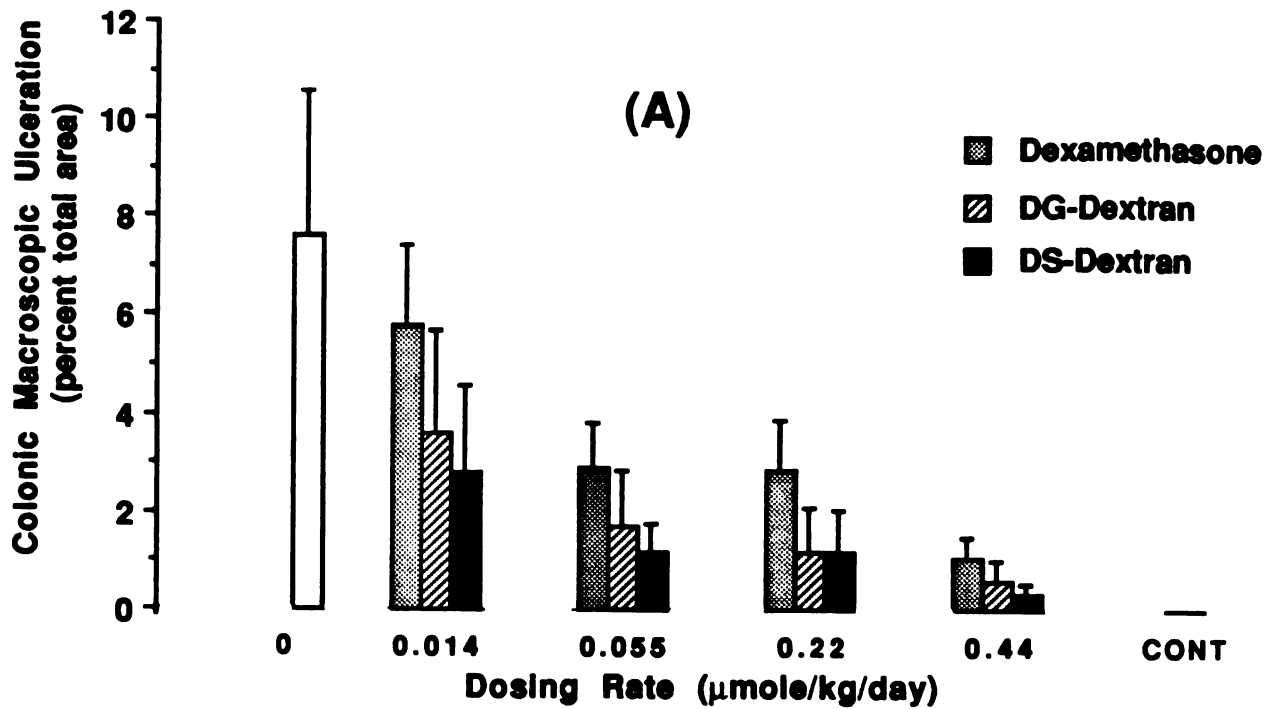
Fig. 8-5   Colonic macroscopic ulceration measured in control animals and in those

treated with acetic acid. Dexamethasone compounds, (graph A) and

methylprednisolone compounds, (graph B) were administered by gavage

in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours

after acetic acid treatment

MPS-Dextran was 9 times more potent than methylprednisolone. It is interesting to note that dexamethasone and methylprednisolone had almost identical ED_{50} values (0.049 and 0.046 $\mu mol/kg/day$, respectively). DS-Dextran and MPS-Dextran were also quite similar (0.005 and 0.005 $\mu mol/kg/day$, respectively). Thus, it appears that colonic macroscopic ulceration is treated equally well with the two glucocorticoids. In keeping with the colonic fluid absorption data, the dextran conjugates show a fourfold to tenfold increase in activity (Table 8-1).

Colonic Myeloperoxidase Activity

As shown in Figure 8-6, induction of colitis with acetic acid significantly elevated the degree of myeloperoxidase activity. Histologic assessment of this colitis model confirmed that neutrophils were the predominant cellular infiltrate. Increasing doses of dexamethasone (graph A) and methylprednisolone (graph B) or their dextran conjugates resulted in reductions in myeloperoxidase activity to near normal levels at the highest doses administered ($p < 0.05$), however, there was no significant difference among the conjugates and glucocorticoids.

