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THE UNIVERSITY OF ALBERTA

The Recovery of the Rat Intestine from Acute Radiation Damage

by

Allan R. Baer

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Abstract

Studies were designed to determine how the intestine responds to acute radiation injury, and what factors are required for the repair of damage. Tests used to monitor recovery from injury included *in vivo* transport, ornithine decarboxylase (ODC) activity, and myeloperoxidase activity. ODC has been implicated in the regulation of DNA synthesis, and myeloperoxidase was used as a measure of tissue leukocyte infiltration. Rats received 6 Gy of gamma radiation to the adbomen, and were studied 3, 7, or 14 days later.

No change occurred in the passive uptake of L-glucose or D-leucine after irradiation, but active D-glucose and L-leucine uptake was reduced at 3 days post-irradiation. Only L-leucine uptake remained slightly reduced at 7 days. Increased sensitivity to phloridzin was seen at 3 days. Thus, care is required in using this glucose transport inhibitor when intestinal damage is likely to be present. The potential difference generated by a hyperosmotic load, indicative of mucosal permeability, was increased at 7 days only. ODC activity was elevated at 3 days and was still slightly increased at 14 days, whereas myeloperoxidase activity was depressed until 14 days. Thus the transport of substrates *in vivo* may not be a good indicator of intestinal recovery, as other parameters remain altered for a longer period.

Villus height in all regions of the small intestine was reduced 3 days post-irradiation, but were normal by 7 days. Mean enterocyte migration rate, assessed by autoradiography, was unchanged from controls at 3 days, increased at 7 days, and normal at 14 days. This suggests increased cell loss at 3 and 7 days, with adaptation occurring by 7 days such that normal villus height is maintained.

To determine if decreasing food intake could account for the changes in transport caused by irradiation, control and irradiated animals were starved for 3 days. Glucose uptake in both groups was reduced to a similar extent. The ODC peak in irradiated rats was abolished by starvation, and myeloperoxidase levels were higher than in fed rats. Thus, feeding is required for ODC induction in the intestine. The importance of ODC to normal function and to recovery was assessed by feeding the animals difluoromethylornithine (DFMO), an inhibitor of ODC. Both starvation and DFMO reduced villus heights in control and irradiated animals. Despite continued food intake, DFMO administration reduced glucose uptake in irradiated and non-irradiated rats. DFMO also increased myeloperoxidase levels compared to those in non-irradiated rats. ODC is therefore important to normal intestinal function and morphology, and its inhibition delays recovery from injury.

Several drugs were used to determine if recovery from intestinal radiation damage could be influenced. Aspirin

reduced glucose uptake and increased ODC activity in control rats. No further change in uptake or ODC activity occurred in irradiated rats receiving aspirin, but intestinal adhesions were prominent. This was not due to weak acid effects, as p-aminobenzoic acid (PABA) caused no change in glucose uptake or adhesion appearance compared with controls. However, the ODC peak in irradiated rats was eliminated by PABA, suggesting a requirement for increased intracellular pH in the induction of ODC. Prostaglandin blockade was not involved in the additional damage seen with aspirin, as indomethacin increased glucose uptake in irradiated rats, without adhesion formation. Prednisolone increased glucose uptake and ODC activity in control rats but caused no further change in irradiated rats. Allopurinol, an inhibitor of xanthine oxidase, improved glucose uptake in irradiated rats, and abolished the ODC peak.

Refeeding after starvation induces ODC. Intestinal perfusion with specific nutrients into starved animals showed that D-glucose, L-glucose, 3-O-methylglucose and D-galactose increased ODC levels, but D-fructose, L-leucine or phloridzin combined D- or L-glucose were without effect. Intravenous glucose also failed to stimulate ODC activity. Thus, intestinal ODC induction requires nutrient on the luminal side of the enterocyte, and does not require active transport, or intracellular metabolism.

ACKNOWLEDGEMENTS

The writing of a thesis is by its nature a difficult undertaking, for it is subject to great expectations as well as unforseen hazards. One always hopes to push back the frontiers of knowledge and in so doing, contribute in some way to the future alleviation of the suffering of human disease. Ideas for scientific enterprise, however, do not simply drop into one's lap. Pasteur's comment that "chance favours a prepared mind" has been particularly relevant in this thesis, as several important ideas began simply as unexpected findings in the course of research into other areas. Whether these ideas prove to be important in the crucible of scientific enquiry, however, remains to be seen.

Much of the credit for the "prepared mind" must go to my mentors, Drs. Chris Cheeseman and Alan Thomson. The timely advice of these two scholars, the critiquing of untried ideas, and the patience shown during tedious times when new techniques were being worked up and data not immediately forthcoming, all contributed to the successful development of new avenues of research and their description within this thesis. Although it may have seemed that their recommendations sometimes fell on deaf ears, in the fullness of time, all advice has been taken to heart. In short, this thesis would not have been possible without these two advisors. and I will be forever in their debt.

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If this thesis were to have a dedication, it would be to the thousands of fellow Canadians with Crohn's Disease. If our problems often appear to be neglected by the bulk of medical researchers, perhaps in some small way the clues revealed in this work may eventually lead to a more effective treatment, and offer some hope for a brighter future.

Table of Contents

List of Tables

List of Figures

I. Introduction and Objectives

Within the past decade, several exhaustive reviews and symposia have been published documenting the extensive changes in transport function and cell proliferation in the intestine as it adapts to large fluctuations in its environment and functional workload (114, 249, 250, 61). Resection (62), starvation (3), lactation (72), diet change, and diabetes (114) are some of the experimental manipulations that have been employed to elicit what is known as intestinal adaptation. Explicitly excluded from consideration, however, has been the adaptation of the intestine to pathological situations producing damage to the gut, on the basis that injury evokes entirely different responses (61). Yet in order to survive, the intestine must compensate for increased cell death by altering the rate of cell proliferation as well as that of transport, if it is to meet the functional demands on the tissue. It would be reasonable to expect therefore, that at least some of the mechanisms regulating intestinal adapt[illegible] would also be involved in the adaptation to inju[illegible], [illegible]h shall be referred to below as repair.

The precise nature of the signals tri[illegible] repair, as well as the cellular and biochemical mec[illegible]able to the gut in order to effect repair, have large[illegible] remained a mystery. To date, no comprehensive review has appeared dealing specifically with the intestine's response to injury. In fact, progress in general has been slow in

developing a scientific (rather than empirical) basis for effective treatment for intestinal disease of non-microbial origin. A large part of this may lie in the difficulty in developing adequate animal models, especially for the idiopathic inflammatory diseases. Several models do exist for study, however. These include intestinal ischemia (201), radiation-induced injury (183), experimental uremia (221), and damage by cytotoxic drugs (146) or parasitic or bacterial infestation (225). In human patients, studies on proliferative and histological changes as well as limited studies on *in vivo* transport in certain diseases, most notably celiac disease (177), have also provided important information on the disease process.

The model of intestinal damage chosen for investigation in the following studies was that of acute radiation injury, a damage state for which a large background of data has accumulated and can be built upon. The intention of the studies was to use the model to approach the general question of how the intestine responds to injury, as well as to answer the following more specific questions:

1. What is the extent of damage and the time course of recovery for intestinal transport? What tests can be used as reliable measures of injury? Are there aspects of function or morphology that recover from injury more rapidly than other parameters?

2. An important mode of repair for the intestine is the

stimulation of cell renewal. Could a consequence of injury and of subsequent rapid cell renewal be the repopulation of the intestine with immature cells?

3. The enzyme ornithine decarboxylase is important in the regulation of DNA synthetic activity (190). Could this enzyme also be important in the recovery process? Is it obligatory for recovery or is it supplementary? If its activity is changed by damage, does the time course of its recovery match that of tissue repair? What are the stimuli for the activation of the enzyme?

4. Can the course of recovery be influenced by pharmacological agents?

II. Literature Review

A. Radiation - Physical Considerations

The description of the effects of radiation damage to tissue calls for a cursory understanding of the nature of the damaging agent. While the electromagnetic spectrum covers several types of radiation, only two will be focused on here, X-rays, and gamma rays, due to their damaging effect on tissue and the frequency of their use in therapeutic regimes.

Unlike alpha or beta rays, which have a component of mass (the equivalent of a helium nucleus or an electron, respectively) as well as energy, X- and gamma rays possess only energy (89). X-rays, first discovery by Wilhelm Georg Roentgen in 1895 (and initially referred to as "Roentgen's rays") are artificially produced in vacuum tubes when electrons are accelerated in an electric field and collide with a solid body, usually a tungsten target. As it approaches the target nucleus, the electron decelerates, resulting in a loss of energy which is emitted from the target material in the form of energy-possesing photons. Gamma rays, on the other hand, are products of the decay of the atomic nucleus, or "natural radioactivity" as first described by Henri Becquerel at the end of the last century. X-rays are generally described as those rays having wavelengths between 10^{-2} and 10^{-1} nm, and gamma rays those with wavelengths from 10^{-5} to 10^{-2} nm, although X-rays in

the gamma range have also been reported (89).

The two most common radionuclides used in radiotherapy include ^{60}Cobalt and ^{137}Cesium. Both emit betas as well as gamma rays, but the beta particles are generally absorbed within the radioactive source resulting in the interaction with atomic nuclei to form X-rays (119). These X-rays do not contribute appreciably to the delivered dose, however, as they are strongly attenuated within the radioactive material and its capsule. Compared to ^{137}cesium, ^{60}cobalt has a higher energy, emitting two photons per disintegration with energies of 1.17 and 1.33 MeV. ^{137}Cesium emits only one photon with an energy of .66 MeV. In addition, the half life of ^{60}cobalt is shorter than that of ^{137}cesium (5.26 versus 30 years respectively). The shorter half life of ^{60}cobalt necessitates more frequent replacement, and therefore higher cost, and as a result is not currently as frequently used as ^{137}cesium.