Serum Corticosterone

Figure 8-7 shows serum corticosterone levels in dexamethasone (A) and methylprednisolone (B) treated animals. The levels were mainly determined at higher doses (0.22, 0.44 $\mu mol/kg/day$) where adrenosuppression was expected to occur. The effect at 0.0137 $\mu mol/kg/day$ was also determined for the dexamethasone compounds. In animals that had colitis induced with acetic acid, but received no glucocorticoid treatment, there was a significant elevation in serum corticosterone levels compared to sham operated controls ($p < 0.01$). As seen in graph A of Figure 8-7, treatment with dexamethasone (0.44 $\mu mole/kg/day$) reduced corticosterone to subnormal levels.
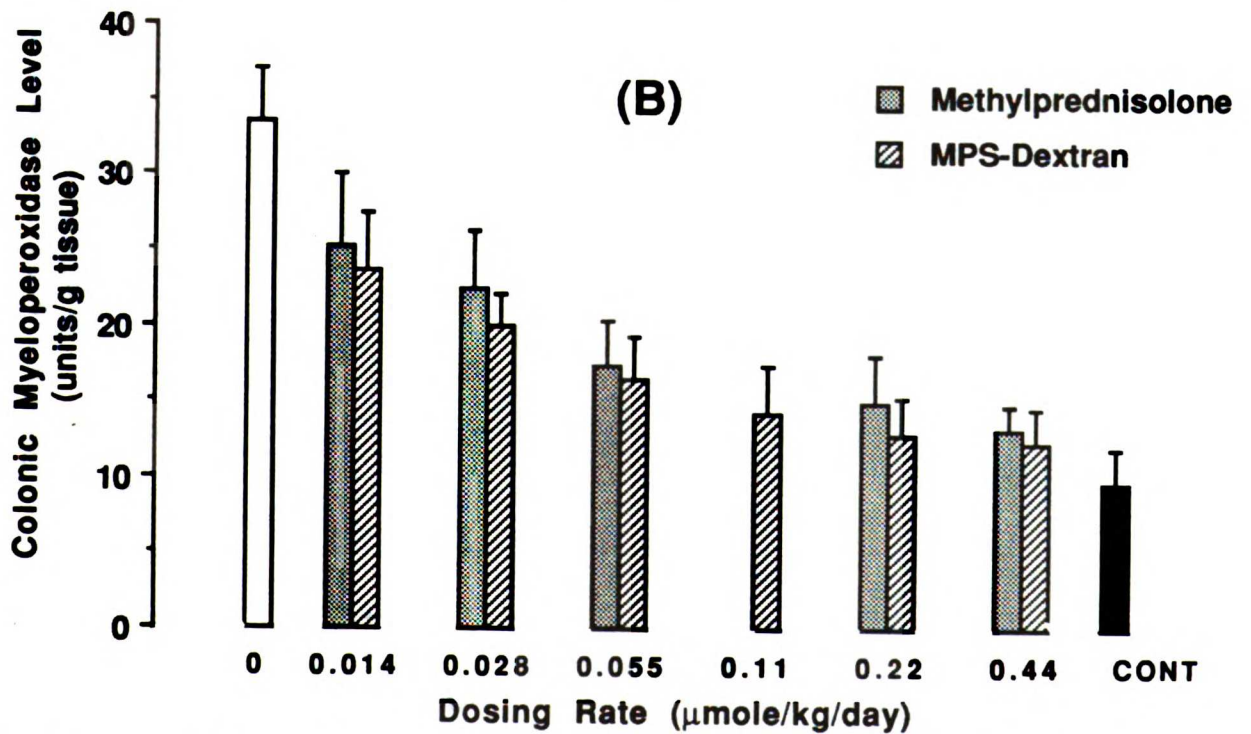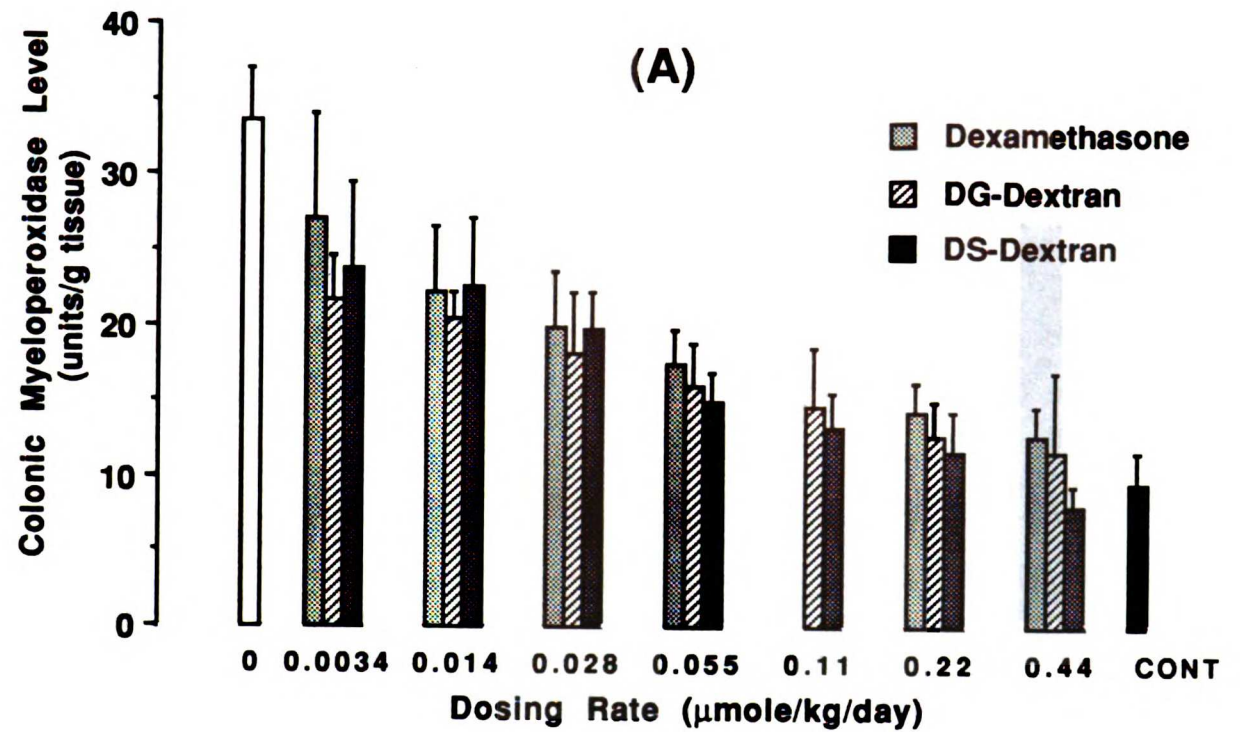
Fig. 8-6   Colonic myeloperoxidase activity measured in control animals and in those

treated with acetic acid. Dexamethasone compounds, (graph A) and

methylprednisolone compounds, (graph B) were administered by gavage

in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours

after acetic acid treatment

125
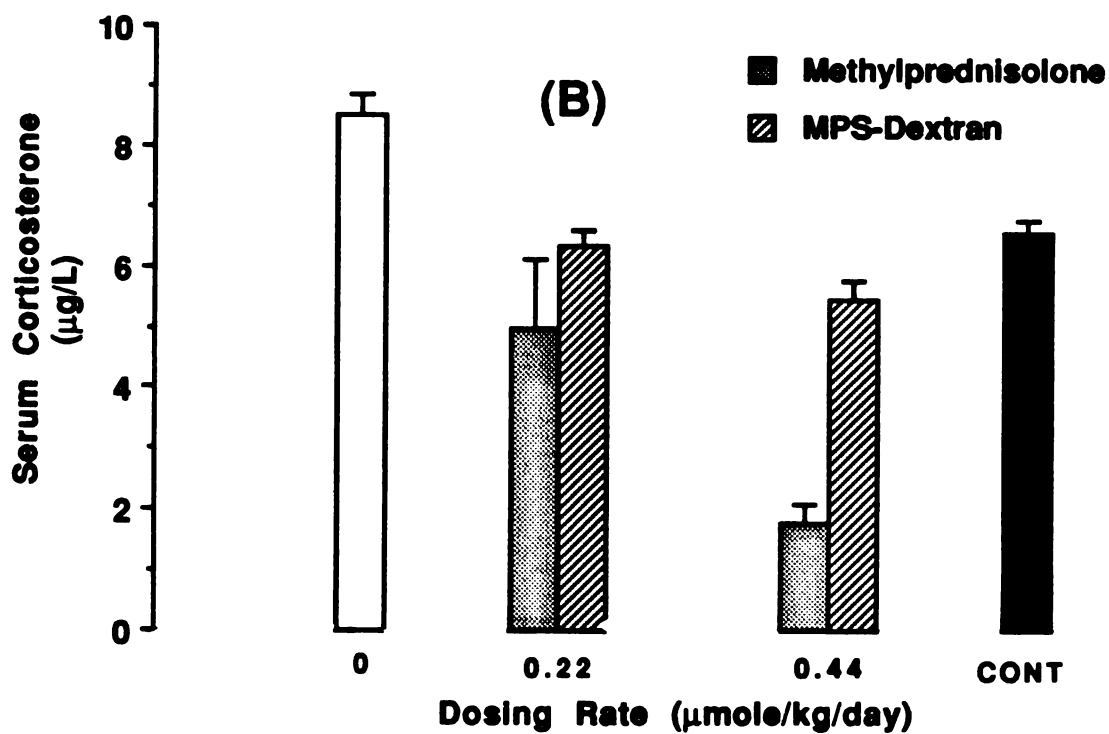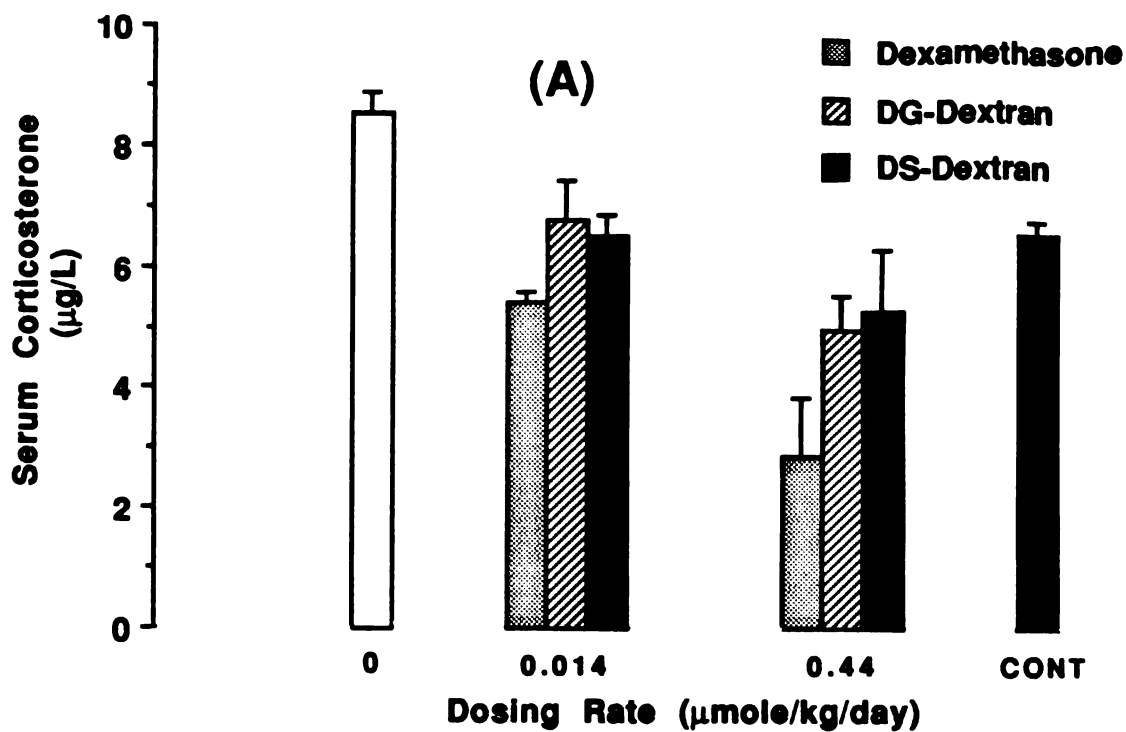
Fig. 8-7   Serum corticosterone levels measured in control animals and in those