B. Dose Representation

Radiation doses were originally expressed in roentgens, introduced in 1928. A measure of ionization in air, one roentgen (R) was originally defined as 1 electrostatic unit per cm^3 air at standard temperature and pressure. This is equivalent to the current definition, i.e., 1R = 2.58 x 10^{-4} coulombs per kilogram air (119). Common use of this measurement was superceded in 1953 by the establishment of the rad, which in contrast to the roentgen

(which refers to the exposure dose), describes instead the dose absorbed by a material. 1 rad represents the absorption of 100 ergs per gram of irradiated material or 10^{-2} joules per kilogram (119). Considerable confusion can arise in reading the literature, from the similarity of abbreviations used for the roentgen (R) and the rad (r). In a more recent update, the Gray has been adopted as the unit of absorbed dose, 1 Gray (Gy) being equal to 100 rads.

C. Radiation - Interaction with Matter

As it passes through matter, radiation loses its energy. In the case of ultraviolet light, this may simply involve displacing electrons from one orbital shell to another shell of higher energy, thus "absorbing" the energy of the radiation. X-rays and gamma rays, however, dissipate their energy by ejecting electrons from atoms in their path, a process referred to as "ionization". This is not a selective process, as any atom or molecule in the path of the radiation may be ionized. In the cell, however, water makes up 70-80% of the tissue and is therefore statistically most likely to be ionized (40).

Under normal circumstances, electrons in molecules generally occur in pairs, each having a spin in a direction opposite to its pair. When radiation ejects an electron, the resultant compound is left with an uneven number of electrons, an inherently unstable configuration referred to as a "free radical". In the case of water, $H_2O^{\cdot}$ is formed by

the loss of an electron, and this compound undergoes a series of rapid reactions producing products which include OH· and H·. (40). In this case, the hydroxyl radical, OH· carries no charge, because the number of protons and electrons is the same (i.e., 9), but since there is an uneven number of electrons, the compound is referred to as a radical. On the other hand, the superoxide radical, $O_2^{-}\cdot$, formed by the acceptance of an electron by molecular oxygen, is negatively charged, yet is also a considered a radical since it too has an uneven number of electrons (i.e., 17).

Free radicals differ substantially in their reactivity, but primary radicals such as the hydroxyl radical are highly reactive, and only diffuse short distances before reacting with other compounds. Hutchison (108) estimated this distance to be 3 nm.. Secondary radicals, such as superoxide, are less reactive, but may still react with compounds such as hydrogen peroxide to regenerate the more reactive hydroxyl radical (39, 54). Although attempts to directly demonstrate this reaction have failed, hydroxyl radical regeneration in biological systems may proceed through the mediation of metal catalysis, with compounds such as porphyrin iron (39).

Hydroxyl radicals may react with other hydroxyl radicals to produce hydrogen peroxide, or react with organic molecules, for example by the addition of the hydroxyl across a carbon-carbon double bond to form an alkoxyl radical, or by abstracting a hydrogen to produce H_2O and a

carbon radical (39). Unsaturated lipids are among the molecules vulnerable to this radical reaction, and the resulting radical may react with neighbouring lipid molecules to propagate the process, or with oxygen to produce a lipid peroxide. It has been estimated that 60-70% of cell inactivation may be attributed to OH· and H·, and 30-40% to the direct effects of the radiation (40). The importance of these indirect, hydration-related effects can also be seen in the requirement of 2 to 50 times the dose to obtain similar enzyme deactivation in wet versus dry yeast cells (109).

Oxidizing radicals can be responsible for the inactivation of enzymes and those with sulfhydryl (SH) groups are especially vulnerable. In 1950, Barron (14) showed that enzymes damaged in this way could be reactivated by supplying a compound such as glutathione, which possesses thiol groups, although such reactivation did not take place if damage other than that of the SH groups occurred. Substances which compete for radicals, referred to as scavengers, reduce enzyme inactivation, and have been much studied since Dale (50) published his classic experiments using leucylglycine as a scavenger in 1942. Dimethylsulfoxide (DMSO) and cysteamine are two such scavengers that have received considerable attention (40).

Hevesy (102) was one of the earliest investigators to publicize the extreme radiation sensitivity of cells actively synthesizing nucleic acids. The effect tends to be

greater with DNA than with RNA because the DNA presents a larger target, and because new RNA can be synthesized if the DNA remains intact. Radiation affects both the structure and the synthesis of DNA. Hevesy, and later, Nygaard (172), demonstrated decreased incorporation of radioactive precursors into DNA as a result of irradiation. Lett and co-workers (137), working with mammalian cells, used the sedimentation rate of DNA in an alkaline sucrose gradient to show that the size of isolated DNA decreased after irradiation. This returned to normal if the cells were incubated with nucleic acid precursors to allow for further DNA synthesis, thus indicating a repair process at work.

One of the results of DNA damage is the delay of cell cleavage (89). The duration of the delay increases with increasing doses of radiation, and the block has usually been ascribed to the G_2 phase of the cell cycle. Progress through mitosis may be delayed by delivering radiation at any point in the cell cycle, however. Certain phases, such as the S (synthesis) phase, appear to be less sensitive than others (91).

D. Intestinal Effects of Radiation

In 1897, only two years after the discovery of X-rays, Walsh made the first report of the detrimental action of ionizing radiation on the gastrointestinal tract, noting vomiting and diarrhea in a human patient (241). Pioneering work by Warren (243) established that radiation injury was a

result of direct injury of the intestine by the radiation, rather than a secondary effect arising from systemic influences. This was established by the observation that the diarrhea and ileal necrosis that occurred in dogs after abdominal irradiation was not seen if only the chest was irradiated.

Among the changes seen in experimental animals receiving radiation treatment was weight loss (29), which was partially attributed to delayed gastric emptying (167, 223). Smith (217) experimented with several doses of radiation and found that weight loss in rats increased with the dose administered. At 500 R, 3 days was required to regain the original weight. A number of interesting anomalies were noted, however. For example, if the irradiated animals were starved, weight gain on refeeding was faster than starved controls despite identical food intake. Further, if irradiated animals were force fed, significant mortality resulted. This hinted at two important but largely overlooked points: first, that the recovery process might be more than a simple resumption of mitosis and second, that overtaxing the compromised function of the injured gut could itself be detrimental.

Five years later, Kay (118) noted that although no changes occurred in the weight of the muscle layer of the small bowel, mucosal weight declined after 600 R of X-irradiation. A subsequent increase in mucosal weight four days later, to a level greater than in controls, was

attributed to cell swelling, as no concurrent DNA overshoot was observed. Kay also found that fasted rats receiving radiation treatment gained weight more rapidly than non-irradiated animals. A similar overcompensation for weight loss was also found by Detrick (57).

By the end of the Second World War, two major approaches had been established in research on intestinal radiobiology. One group pursued the influence of radiation on transport ability (222). The second group was primarily concerned with the effects of radiation on cell proliferation and its ability to delay mitosis and even to produce cell death (189). Many of these studies were sponsored by the military, for the sensitivity of the intestine to radiation damage seemed to be a major factor in acute mortality resulting from the massive radiation exposure associated with nuclear blasts. Such sponsorship did restrict the publishing of some data, as seen in Friedman's comment in 1945 that further experiments were "brought to a close by the war and cannot be reported at this time" (78).

Studies by Friedman (77) and those of Knowlton (125) had established that mitosis was suppressed by radiation and that the depression in mitotic index was dose dependent. Leblond's pioneering studies published in 1948 drew attention to the high rate of cell proliferation in the intestine, showing that new cells were produced in the crypts of Leiberkuhn, and migrated up the villus to be

exfoliated at the tips. He estimated the time required for the process in the rat intestine was 38 hours in the duodenum, compared to 32 hours in the ileum, where villi were shorter (132).

There appeared to be a lag time, however, between mitotic interruption and villus changes. Montagna (164) noted that the villi were histologically normal for 96 hours until massive cell loss occurred which coincided with the bulk of the deaths resulting from the treatment. Other authors also found the greatest morphological changes occurring three to four days after irradiation in the form of blunted villi (11, 238, 222). This occurred, however, at a time when mitosis had resumed, and often exceded the original proliferative rate. This led Quastler (189) to speculate that the newly formed cells were being aborted prior to reaching the villus. However, it was later noted (215) that cell migration up the villus still took place during the first 12 hours after irradiation, during a period when no cell production took place, as determined by radioactive thymidine incorporation or the presence of labelled mitotic figures. No movement out of the crypt occurred at 48 hours, however, which was attributed to crypt cell depletion. This appeared to alter the usual proportion of proliferating and differentiating cells in the crypt.

Under normal circumstances, most proliferation occurs at the base of the crypts, referred to as the "proliferative zone", while the upper crypt, termed the "maturation zone",

seems to be more related to cell differentiation (84). After irradiation, the latter zone disappears as the proliferative zone moves upwards (136, 130). Most authors noted, however, that cell proliferation had usually increased by the time gross changes in villus morphology were apparent (36, 222). The inherent advantage of continued cell migration despite interrupted mitotic activity is evident. As cells are lost from the villus, they are replaced by migrating cells. By the time that the crypts have been depleted of their reserves, cell regeneration has generally resumed.