treated with acetic acid. Dexamethasone compounds, (graph A) and

methylprednisolone compounds, (graph B) were administered by gavage

in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours

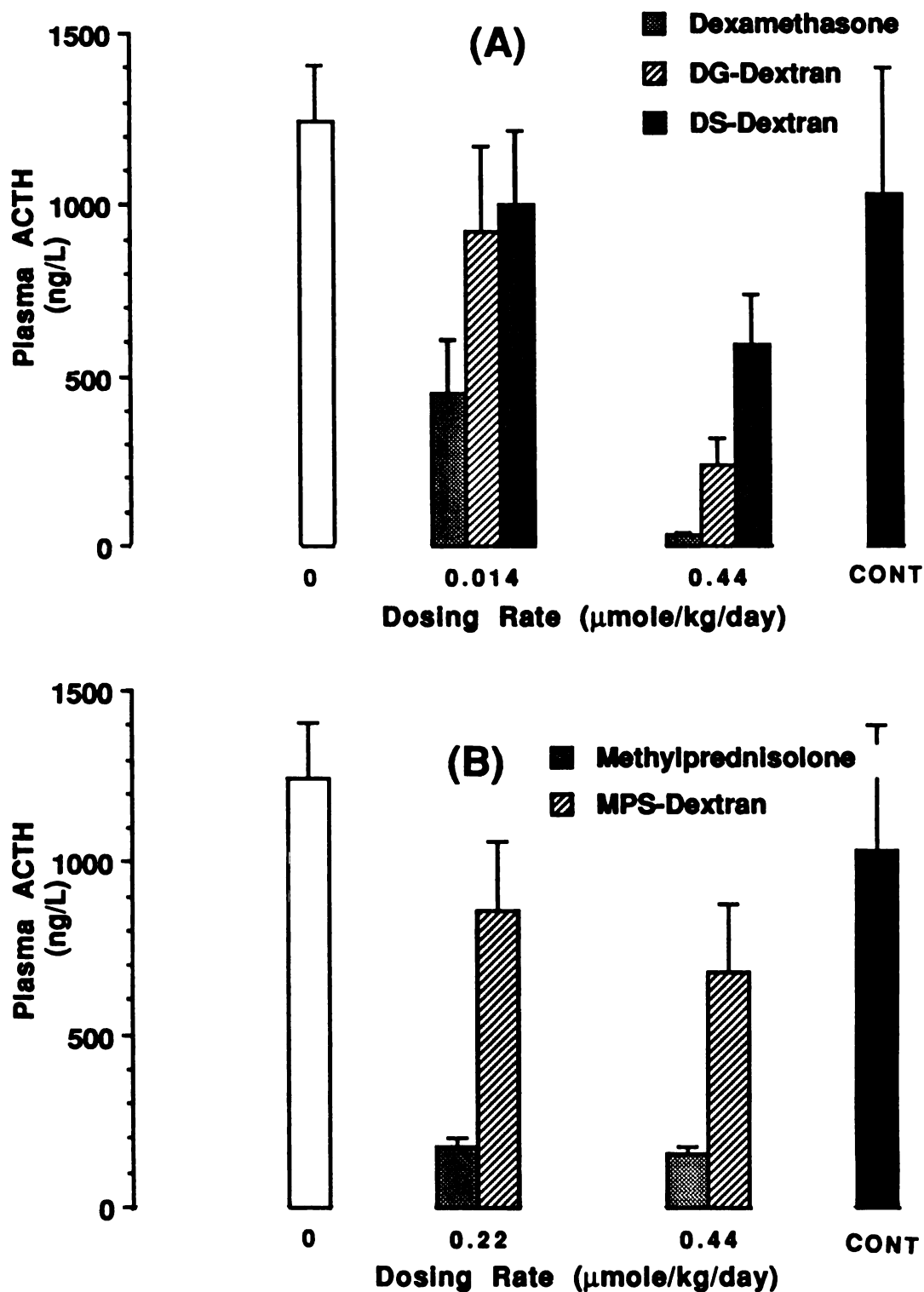after acetic acid treatment

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Fig. 8-8   Plasma ACTH levels measured in control animals and in those

treated with acetic acid. Dexamethasone compounds, (graph A) and

methylprednisolone compounds, (graph B) were administered by gavage

in doses, ranging from 0 to 0.44 μmole/kg/day, 24 and 48 hours

after acetic acid treatment   127

In contrast, treatment with the dextran-conjugates returned corticosterone levels to normal values, with nonly mild adrenosuppression occurring at the highest dose (0.44 μmol/kg/day). Graph B of Figure 8-7 shows similar findings for methylprednsiolone and MPS-Dextran.

Plasma ACTH

Data shown in Figure 8-8 demonstrate the effect of dexamethasone (Graph A) and methylprednisolone (Graph B) on plasma ACTH levels. The levels were similar in both sham operated and control animals that had colitis induced by acetic acid. Plasma ACTH levels were reduced after administration of dexamethasone and methylprednisolone at doses. By contrast, the dextran conjugates did not suppress ACTH levels to the same degree.

Discussion

Recent advances in the medical therapy of ulcerative colitis have led to the development of colon-specific mesalamine (5-aminosalicylic acid) preparations. However, these preparations have generally been shown to be less active than glucocorticoids in the treatment of idiopathic ulcerative colitis. Oral glucocorticoids thus remain the treatment of first choice for moderate to severe colonic disease. Nevertheless, the beneficial effects of oral glucocorticoids are constantly balanced against their systemic side-effects, particularly adrenosuppression. In an attempt to reduce glucocorticoid side-effects, topical preparations (enamas, foams, suppositories) have been developed. While leading to less toxicity, these preparations are poorly tolerated relative to oral preparations and are useful only in distal colonic disease. The development of glucocorticoid-dextran-conjugates which limit drug glucocorticoid absorption in the small intestine, but provide controlled release along the entire length of the colon represents a promising therapeutic approach to the treatment of idiopathic ulcerative colitis in humans.

Comparing ED$_{50}$ values in Table 8-1, dexamethasone is 3.3 times more potent than methylprednisolone in accelerating healing of colitis as determined by improvement in net colonic fluid absorption. Dexamethasone has been shown clinically to have stronger (approximately five times) antiinflammatory properties than methylprednisolone (Haynes and Murad, 1980). The agreement in the relative potencies indicates that this colitis model may correlate with clinical observations in humans.

All of the dextran-conjugates were more potent than the corresponding glucocorticoids in healing macroscopic ulceration and improving colonic fluid absorption in animals with colitis. Indeed, colonic fluid absorption returned to control values after the administration of only two doses of DS-Dextran (0.44 μmol/kg/day). Thus, the diarrhea associated with idiopathic colitis may be rapidly reversed by administration of the dextran-conjugates.

Empey et al. (1989) have shown that changes in ileal fluid absorption after induction of colitis are most likely a neuro-inflammatory reflex process, and not by reflux of acetic acid into the ileum. Thus, healing of the colitis causes concommittant improvement in ileal fluid absorption.

Induction of colitis did not affect fluid absorption from the jejunum. Glucocorticoids and dextran-conjugates had no effect on fluid absorption from the jejunum in rats with colitis or in sham operated control rats. These results suggest that the glucocorticoids and dextran-conjugates alter fluid absorption in the ileum and colon by reducing inflammation and accelerating mucosal healing, but do not cause hyperabsorption of fluid.