That DNA synthesis is the "weak link in the chain" in the ability of a cell to avoid radiation damage can be seen in experiments with hibernation. In the hibernating dormouse, irradiation during hibernation leads to a postponement of early radiation death until after arousal (2). In the intestine, mitosis is blocked during hibernation at the G_2 phase. This is unlike starvation, where the block occurs in the G_1 phase, having more phases to proceed through before mitosis occurs. Some DNA synthesis still occurs during hibernation, although only at 4% of control rates. The association of death with the resumption of mitosis, therefore suggests that DNA destruction does not become lethal until the cell divides.

The enterocyte is not equally sensitive to DNA alteration at all stages of the cell cycle. Of the G_1, S, G_2, and M phases, the S phase appears to be the most radioresistant (19, 91). This has been elegantly shown by

altering the amount of time the cells spends in various parts of the cell cycle. Intestinal resection has been shown to increase cell proliferation in the gut (60). Hanson (95) demonstrated that this is accompanied by a greater proportion of cells in the S phase by their greater sensitivity to hydroxyurea, an S phase specific cytotoxic drug. By increasing the proportion of cells in this phase, he was able to increase the survival of irradiated intestinal crypt cell cultures.

It appears that the time required for cells to travel through the villus compartment is a critical determinant of survival time after large radiation doses. Cell transit time to the villus tip is increased in the gerbil (171), in germfree mice (84), and in mice fed an elemental diet (135). Immediately after irradiation, there is generally no change in the rate of migration, but in germfree and elementally fed animals, there is a delay in the increase in proliferative activity usually seen after irradiation. In the gerbil, there is a delayed appearance on the villus by cells labelled in the crypt during mitosis (171). This is also associated with increased survival from potentially lethal radiation doses. The conclusion generally drawn from such results is that the radiosensitivity of the mucosa can be decreased by factors which normally decrease cell exfoliation.

Studies on animals with lower metabolic rates such as the goldfish (110) and the amphibian Necturus maculosus

(188) often revealed that enterocyte proliferation was lower than for animals such as mice or rats. In both cases, this was associated with an increase in the rate of survival after high doses of radiation. Experiments with goldfish revealed that as the temperature of their environment increased from 6° C. to 25° C., cell migration rate increased and histological damage developed more rapidly. Similarly, very high radiation doses could be administered to the necturus, which is normally kept at 7° C., without visible intestinal pathology. In both cases, it was suggested that the lower metabolic rate was important in determining survival success. It is interesting to speculate that cell proliferation could be stimulated by cell loss, which in turn is influenced by the metabolic rate.

As radiotherapy came to play an increasing role in the treatment of pelvic tumours, damage arising from concurrent injury of normal intestinal tissue underscored the importance of the work with experimental animals. Vomiting and diarrhea were frequently reported, in some cases occurring intratherapeutically, but often not being evident for up to ten years after radiation treatment (204, 230).

As early as 1931, Buchwald reported decreased absorption of glucose, fructose, and mannose from *in situ* loops of rats after radiation exposure (34). In these early studies, doses often exceded 15 Gy, and recovery responses were often obscured by death. Nevertheless, the functional response after radiation is usually a depression in

substrate uptake by the third day, and recovery by about six days, whether assessed using *in vitro* (222, 182, 184) or *in vivo* (162, 163, 183) techniques. In human patients, reduced disaccharidase activity (232) and glucose malabsorption (51) have been observed.

Several shortcomings existed in early studies. The tendency to use a single concentration to assess transport (183, 162) makes it impossible to draw any conclusions concerning kinetic changes which might have occurred, such as a change in the affinity of a transporter without affecting its maximal transport rate. Also, the use of volume markers have frequently been neglected. Thus, the decrease in the passive absorption of sorbose reported by Moss in 1957 in rats receiving 7 Gy (167) could have been an artifact arising from decreased fluid absorption.

Some discrepancies in uptake studies have been noted. For example, Perris (182) found no change in galactose, 3-O-methylglucose or histidine uptake *in vitro* after 6 R, but found a depression *in vivo* in all these substrates (183). D-glucose uptake, on the other hand, was depressed in both situations. In addition, histological changes are not always associated with transport changes. Decreased glucose uptake has been observed in rat everted sacs four days after receiving 6 Gy despite normal histology (57). Curran (49) reported a reduction in ileal sodium and water transport *in vivo*, as early as six hours after irradiation, well before morphological damage had appeared, and suggested that

transport synthesis had been inhibited. Sullivan (222) found that absorption of 290 mM glucose *in vivo* decreased with increasing doses, but over 9 Gy, no further depression in transport could be seen, despite evidence of greater histological damage. Indeed, the work of Cheeseman *et al.* (41) indicates that three days after irradiation, D-glucose and glycyl-L-leucine uptake (expressed per enterocyte) was actually elevated rats which had received 6 Gy to the abdomen.

Fluid and sodium absorption have also been reported following radiation treatment (49, 222). Perris (183) observed that glucose stimulated fluid flux *in vivo* to the same degree in irradiated rats as in controls, but the baseline absorption was lower in the former. An osmotic gradient on the serosal side created with sorbose was able to increase serosal fluid accumulation. Several other authors have suggested that increased mucosal permeability contributes to excessive fluid loss after radiation, usually assessed by the ability of poorly permeable probes such as mannitol (240), or non-permeating compounds such as polyvinylpyrrolidine (105) to penetrate the damaged mucosa.

Attributing changes in absorptive parameters such as fluid flux solely to permeability changes provide certain problems, however. In general, it is difficult to differentiate between changes in permeability arising in the intestinal vasculature versus a "leakiness" of the tight junctions between enterocytes. Since fluid flux across the

intestine is governed by Starling forces (86), it is quite conceivable that increased serosal pressure arising from tissue edema could result in fluid secretion even in the absence of mucosal permeability changes. Any such mucosal permeability changes would only modulate underlying forces. Lifson (144), for instance, demonstrated that increased venous pressure could result in luminally directed fluid flow.

Several vasoactive compounds such as kinins (168), histamine (251), serotonin (88) and prostaglandins (30), may be released by damage. The resulting tissue edema could cause a net pressure gradient directed towards the lumen and result in fluid loss (86). In the rat, intestinal histamine content is reduced within 24 hours of exposure to 15 Gy., indicating release from intracellular stores (251). Histamine is capable of stimulating substantial fluid secretion in normal dogs, an action secondary to its vasodilatory properties (134). However, antihistamine drugs are only able to decrease initial changes in permeability after radiation treatment (251). In the case of serotonin, intestinal tissue levels of this agent are reduced after administering 725 R. to the rat (77), and serotonin-containing Kulchinsky cells in the gut appear depleted (133). These findings suggest that more extended changes in intestinal permeability are mediated by other agents.

Studies from the vascularly perfused rat show that decreased blood flow results in decreased water absorption (253). Microangiographs of the irradiated rat intestine reveal substantial arteriole occlusion as well as a reduction in the number of vessel present 24 hours after the exposure (66), albeit to rather large (1460 R) doses. This appears to give way to marked vasodilation by the fourth post-exposure day. On the other hand, the capillary filtration coefficient, which provides an indication of the capillary area open to circulation, is reduced in cats four days after 15 Gy, and although some recovery occurs, the coefficient remains below normal for some time (69). At best, then, data on blood flow in the irradiated intestine is sketchy, and definitive statements on the effects of changes in blood flow on fluid transport await further characterization, especially at lower, less lethal radiation doses.

E. Comparison with Other Intestinal Damage Syndromes

Intestinal damage produced by a wide variety of agents often elicits similar symptoms and most, if not all of them are also shared by radiation damage. These include decreased villus height, decreased activity of brush border enzymes as well as glucose and amino acid transport, and either diminished fluid absorption, or net fluid secretion.

Villus height is reduced after damage produced by lactic acid (93) or formalin instillation (160), by ischemia

(198), administration of the cytotoxic drug methotrexate (229, 237), the estrogen antagonist, triparanol (196), infection by bacterial or parasitic agents (225), and in celiac disease (256). In celiac disease, evidence for an ongoing damage process may be seen in the increased loss of DNA into the intestinal lumen (48). Appropriately enough, cell proliferation is substantially increased, which likely enables the intestine to compensate for the increased cell loss (48, 244). Increased cell production has also been observed following intestinal ischemia (198), as well as ulcerative colitis (211) and giardia infection (120). In the latter case, villus histology appeared normal, which would be consistent with a new balance being achieved between increased cell loss and increased cell proliferation, such that no net change in villus height occurs.

A decrease in the functional activity of the intestine is another hallmark of intestinal injury, although recovery is generally rapid, returning to normal in a matter of days. Three days after two hours' ischemia, 3-O-methylglucose and phenylalanine accumulation by mucosal slices is decreased, but has returned to control levels by seven days (200, 202). Brush border enzyme activity, including that of sucrase, alkaline phosphatase, and leucine-aminopeptidase, is also reduced, but return to normal in a similar time span (32, 199). Decreases in glucose absorption *in vitro* have been reported following formalin injury, and three weeks or more may be required for repair (160). In humans, celiac disease

is accompanied by decreases in glucose and amino acid absorption as well as in disaccharidase activity (27, 185).

As with radiation injury, decreases in enzyme activity and transporter function may result in much more rapid cell loss, leaving the villus populated with immature cells. For instance, immediately after a period of intestinal ischemia, cells appear to be shed intact into the lumen. Quantitative histochemical studies on desquamated cells reveal normal levels of enzyme activity (82). As recovery procedes, a greater proportion of cells in the post-ischemic crypt show mitotic activity, resulting in a smaller maturation zone (198). Non-specific esterase and neutral glucosidase activities on the villus, also determined histochemically, decrease as cell proliferation increases. Increased proliferative activity, therefore, appears to interfere with normal maturation processes.