Fitting the Hill equation to macroscopic ulceration data yielded results which were similar to those obtained with the colonic fluid absorption data. The dextran-conjugates were four to ten times more potent than the glucocorticoids in healing colonic macroscopic ulceration. Methylprednisolone had the same potency as dexamethasone in healing colonic macroscopic ulceration (0.046 vs. 0.049 μmole/kg/day, Table 8-1), a different result from

that obtained by measuring colonic fluid absorption. Macroscopic ulceration was evaluated by inspecting the colon surface. Perhaps superficial ulceration was healed equally well by both methylprednisolone and dexamethasone while the underlying tissue had not yet returned to normal. This could explain the difference in potencies of the glucocorticoids. Administration of higher doses of the dextran-conjugates almost completely healed the formation of ulcers, Figure 8-5.

Chapter 7 described the steady-state blood levels of dexamethasone after intragastric infusion of DS-Dextran and DG-Dextran and subcutaneous infusion of dexamethasone-phosphate. Dexamethasone was poorly available from DS-Dextran and DG-Dextran (28% and 16%) compared to dexamethasone-phosphate. Adrenosuppression is directly related to blood levels of exogenous glucocorticoid (English et al., 1975; Loew et al., 1986). Thus, we hypothesized that dosing with dextran-conjugates should cause less adrenosuppression than the same dose of glucocorticoid. In humans ACTH directly regulates the production of cortisone, whereas in the rat ACTH regulates the production of corticosterone. Thus, measurement of ACTH and corticosterone is a sensitive means of determining the degree of adrenosuppression in rats. Figures 8-7 and 8-8 show that the dextran-conjugates only suppressed corticosterone levels at higher doses (0.44μmole/kg/day) whereas dexamethasone and methylprednisolone caused suppression at all doses. The dextran-conjugates did not depress plasma ACTH levels to the same degree as the glucocorticoids. Adrenosuppression is a serious side-effect of glucocorticoids and limits the use of this pharmacologic class of drugs.

Thus, the effective dose of glucocorticoid is lowered because of specific delivery to the colon and the systemic side-effects are reduced by a decreased systemic exposure.

Conclusions

The salient findings of this dissertation were as follows:

Synthesis

The dextran conjugates were readily purified in high yields (>90 %). The purification method enabled rapid production of large batches of the conjugates. HPLC analysis of the conjugates indicated that the amount of free drug (non-covalently bound) was less than 1 % of the total drug content.

Chemical Stability

Hydrolysis of the ester bonds of the dextran conjugates increased at pH values above and below pH 3. In all cases, the release rate of hemiester was faster than the release of glucocorticoid. This difference was most pronounced for the dexamethasone conjugates. Acyl migration of the 21-hemiester group to the 17-position increased with pH; however, only very small amounts (0.3 of 50 μmoles) of 17-hemiester were produced during incubations at pH 6.8. The half-lives of the dextran-conjugates, as measured by release of hemiester and glucocorticoid at pH 6.8 and 37°C were as follows: MPS-Dextran, 82 hours; DS-Dextran, 75 hours; and DG-Dextran, 103 hours. These values indicated that the dextran-conjugates should only undergo minor nonenzymatic hydrolysis during passage down the gastrointestinal tract.

Incubations in Vitro

The hemiesters were rapidly hydrolyzed in the proximal small intestine (PSI) and the rate declined progressively further down the GI tract. The enzyme distribution suggested that the esterase was either secreted in bile or released from the mucosa of the

small intestine. Most of the dextran conjugates hydrolysis occurred in the cecum and colon. Dextran appeared to protect both linker bonds from hydrolysis by esterases in the PSI and DSI. Most drug was released from the dextran-conjugates as glucocorticoid-hemiester while virtually no glucocorticoid was hydrolyzed directly from the conjugates.

The cecal enzyme which hydrolyzed the glucocorticoid-hemiesters was postulated to be a type-A carboxylesterase, possibly of pancreatic origin.

Dextranase rapidly hydrolyzed both dextran and MPS-Dextran as indicated by the change in molecular weight distribution as determined by size-exclusion chromatography.

Permeability Experiments

Caco-2 cells were highly permeable to both dexamethasone and methylprednis-olone. Dexamethasone crossed the cells more rapidly than methylprednisolone. The cells were significantly more permeable to dexamethasone and methylprednisolone than to the hemiesters. The permeability of dexamethasone-hemiglutarate was greater than dexamethasone-hemisuccinate and methylprednisolone-hemisuccinate. The permeability of rabbit colon mucosa to methylprednisolone-hemisuccinate was an order of magnitude lower than that previously published for another glucocorticoid, hydrocortisone. Dexamethasone and methylprednisolone had relatively high logarithmic partition coefficients (1.55 and 1.73, respectively) compared to methylprednisolone-hemisuccinate, dexamethasone-hemisuccinate and dexamethasone-hemiglutarate (-0.57, -0.91 and -0.26, respectively).