A similar relationship between maturation and proliferation also holds in other damage states. Ten days after innoculation with Nippostrongylus brasiliensis, when parasite counts are highest, the enterocyte population is actually increased, yet maltase, leucine aminopeptidase and alkaline phosphatase activities are decreased (226). In addition, the activity of succinic dehydrogenase, a cytosolic enzyme, is also decreased. Changes in enzyme function are therefore not due simply to the proteolysis of disaccharidases on the membrane surface by increased pancreatic protease action (5), as intracellular enzymes are

affected as well.

The activities of many mucosal enzymes such as disaccharidases (52) alkaline phosphatase (245), and adenosine deaminase (111) increase in enterocytes as they approach the villus tip. Provided that the cell migration rate does not decrease, conditions involving increased cell loss therefore result in the exfoliation of younger cells, allowing less opportunity for full expression of the enzymes required to meet the functional workload of the villus. Premature appearance on the villus of cells labelled during mitosis in both celiac disease (177), and after Nippostrogylus infection (224), also tends to decrease the average age of cells at the top of the villus. This again resembles the pattern of cell maturation observed after radiation injury, i.e., cell migration continues despite cell damage, leading to greater recruitment of new cells from the upper crypt, which reach the villus tip earlier than normal (215).

Fluid absorption *in vivo* is decreased after ischemia, and recovery of normal water uptake can be more retarded than the recovery of the active transport of glucose and amino acids (201). This has been attributed to increased intestinal permeability, as some investigators have demonstrated enhanced passage of normally impermeable macromolecules such as inulin, PVP and creatinine in the rabbit intestine (122). On the other hand, Robinson (201) found that phenol red injected intravenously into the dog

did not appear in the lumen of the ischemic intestine. Nevertheless, there is some evidence that increased vascular permeability is detrimental to the ischemic gut. When aminoguanidine is used to inhibit histamine metabolism by diamine oxidase (DAO) in rabbits or dogs with experimental intestinal ischemia, histamine levels increase. This is associated with a substantial increase in mortality of the animals, likely as a result of the alteration in vascular permeability induced by histamine (129). It should also be noted that DAO metabolizes other molecules, notably polyamines, which will be discussed in greater detail below.

The association of increased fluid loss with an increase in intestinal permeability has also been noted in other damage models, notably parasitic infestation in rats (43) and in celiac disease (44). Bjarnason and Peters (26), using a series of polyethylene glycol (PEG) molecules of different molecular weights, found increased permeability to molecules under a molecular weight of 1500 daltons in patients with celiac disease, which persisted even after gluten withdrawal. Such permeability changes, however, may only affect fluid movement indirectly. Noting that immunogenic fragments of gluten had a size of approximately 1000 daltons, the authors suggested that the effect of the increased permeability was to make the intestine more susceptible to invasion by antigenic molecules which could then provoke an immune reaction. It could be that the immune response, and the accompanying tissue edema could serve as

the driving force for fluid efflux. This bears considerable similarity to intestinal radiation damage, in which increased susceptibility to bacterial infection has been attributed to permeability changes resulting in increased invasion by the pathogen (240).

One explanation for the decreased fluid absorption is that it is simply due to a reduction in the surface area available to diffusion as a result of the loss of villus cells (27). Robinson (201) has further refined this by suggesting that a greater proportion of cells remaining after damage are secretory in nature. He noted that some investigators (59, 246) have claimed that crypt cells secrete sodium and contribute to luminal fluid flux, whereas villus cells predominantly take up sodium and are responsible for water absorption. Since most histological damage appears to occur in the villus tip, loss of tip cells would result in an increased proportion of secretory cells without any change in the nature of the remaining cells. In support of this, Robinson (201) found that unidirectional serosal-to-mucosal sodium flux was largely unaltered in the ischemic dog intestine, whereas flux in the opposite direction was considerably reduced, producing a net sodium secretion and fluid movement into the lumen.

The fact that at least residual active transport of sugars and amino acids remains after injury is an important factor in the control of excessive fluid loss. Glucose is co-transported with sodium independently of underlying

sodium fluxes (76) and is able to increase sodium uptake and water absorption even in the presence of fluid secretion. This substrate-coupled stimulation of fluid absorption in the presence of injury has also been noted after ischemia (106), and after cholera toxin administration (74).

Recently it has been suggested that oxygen free radicals may play an important role in the pathogenesis of ischemic injury (87, 178). While the generation of oxygen radicals might appear to be improbable in such an oxygen-deficient state, damage is often not observed until the tissue is reperfused. Using increasing protein content in lymph as an indication of intestinal damage, Granger found that ischemic injury in the cat intestine could be prevented by administering dimethylsulfoxide, a free radical scavenger, or superoxide dismutase, an enzyme which removes the superoxide radical.

McCord (157) noted that the tissue content of xanthine oxidase, which converts ATP breakdown products into uric acid and superoxide, was increased when tissue damage was present. By administering allopurinol, an inhibitor of xanthine oxidase, Granger (178) could substantially decrease ischemic damage. The increase in ATP catabolism in an energy-deficient tissue in tandem with increased xanthine oxidase activity following ischemia would therefore result in increased superoxide production and tissue destruction. This mechanism could potentially offer an explanation for the ability of intraluminal mannitol to ameliorate the

effects of intestinal ischemia in some cases (201), since mannitol can act as a free radical scavenger (192). The low permeability of mannitol could be a limiting factor in such protection, however, as extensive damage may have to occur before the cell allows sufficient mannitol influx.

Other systems are also capable of generating free radicals, and probably the most physiologically relevant to the current discussion is that of NADPH oxidase, found on the exterior surface of polymorphonuclear leukocytes (8, 155). Important in its bacteriocidal action, patients unable to reduce oxygen with this enzyme suffer recurrent bacterial infections which may prove fatal (8). It has been suggested that increased superoxide release by inflammatory cells plays an important role in the inflammatory response in chronic diseases such as ulcerative colitis and rheumatoid arthritis (54, 156).

The extracellular nature of free radical generation by NADPH oxidase is of special importance in the pathogenesis of inflammatory diseases, because enzymes contributing to the breakdown of free radicals such as superoxide dismutase, are predominantly intracellular (155). This makes connective tissue and vascular elements vulnerable to peroxide attack. Del Maestro (55) has documented hyaluronic acid degradation, increased microvascular permeability and increased polymorph-endothelial cell adhesion in the presence of free radicals. These radicals were generated using artificial systems, usually with xanthine/xanthine oxidase.

Unfortunately, the difficulty in quantifying tissue peroxide or radical content has thus far stood in the way of establishing whether peroxide levels in diseased tissue are substantially different from normal tissue.

Nevertheless, inflammatory status likely has some influence on the function of intestinal cells. Hessel and coworkers (101) found that primary infection of rats by trichnella spiralis produced no changes in α-methylglucoside uptake by intestinal segments *in vitro*. By comparison, a 20% decrease in absorption occurred in immunized rats within 30 minutes of innoculation. Other evidence also suggests that inflammatory infiltration and decreased enterocyte function occur in tandem. The activity of peroxidase, a useful enzyme marker of leukocyte infiltration, is elevated following infection or by acetic acid injury to the intestine (128), over a period of time which coincides with a depression of maltase activity. Finally, inhibition of inflammatory activity by cortisone is known to be effective in restoring normal glucose uptake in celiac disease (117). Cortisone, however, can increase glucose uptake *in vivo* in the normal bowel (16, 210), so it remains uncertain if the effect in celiac disease is a direct or an indirect one.

It can be seen, therefore, that free radical generation results not only from exposure to radiation, but also occurs when damage has been produced by unrelated means. Indeed, an unexplored avenue in radiation damage is the extent to which free radical generation from sources

other than the initial radiation insult itself may contribute to the overall damage picture.

F. Polyamines and Cell Growth Regulation

The short lifespan of the average enterocyte means that the ultimate defence of the intestine against damage and accelerated cell loss is the stimulation of cell regeneration. Polyamines play an important role in the pathway regulating DNA synthesis and cell proliferation (190, 227), and thus are of considerable interest in the study of the repair process in the injured intestine. Due to the massive amount of work now available on the subject, it would be impossible to present a comprehensive review of the field, and therefore, only a cursory overview is provided here.

"Polyamine" is a collective name given to any of the compounds putrescine, spermidine, and spermine. These small, cationic molecules are found in all tissues of the body. Leewenhoek is generally credited with the first observation of spermine crystals in human sperm in 1677 (227), but the chemical structures for spermine and spermidine were not elucidated until 1926 and 1927 respectively (63, 64). Moreover, although the details of their synthesis and degradation pathways have been known for at least three decades, sources as recent as 1961 relied primarily on data from bacterial systems (227), and the biological function of polyamines in mammalian systems was largely speculative.

The pathway for the synthesis and degradation of polyamines in mammalian cells is outlined in Figure 2.1. Ornithine decarboxylase (ODC) is considered to be the rate limiting step in the pathway, owing to its low level of activity in quiescent tissue relative to that of the spermidine and spermine synthases (191). Among the remarkable features of the enzyme is its short half-life, which has been estimated to be as brief as 11 minutes (208), making it the shortest among enzymes studied to date. The enzyme is dependent on the presence of pyridoxal phosphate for activity. When assayed, ODC also requires thiol containing reagents such as dithiothreitol for full activity, being an inactive dimer in its absence (79). Normally present in small amounts in quiescent cells, the distribution of ODC varies throughout the body, with one of the highest concentrations found in the intestine (191), likely a reflection of the high rate of cell production that normally occurs in this tissue.

Some of the earliest data to indicate the importance of polyamines in cell growth came from the isolation of bacterial mutants which lacked ODC and required exogenous putrescine for continued growth (99). More recently, a particular line of mutant Chinese Hamster Ovary (CHO) cells has also been described in which ODC is also absent, and likewise requires exogenous polyamines for growth (220), thus establishing that a similar pathway exists in mammalian cells.