Experiments in Vivo

Pharmacokinetic principles were used to assess the colon-specific delivery of dexamethasone from dextran-conjugates. DS-Dextran increased concentrations of dexamethasone in the large intestine threefold and decreased blood concentrations threefold. Thus, this dextran conjugate should both increase efficacy and reduce systemic

toxicity. DG-Dextran gave a fivefold decrease in blood concentrations while maintaining similar colonic concentrations. In addition, subcutaneous administration of methylprednisolone as the disodium phosphate ester resulted in high concentrations of methylprednisolone in both cecum and colon, possibly as a result of enterohepatic cycling.

Pharmacodynamic Testing

Dexamethasone was 3.3 times more potent than methylprednisolone in its effect on colonic fluid absorption. All of the dextran-conjugates were more potent than the corresponding glucocorticoids in altering colonic fluid absorption in animals with colitis. Macroscopic ulceration data yielded similar results. The dextran-conjugates did not affect either ACTH or corticosterone levels at all doses tested. This lack of effect was in contrast to dexamethasone and methylprednisolone which caused marked adrenosuppression. Thus, the effective dose of glucocorticoid was lowered because of specific delivery to the colon and the systemic side-effects were reduced by a decreased systemic exposure.

Future Experiments

Further investigations into the postulated mechanism of enterohepatic cycling of methylprednisolone after parenteral administration.

Predosing the rats with dextran to induce dextranases and to determine whether this affects the degradation of the dextran-conjugates.

Measure the pharmacokinetics of glucocorticoid released from the dextran-conjugates after administration to humans. Clinical trials in patients to evaluate the efficacy of the conjugates in treating chronic colitis.