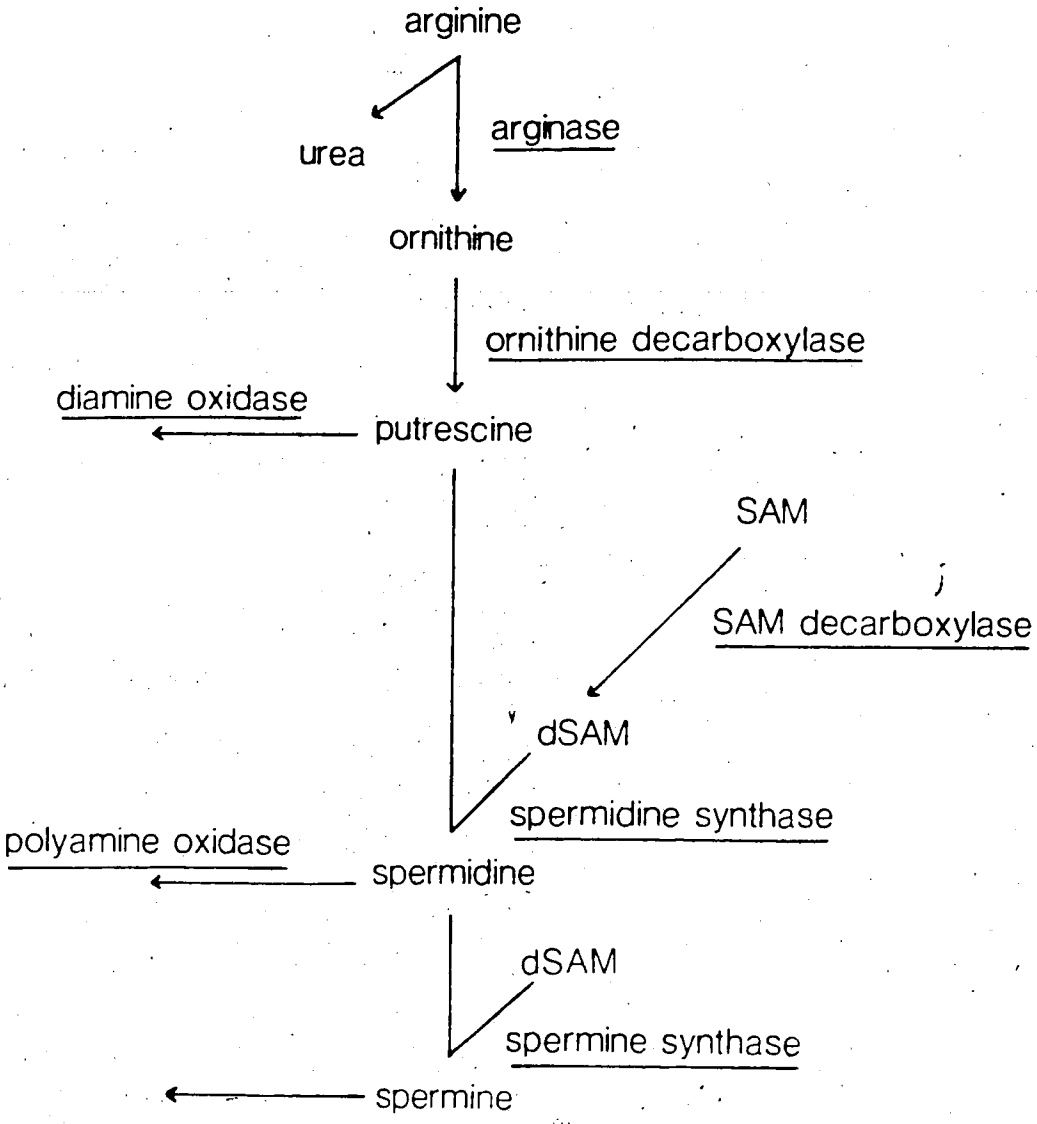

Figure 2.1 The polyamine pathway. Arrows on the left of the figure indicate catabolic processes. Arrows on the right of the figure point to synthetic reactions. SAM = S-adenosylmethionine, dSAM = decarboxylated S-adenosylmethionine.

Intracellular polyamine content is usually elevated when the rate of cell proliferation is increased (98, 113). Friedman (80), working with synchronized Don C cells, and Heby and Anderson (96, 98) using CHO cells have shown that ODC activity and polyamine content peak in early G_1/S phase, immediately prior to DNA synthesis. Polyamines bind tightly to polynucleotides, and are involved with the enzymes of DNA synthesis, stabilizing the correct helical structure of DNA polymerase I (124). Polyamines also play an important role in protein synthesis. Spermidine binds tightly to the tRNA molecule (227), and decreases the rate of misincorporation of precursor amino acids into new proteins. It also increases the initial rate and prolongs the linearity of protein synthesis reactions (1). ODC also appears able to activate RNA polymerase I directly (207).

While polyamine content and ODC activity is generally low in non-dividing cells, this changes dramatically when cells are stimulated to proliferate. Much of the current interest in the role of polyamines in mammalian cells stems from the experiments of Dykstra and Herbst (65), who found that increases in spermidine occured after partial hepatectomy in rats which parallelled RNA synthesis. Schrock et al. (209) demonstrated that increases in ODC activity of 50-fold or more were associated with partial hepatectomy and liver regeneration. Sham operation alone did not appear to increase hepatic ODC. Similar stimulation of ODC activity was also documented in chick embryonic development (206) and

in malignant cells (113), both conditions being associated with rapid cell growth. These increases in the activity of the enzyme are dependent on new protein synthesis, and can be inhibited by protein synthesis inhibitors such as cycloheximide (80) and puromycin (71).

Considerable advances in the study of polyamines have been made due to the introduction of specific inhibitors of ODC such as α-methylornithine (a reversible inhibitor) and α-difluoromethylornithine (DFMO, a non-reversible inhibitor), and of S-adenosylmethionine decarboxylase such as methylglyoxal (bis) guanylhydrazone, (MGBG) (152, 153). The growth of cell cultures is arrested by DFMO, but resumes when the inhibitor is removed from the medium (152), indicating that the compound is not cytotoxic. Cell proliferation can also be reversed by the addition of exogenous putrescine. Although previous authors had found no effect of polyamine inhibitors on RNA and DNA synthesis in HTC cells, Mamont (153) demonstrated that a decrease in DNA synthesis occurs only after the first round of division. The existence of a spermidine pool in the cell apparently delays polyamine depletion and probably accounts for the latency of the effect of inhibitors on proliferative activity.

A wide variety of hormonal agents have the ability to stimulate ODC activity. Catecholamines (151), cortisone (21, 45), cyclic AMP (21) and prostaglandins (9), are among the many agents which elevate tissue ODC levels. Phorbol esters, which are widely used tumour promoters, are also effective

in inducing the enzyme (33, 116, 239).

The mechanism by which ODC stimulation occurs is unclear. Using a line of CHO cells deficient in protein kinase, Lichti and Gottesman (143) could show that phorbol esters could still stimulate ODC activity. The locus of the stimulus from this agent at least, must therefore be distal to the cAMP/protein kinase system, which itself can induce ODC (9, 21). Prostaglandins are also potential mediators of ODC stimulation, and prostaglandin E accumulation in epidermal cells is promoted by phorbol esters (7). Indeed, inhibitors of prostaglandin synthesis such as indomethacin or aspirin have been reported to also block ODC stimulation (33, 239).

Intracellular calcium levels have been implicated in the induction of ODC activity, which is consistent with the role of this ion in the initiation of cell proliferation (112, 248). ODC can be stimulated in guinea pig lymphocytes by the use of the calcium ionophore A23187 (176), and increased extracellular calcium stimulates the activity of the enzyme in cultured epithelial cells (131). Increased medium calcium levels are also capable of stimulating phospholipase A_2 activity in the membrane (70), which promotes prostaglandin release by the provision of its precursor, arachidonic acid. This may lead to an indirect stimulation of ODC via increases in cell or tissue prostaglandin levels.

Phorbol ester induction of ODC can be inhibited by mepacrine, a phospholipase A_2 inhibitor. Whereas indomethacin inhibition of ODC induction can be overcome by PGE, exogenous prostaglandins are unable to stimulate ODC activity after mepacrine treatment (116). This suggests that the products of the lipoxygenase pathway, the leukotrienes, could serve as an important ODC stimulus, as lipoxygenase is less affected by indomethacin than is cyclo-oxygenase, the initial enzyme of prostaglandin synthesis.

In addition to their regulation of cell proliferation, polyamines may also be important in influencing cell differentiation. The differentiation of cultured Friend erythroleukemic cells can be stimulated by dimethylsulfoxide (DMSO), and this process is accompanied by an increase in ODC activity. This differentiation can be inhibited by polyamine inhibitors such as MGBG (85). Similarly, fibroblast conversion to adipocytes in the presence of insulin and methylisobutylxanthine is accompanied by an increase in intracellular spermidine levels and has been reported to be blocked by DFMO (23). Cultured mammary cells from virgin mice normally do not undergo milk protein synthesis, but may be stimulated to do so in a medium containing prolactin, insulin, and cortisol. It was found that spermidine could replace cortisol in the stimulation of mammary gland development. Further, casein production could be blocked by DFMO, which in turn could be overcome by the addition of exogenous spermidine (174, 175).

G. Role of Polyamine Metabolites

Some of the effects of polyamines may be due not to the original polyamine molecule, but rather to an acetylated or oxidized form. Several synthetic diamines have been found to possess potent capability to induce differentiation in Friend erythroleukemia cells (194). Removal of an amine group by acetylation could enhance polyamine exit and entry into cells. Since they contain fewer positive charges, these acetylated derivatives could compete with nuclear polyamines to destabilize DNA structure, an interesting possibility partly confirmed by the fact that the effects were inhibitible by putrescine. In addition, acetylation may make the molecule more lipid soluble, and therefore more capable of penetrating the membrane's lipid bi' er. Since polyamine acetylation is a normal process that occurs in the cell (181), the effects observed may reflect a physiological phemomenon.

Polyamines are metabolized to aldehyde derivatives by the enzymes diamine oxidase (DAO), first described by Best in 1929 (22), and polyamine oxidase, with the concurrent production of H_2O_2 (10). The intestine contains the body's highest concentration of DAO, although it may also be released into the circulation, especially after injury (146). Polyamine oxidase is found predominantly in the blood. As a result of the action of these two enzymes, only low levels of free polyamines appear in the blood. However, polyamine content of both urine and plasma is much higher

after hydrolysis, indicating the formation of polyamine conjugates (205). Intravenously injected polyamines are rapidly conjugated. Polyamine conjugates may be important in the inhibition of cell growth. While exogenous spermine may itself inhibit cell growth *in vitro*, polyamine-containing conjugates were ten times more potent in inhibiting the growth of JB-1 cells in culture than spermine (13).

Efflux of spermidine from cultured cells occurs when they reach confluence (159), resulting in the repression of further growth of the culture. This can be reversed by placing the confluent cells in fresh medium. The export of polyamines into the surrounding medium may not be the only means of depressing growth in surrounding cells. The ability of the fluorescent compound fluorescein (MW = 330) to pass between adjacent cells in the Chironomus salivary gland suggests that cell junctions might play a role in the passage of regulatory molecules between cells (203). Even the largest of the polyamines is small enough (spermine MW = 202) to take advantage of this route to inhibit growth in neighbouring cells.

Junctional permeability appears to depend on intracellular calcium concentrations. Microinjection of a series of fluorescent compounds of different molecular weights indicates that the decrease in junctional permeability occurs in a graded fashion as intracellular calcium levels are increased. Even the largest molecules tested are passed between cells when EGTA, a calcium

chelator, is present (203). It would be expected, therefore, that cell division, when intracellular calcium levels are elevated (112, 248), would coincide with low junctional permeability. Indeed Slack and Palmer (216) found that fluorescein did not move between neighbouring cells of the embryo of the amphibian xenopus laevis. Smaller molecules, such as sodium, were still permeable, however, for current movement could still be detected by microelectrodes in adjacent cells. As embryonic tissue also has a high polyamine content (97), intercellular movement of these molecules would also likely be blocked.

Such a control of the passage of regulatory molecules between neighbouring cells would be of substantial importance in the intestine, in which the rate of cell proliferation can be rapidly altered by a wide variety of environmental factors. Increasing the crypt mitotic index leads to a disorganization of the enterocyte's tight junctions, while the opposite occurs when the cell proliferation rate is decreased (236). This is consistent with the ability of elevated intracellular calcium levels to interfere with microtubule assembly, which is involved in junctional organization (158).

H. Polyamines and Inflammation

Inhibition of mitogenic transformation of lymphocytes and inhibition of lymphocytic proliferation are other possible functions of polyamine metabolites. Incubation of

Bri8 leukemic lymphocytes with putrescine in the presence of DAO produced growth stasis, whereas histamine, which is also metabolized by DAO, or gamma-aminobutyric acid (GABA), which is produced after several subsequent metabolic steps, were without effect in the same system. That the lymphocyte inhibition could be reversed by aminoguanidine, a DAO inhibitor, is consistent with the idea that DAO products, and not polyamines themselves were responsible for the effect (35).

Other evidence, however, suggests that it is the higher order polyamines that play a major role in interaction with immune elements. Thymus extracts containing complexes incorporating polyamines are able to inhibit mitogen-stimulated lymphocyte transformation in the presence of bovine serum (4), which is known to contain polyamine oxidase. Hydrolysed samples, which contained free polyamines, could also inhibit transformation, but putrescine could not, suggesting an effect, in this case, specific to the higher polyamines. On the other hand, human serum, which seems to lack or contain very low levels of polyamine oxidase under normal circumstances, did not prevent the transformation. *In vitro* stimulation of DNA synthesis in lymphocytes by concanavalin A can be abolished by spermine and spermidine in the presence of bovine, but not human or mouse sera. The addition of either of the above polyamines, but not putrescine can effectively inhibit DNA synthesis up to 24 hours after concanavalin A addition,

although greater amounts are required the later the polyamine addition occurs (35). The main difficulty in assessing this evidence lies in the fact that information obtained from *in vitro* systems may not account for the full range of interconversions among different forms of polyamines that could occur *in vivo*.

The resistance of the fetus to maternal rejection despite the presence of paternally inherited histocompatibility antigens has been a research area of considerable interest. Morgan and Illei (165) found high levels of polyamine oxidase activity in retroplacental blood which could significantly suppress ^{3}H-thymidine incorporation by human lymphocytes in the presence of exogenous spermine. Serum concentrations containing higher polyamine oxidase activity than that found in peripheral maternal serum during pregenancy were required, however. This appears to indicate that the effect occurs only at a local, i.e. tissue level, thereby avoiding inhibition of immune function elsewhere in the body, where defence against disease takes greater priority. This may also be reflected in the low polyamine oxidase activity in the sera of mice and rabbits, whereas high activity is found in the liver and spleen of these animals. DAO may also have some role to play in avoiding fetal rejection, in addition to that played by polyamine oxidase, as plasma DAO levels rise throughout pregnancy, but remain low in women who abort spontaneously (83).

If enzymes such as DAO in part serve to protect tissues from inflammatory attack, one might be led to conclude that injured tissues with reduced DAO activity might be especially vulnerable to infiltration by immune elements. While DAO activity may be lower, however, the damage may provide more substrate by inducing ODC activity. The net effect may then still be to prevent inflammatory infiltration. What needs to be underscored here, however, is the relative paucity of data dealing with the effects of polyamines on immune elements in normal and injured tissue *in vivo*, especially in the intestine, which normally has a high content of inflammatory cells (38).

I. Polyamines in the Intestine

The intestine is an extremely dynamic tissue capable of substantial morphlological changes in response to changes in its functional load. Low demand states such as starvation (3, 6, 138), intravenous feeding (126, 127, 107) or consumption of an elemental diet (which is totally absorbed within a small portion of the upper gut (166)) result in tissue atrophy associated with decreased transport activity, and reduced cell proliferation and migration rates. On the other hand, high demand results in a hypertrophic condition in which villus height, cell proliferation and active transport are increased. Jejunectomy (62, 72, 172) and lactation (67, 68) are among the circumstances under which such changes can occur.

If polyamines are closely linked with rapid cell growth, it would be reasonable to expect increases in intestinal ODC activity to occur when demand is high in this tissue. Indeed, even in periods of normal demand, the importance of ODC in the intestine can be seen in the fact that this tissue contains the highest content of ODC in the body, next to the prostate (190). The importance of ODC in intestinal adaptation has been demonstrated in a series of papers by Luk and his colleagues. Jejunectomy, thought to result in the exposure of the ileum to nutrient normally absorbed in the proximal gut, coincides with an increase in ODC activity (147). Inhibition of this increase by DFMO prevents the hypertrophic response to resection. Similarly, intestinal hypertrophy that occurs during lactation can also be inhibited by DFMO (257). Weaning results in an increase in ODC activity as well as disaccharidase activity, and both these changes are inhibited by DFMO (146). Proliferative activity in starved animals increases upon refeeding (89) and this coincides with a rapid increase in ODC activity (154).

Intestinal injury by the cytotoxic drug arabinofuranosylcytosine (ARA-C) results in a decrease in disaccharidase activity which gradually returns to normal over several days. By comparison, ODC activity is substantially elevated shortly after the injury and ODC inhibition by DFMO prevents the recovery of other intestinal enzymes (146). That this increase in ODC activity occurs

when that of many other mucosal enzymes is depressed indicates that the induction of the enzyme is not simply a result of a generalized increase in protein synthesis. It is interesting to note that among the intestinal enzymes depressed by ARA-C injury is diamine oxidase (148, 149), an enzyme involved in the degradative metabolism of putrescine, the product of ODC. More rapid recovery of the synthesis compared to the degradation of polyamines may be a significant part of the repair process.

It has been suggested that the localization of ODC activity in the intestine follows a distinct pattern. Using cell fractionation techniques, Baylin (17) found that a gradient in the activity of this enzyme existed on the villus, with the highest levels in the villus tips. On the other hand, Ball (12) using a tissue planer, found little difference in ODC content between the villus and crypt. Even if levels in the tips were similar to those in the crypt, however, they would still be unusually high for non-proliferating cells.

The levels of other enzymes in the polyamine pathway may be important in understanding this phenomenon. Diamine oxidase, which degrades putrescine to an aldehyde derivative, is also found to a larger extent in the villus tips (17, 213), suggesting that a larger percentage of the putrescine synthesized in the crypts is available for the synthesis of higher polyamines. In addition, S-adenosylmethionine decarboxylase activity, which is

necessary for spermidine and spermine synthesis, is highest in the crypts (186), as is the intracellular concentration of spermidine and spermine. This may indicate a specialized function for different polyamines in the control of either proliferation or differentiation, and this may be reflected in their localization.