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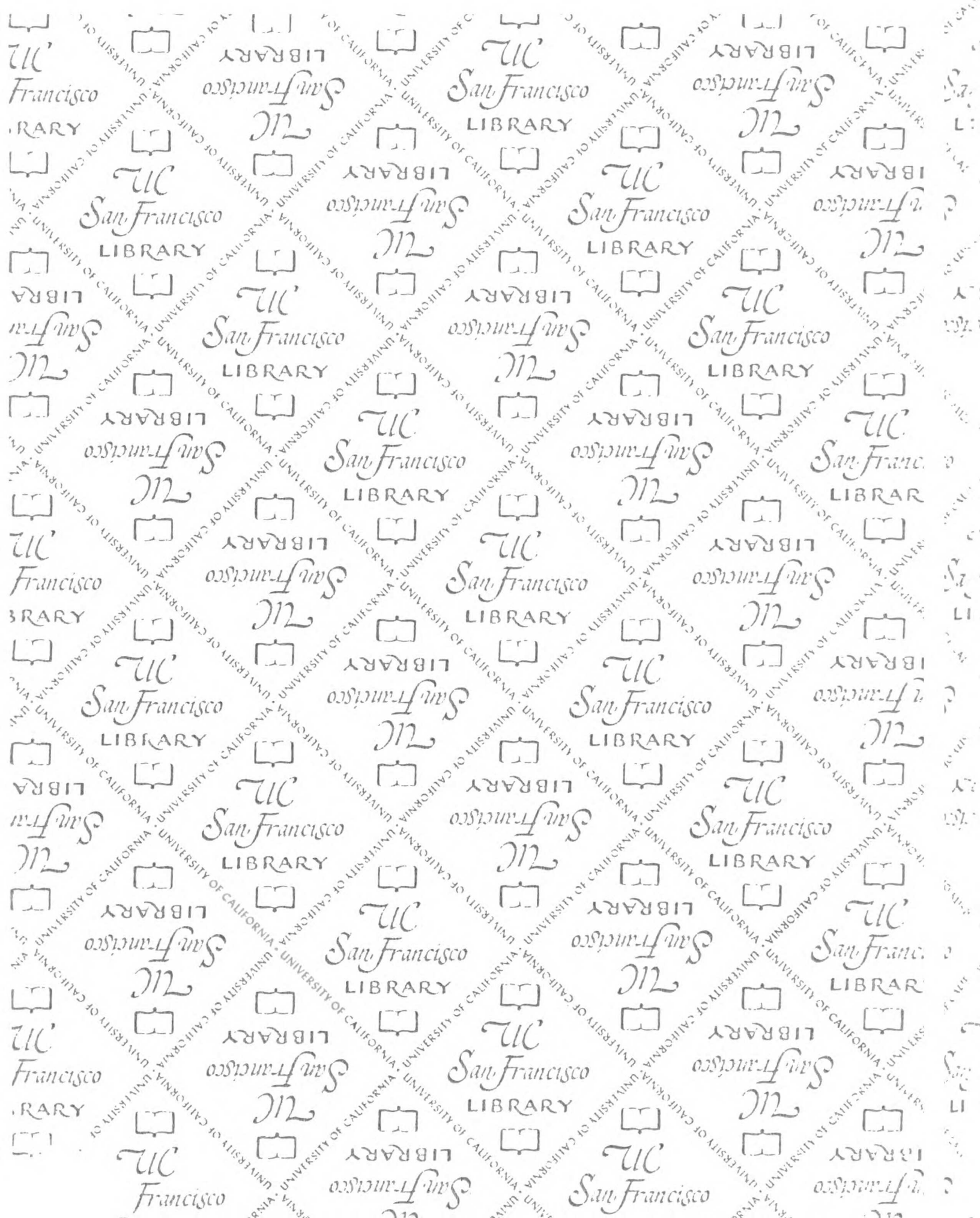
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