J. Methodological Considerations for Transport Studies *in vivo*

Considerable advances have been made in the last decades in the study of intestinal transport mechanisms using *in vitro* techniques, most notably those employing either brush border vesicles or isolated enterocytes (121). Removal of the intestine from the body, however, disrupts several factors which would normally contribute to the control of absorption of fluid, electrolytes and substrate. Levine observed rapid histological deterioration of everted intestinal sacs *in vitro* (141), although this particular preparation may represent an extreme example of tissue viability. As extensively reviewed by Tapper (231), neural influences play an important role in fluid absorption. Adrenergic influences stimulate fluid and electrolyte absorption, whereas cholinergic elements mediate secretion (187).

The absence of a blood supply is also likely to have drastic effects on uptake. Studies using the vascularly perfused rat reveal increased water uptake as blood flow is

increased, and the reverse when blood flow is decreased (253). Likewise, both L-phenylalanine and 3-O-methylglucose uptake have been shown to decrease when blood flow is decreased (254). The continued removal of absorbed substrate from the serosal surface of the enterocyte appears to facilitate absorption by maintaining the chemical gradient across absorptive membranes. In addition, the absorption of glucose itself stimulates an increase in blood flow, thereby facilitating its own removal from the mucosa (28).

Water uptake may also follow a different pattern *in vivo* than with isolated tissue owing to hydrostatic effects. Lifson (144) demonstrated that intestinal secretion could be induced experimentally by elevating venous pressure and suggested that increases in interstitial pressure from tissue edema (86), resulting from the release of inflammatory mediators, could produce a similar effect.

Experimental difficulties using *in vivo* tec iques arise, however, if more than a simple comparison of total absorption is attempted. *In vitro*, for instance, glucose transport can be shown to be an active, saturable process which conforms to the Michaelis-Menten equation (75). *In vivo*, however, some authors have claimed that glucose uptake displays characteristics typical of simple diffusion, i.e. linearity of uptake over a range of concentrations (126, 195). This can now be shown to be a result of the use of low perfusion rates, resulting in a large unstirred layer which acts as a resistance to solute uptake by the mucosa (58,

247).

Unstirred layers arise when any solid surface is superfused by a moving fluid, as a physical property of fluid dynamics. Several reviews and theoretical descriptions of the unstirred layer effect may be referred to for a more complete approach to the topic (58, 247, 234, 235). In the intestine, stationary layers of adherent fluid arise next to the mucosa, forming a resistance to transport in addition to that of the lipid membrane. In the case of some substrates, especially lipid soluble ones, this unstirred layer represents the rate-limiting step to substrate transport (235).

While unstirred layers may be reduced by increasing the turbulance in the system, i.e. by stirring or increasing the perfusion rate (142, 255), they cannot be entirely eliminated, as tissue destruction occurs if stirring is too vigourous (142). In addition, increasing the flow rate *in vivo* can increase intraluminal pressure, which in turn leads to greater fluid uptake as the villi are forced apart, exposing a larger surface area for absorption (160). Whether the resistance attributed to unstirred layers is solely a function of fluid dynamics is itself a matter of controversy (56). Some authors have claimed that adherent mucus and the glycocalyx also make a significant contribution to the resistence of the system to substrate transport across the epithelium (219).

In practical terms, the effect of unstirred layers on estimates of transport kinetics is reflected in the following simplified equation (139, 140):

$$\text{Apparent Km} = \text{Actual Km} + \text{Vmax}(d/D)$$

where Km is the concentration required for half saturation of the system, Vmax is the maximal transport rate, d is the thickness of the unstirred layer, and D is the free diffusion coefficient of the substrate under study. The apparent Km of the transport system is therefore always increased compared to its 'real' value. Further, an increase in the Vmax will result in an artificial increase in the Km as a result of the unstirred layer.

Methods have been described for the measurement of the unstirred layer. The exposure of the mucosa to a hyperosmotic solution (mannitol is typically used) results in an increase in fluid flux directed towards the lumen, which also results in sodium flux in the same direction, owing to solvent drag. This creates a charge separation or potential difference which may be measured by electrodes placed on opposite sides of the tissue. The half time (t1/2) required for this potential to attain a steady state plateau provides an estimate of the dimensions of the unstirred layer (247).

Another technique is to use a series of alcohols with varying chain lengths (103, 233). Highly lipid soluble, and thereby capable of readily crossing lipid membranes, the

uptake of these alcohols will increase with increasing chain length as their lipid solubility increases. A limiting chain length is reached at which no further increase in uptake occurs. At this point, the unstirred water layer is the rate limiting step, and changes in uptake [illegible] this chain length therefore reflect changes in the thickness of the unstirred layer. It must be emphasized that the apparent Km is a function of the free diffusion coefficient of the probe. Since this value varies from substrate to substrate, the effect of the unstirred layer on transport parameters will vary depending on the substrate used.

Other considerations involved in assessing results obtained by *in vivo* techniques involve the method of normalization of the data. Various investigators have expressed uptake on the basis of length of intestine, dry weight, or per enterocyte, with markedly different conclusions. This has been extensively reviewed by both Karasov (115) and Levin (140), who both arrived at the conclusion that the most reliable and physiologically relevant standard was uptake per unit length. They noted that dietary or surgical manipulation often results in changes in intestinal weight, as well as in uptake, which may tend to cancel each other out. Starvation, for example, results in considerably reduced gut weight (115).

Expressing uptake per enterocyte (15), on the other hand, assumes that all cells on the villus transport substrate to a similar extent. In fact, autoradiographic

techniques have shown that cells at the tip tend to transport more substrate than cells in the lower portion of the villus or in the crypt (5, 123, 218). This could be due either to fewer transporters in the cell membrane, or to the greater unstirred layer that the probe must cross in order to reach the cells at the villus base. Expressing uptake as a function of gut length therefore remains the method of choice for normalizing data.

K. Techniques

Early studies on absorption *in vivo* used the simple method of tying off a loop of intestine, filling it with a solution containing a probe, and, after waiting for a given period of time, removing the loop and determining the amount of probe remaining (34). As only a single concentration of substrate was usually used, this method sufferred from the likelihood of overlooking subtle changes in uptake. As intestinal transport of hexoses and most amino acids has often been shown to obey Michaelis-Menten or related kinetics, changes in Km and Vmax could occur such that only minimal changes would be detected at some concentrations, while larger differences may occur at others. Choosing an inappropriate concentration could therefore result in changes being overlooked. In addition, the complete lack of stirring in the system would result in extremely high unstirred layers, which in some cases could be so high as to be the rate-limiting step in transport, rather than

transport across the membrane.

Attempts to deal with the former problem have resulted in the development of the perfused intestine, as described by Sheff and Smyth (214). Using either a pump or a gas lift, solutions, generally covering a range of concentrations, are perfused through the intestine without disturbing the vascular supply. Both re-circulating (53) and single pass (81) methods have been employed with this technique. That unstirred layers remain a significant consideration in this technique is reflected in the increase in uptake that occurs with an increase in pump rate (255). As the rate of flow through the intestine increases, the unstirred layer decreases, resulting in greater absorption of the probe as the resistance to its uptake is diminished. In general, non-absorbed markers of larger molecular weight such as inulin or polyethylene glycol (PEG), are perfused simultaneously with the probe to correct for volume changes resulting from fluid flux.

In vitro experiments have firmly established that the absorption of glucose and most amino acids by the enterocyte occurs by an active, sodium-dependent pathway at the mucosal surface of the cell (121). In the whole tissue, this is expressed by movement of sodium ions (a process coupled to gluc movement) across the epithelium, causing a rapid rise in the transepithelial potential difference. Debnam and Levin (53) have described an *in vivo* technique based on this principle to measure the active (sodium dependent) component

of glucose transport *in vivo* which has been successfully employed in both rats and in man (193). By inserting an electrode (via an agar bridge) into the lumen of the intestine, and using a wick electrode in the peritoneum as a ground, the potential difference can be measured. The transport of glucose out of the lumen results in sodium movement, which is measured as an increase in the potential difference.

Previous attempts to separate active transport from passive diffusion had largely relied on inhibitor studies by using phloridzin, a glucose specific transport inhibitor, to eliminate the active component of transport, leaving a component attributed to passive flux (114, 178, 179). Glucose transport is inhibited by phloridzin in the intestine *in vivo* and *in vitro*, as well as in other tissues, most notably the kidney. Phloridzin consists of a glucose molecule coupled to another molecule, phloretin. In the intestine, phloridzin acts primarily at the mucosal surface, in a competitive fashion, i.e., its effects are reversible by increasing concentrations of glucose. By comparison phloretin is 100 fold less effective at this site, but is the more effective inhibitor in red blood cells, and at the serosal surface of the enterocyte (46).

Potential problems could arise, however, in using phloridzin to assess the active transport component in the intestine under conditions where intestinal function is altered by injury or by other adaptive changes. The

description of a phloridzin hydrolase on the mucosal surface raises questions about the limitations of this inhibitor. This hydrolase is a disaccharidase which has been isolated as part of a complex also containing lactase, in a manner analagous to the appearance of the enzymes sucrase and isomaltase in the same complex (94, 242). Patients with lactase deficiency also display an increase in phloridzin mediated inhibition of glucose transport (145). Although the physiological significance of such an enzyme remains unknown, the effect of a depression in the activity of this enzyme would be the inhibition of glucose transport at concentrations of phloridzin considerably lower than that normally employed.

Other approaches to the study of passive or diffusive transport have involved the use of substrates whose uptake is supposedly not mediated by an active process. Sorbose, xylose and L-glucose have been suggested to fall into this category (53, 252). Even with compounds such as these, it must be borne in mind that some investigators have found evidence for active transport, especially in starved animals (169, 170) and at low concentrations (24, 25, 37). All such reports come from *in vitro* models, however, and it remains to be shown whether findings such as these affect the use of these sugars as probes of passive permeation *in vivo*.