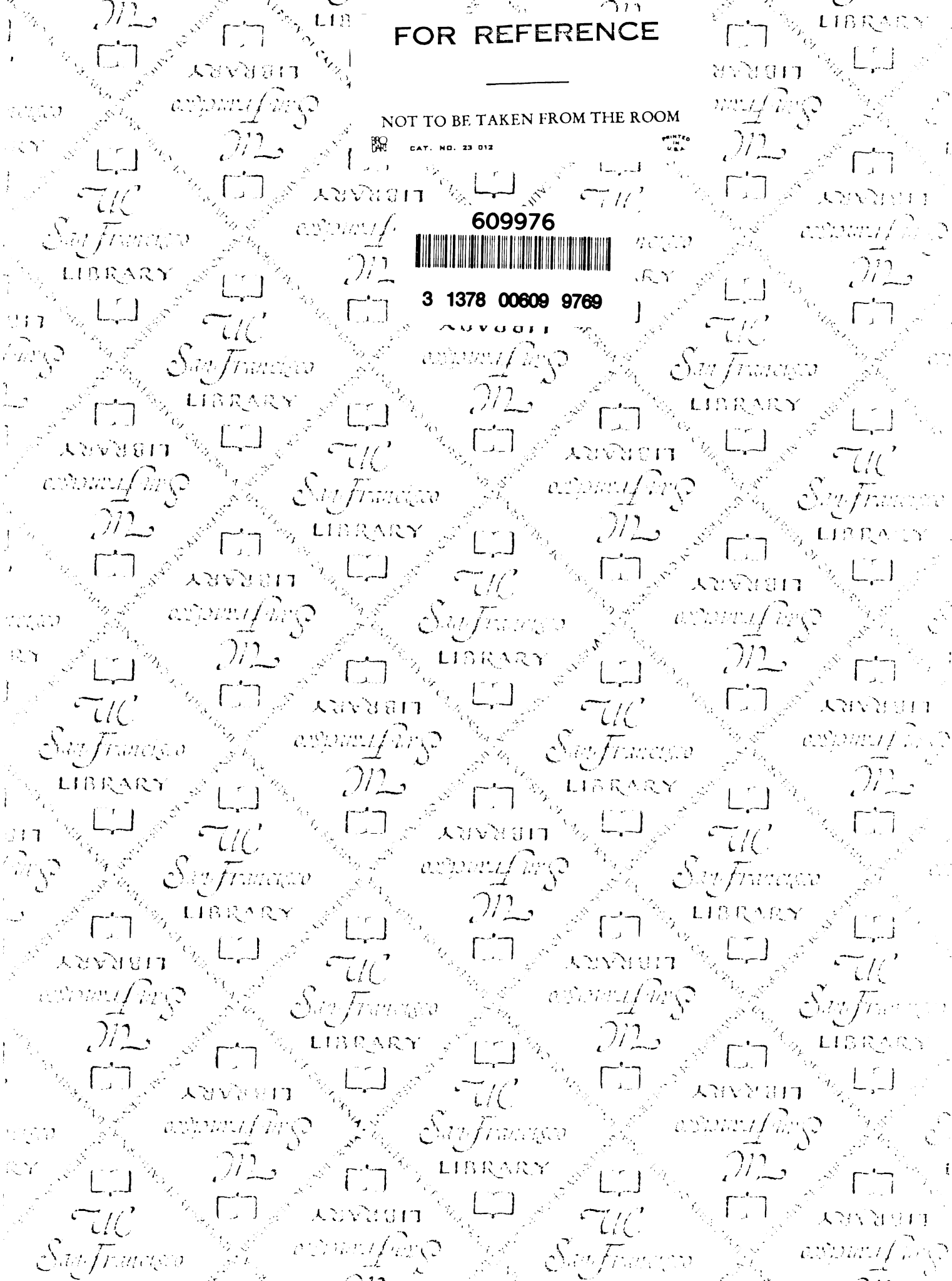Wingerd, B.D. (1988) *Rat Dissection Manual*, The Johns Hopkins University Press, Baltimore, p. 47.