L. An Integrated Approach to Assessing Intestinal Injury and Repair

The study of the recovery from intestinal radiation injury has been complicated by the use of widely varying conditions, including different doses, dose rates, type of radiation, age and species of test animal, and the time frame of study after injury. Changes occur in both intestinal function and structure after radiation damage. However, many previous studies have only reported the changes observed when damage was the greatest, and have not documented the full recovery process. Thus there is uncertainty whether functional changes are correlated with histological ones, especially under similar conditions. Do both these features return to normal over a similar time scale, or does one recover more rapidly than the other? In addition, some morphological changes, such as earlier exfoliation of enterocytes, may not be immediately discernable under the microscope if other factors, such as migration rate, are also changed.

If substrate uptake *in vivo* is to be used as a measure of mucosal injury or repair, it must be borne in mind that intestinal absorption consists of several components, any one of which may be changed by injury, or remain unaltered. As positive results could be masked by changes in the opposite direction by different components of uptake, for example passive and active uptake, a thorough survey of several substrates is warranted to assess whether transport

changes reflect the degree and duration of damage phenomena. Consideration should also be made for possible changes in unstirred layers, and for using a range of substrate concentrations large enough to detect changes in kinetic parameters. Such changes could be overlooked if, for example, they occurred predominantly at high concentrations when only low concentrations were monitored. In addition, further study is required to determine whether the possible reduction of food intake after intestinal injury affects the course of recovery.

In addition to the assessment of transport activity, there is also a need for other tests to monitor the repair of the damaged gut. ODC is involved in the synthesis of DNA, and therefore the activity of this enzyme may also be a useful indicator of the progress of intestinal repair processes following radiation damage, where the interruption of DNA synthesis and cell mitosis is followed by large changes in proliferative activity. To date, however, no study has been undertaken to determine what influence irradiation has on intestinal ODC activity. It would be of particular interest to correlate changes in the activity of this enzyme with alterations in mucosal morphology.

Even under normal circumstances, only one study has been published describing the importance of ODC in the turnover and appearance of the mucosal cell. It needs to be shown, therefore, whether ODC activity is critical to the intestine in both normal and pathological circumstances.

Further the nature of the stimulus for intestinal ODC induction is still not well understood. Examination of the factors which could serve as triggers of this enzyme could be useful in predicting the influence of dietary or pharmacological manipulations on the course of recovery of the bowel after injury. Indeed, the use of pharmacological agents may themselves reveal information about the mechanisms involved in damage and repair processes.

Another neglected parameter of intestinal recovery has been the repopulation of the immune elements in the gut after abdominal irradiation. This has not been approached in a quantitative manner thus far due largely to technical difficulties involved in the microscopic evaluation of granulocyte populations in a heterogenous cell environment. The development of biochemical tests for leukocyte infiltration of the mucosa could be useful in charting the recovery of this component of gut function. Given the influence that polyamines on immune function, it would be especially interesting to determine if ODC activity correlates with infiltration of the gut by immune elements.

Thus, there is a need for a more integrated approach to the assessment of the recovery process after radiation damage that takes into consideration the many parameters that should be affected, as well as the way in which they could interact. By standardizing the damage conditions and following a wide range of indicators over the entire course of recovery after injury, it should be possible to gain a

fuller understanding of the mechanisms involved in intestinal damage and repair - an understanding which to date has only been piecemeal.

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III. Experiment 1: The Assessment of Recovery of the Intestine After Acute Radiation Injury

A. INTRODUCTION

Acute intestinal radiation injury is characterized by a decrease in glucose and amino acid uptake (16, 43, 48), brush border enzyme activity (6, 58) and a reduction in villus height (44, 51), within the first three days following exposure. These parameters subsequently return to normal over the next one to two weeks (43, 48, 57). A variety of *in vitro* (11, 43, 48, 49, 61) and *in vivo* (7, 16, 47, 57) techniques have been employed to monitor the severity of the damage produced by abdominal irradiation and the subsequent course of recovery. Contradictory results have been obtained, however, depending on the technique used. Cheeseman et al. (11) noted that glucose transport actually increased 3 days after rats received 6 Gy to the abdomen. Similarly, Thomson (61) demonstrated that the maximal transport rate *in vitro* increased three days after irradiation once changes in unstirred layers were accounted for.

Previous reports dealing with the effect of substrate uptake *in vivo* contain several technical problems which make it difficult to determine if the effects observed are due to changes to the transporter or to other aspects of intestinal function. Alterations in blood flow result in corresponding changes in fluid and substrate uptake (66, 68) which

conceivably could produce decreased transport activity even in the absence of changes to the membrane carrier. Changes in the resistance of the unstirred layer (60, 64), which have not been determined *in vivo* after radiation injury, could also mask the true response of the tissue. Virtually all studies to date using *in vivo* methods have only used a single concentration of substrate (43, 47, 57). Since uptake at certain concentrations may be more affected than others, depending on whether affinity (Km) or maximal transport rate (Vmax) of the transporter is more affected (36), subtle changes may be overlooked. Finally, the transport of substrates such as glucose may have both active and passive components (17), which have not been examined separately following radiation injury. If one component is affected by injury more than another, or in an opposite direction, a misleading picture of the intestinal response to damage could arise. The present studies were undertaken, therefore, to rectify some of these deficiencies.

The response of the intestine to injury is not restricted to changes in transport, morphology, or brush border enzyme activity, however. Other parameters may not necessarily be affected to the same degree or over a similar time scale as transport changes. Two such parameters chosen for investigation here are leukocyte infiltration of the gut, and ornithine decarboxylase (ODC) activity.

Cellular elements of the immune system are extremely sensitive to the effects of radiation (30). While the gut is

the locus of large accumulations of lymphocytes and granulocytes (10), scant information exists on the ability of such cells to repopulate the intestine after irradiation. Measurements of leukocyte population size have in the past been impeded by the difficulty in obtaining quantitative assessments, which were usually restricted to tedious and time-consuming histological work. However, with the finding that myeloperoxidase activity correlates linearly with leukocyte numbers (34, 54), a useful biochemical tool is now available to provide reasonable estimates of this parameter.

The enzyme ornithine decarboxylase has been implicated in the control of DNA synthesis (52), which is of critical concern in the intestine where cell turnover time is so rapid (21). Intestinal ODC activity can be elevated by mucosal damage produced by the cytotoxic drug ARA-C (41). The profound effect of radiation on DNA synthesis and mitotic activity in the intestinal mucosa (6, 44, 51) makes the study of this enzyme particularly relevant.

B. METHODS

Animals - Male Sprague-Dawley rats weighing 250-300 g. were obtained from a local colony. Animals were housed three to a cage and allowed free access to food (Purina rat chow) and water throughout the course of all experiments. A constant light cycle of 7 a.m. to 7 p.m. was maintained.

Perfusion Solutions - ^{14}C labelled sugar concentrations ranging from 4 to 96 mM and ^{3}H labelled

leucine concentrations from 4 to 48 mM were dissolved in Krebs' bicarbonate saline (NaCl, 70 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 1 mM; pH 7.4) bubbled with 5% CO_2 and 95% O_2. The sodium chloride concentration in these solutions represents a reduction of 96 mM from isosmolarity, the difference between each sugar or leucine concentration and isosmolarity being made up by mannitol, which is minimally absorbed. Corrections for fluid movement were made using a non-absorbable marker. ^{3}H-inulin was used with the sugars and ^{14}C-inulin was used with leucine.

Probes employed included D-glucose, L-glucose, 3-O-methylglucose D-galactose, L-leucine, and D-leucine. In the case of D-leucine, 32 mM L-leucine was also added to the perfusate to avoid the possibility of low affinity transport of the D isomer by the L carrier (31). Fatty alcohols, of increasing chain length from 6 (hexanol) to 12 (dodecanol) were used to assess possible changes in the resistance of the unstirred layer (59).

Perfusion Technique - *In vivo* absorption was determined using an open loop perfusion technique (17). Rats were anaesthetized with pentobarbital (60 mg/kg, i.p.), and the small intestine was exteriorized through a mid-line incision. A loop of jejunum corresponding to an *in situ* length of about 10 cm. was cannulated at both ends, beginning about 5 cm. distal to the ligament of Trietz. The intestine was then placed back into the abdominal cavity.

Body temperature was maintained by placing the rats on a heated water table (temperature maximum: 36° C. - higher temperatures tended to overheat the animal and result in unnecessary mortality). Anaesthesia was maintained by periodically injecting pentobarbital through a catheter in the jugular vein.

The jejunual segments were perfused distally with preheated isosmotic solutions using a peristaltic pump. A single pass method was used, the effluent being collected over thirty minutes in aliquots corresponding to a 5 minute period. The pump rate (0.5 ml/min) was chosen on a pragmatic basis. Although uptake increased with higher rates and decreased at lower rates, indicative of changes in the unstirred layer (64, 67), higher rates were found to result in increased mucus accumulation over the perfusion period, resulting in tubing blockages and unreliable results.

Since uptake occurs over the length of the segment perfused, the initial concentration of the solution in the lumen will differ from the final luminal concentration. Various methods of expressing the mean luminal concentration have been used; the logarithmic mean, determined to be the most accurate (69), was employed in these studies. With this correction, luminal concentration is equal to the following:

$$\frac{Ci - Co}{\ln (Ci/Co)}$$

where Ci is the input concentration and Co is the output concentration. Due to evaporation losses, the calculation of Ci for the fatty alcohols also employed the time of sampling to interpolate the corresponding value of Ci from a logarithmic decay curve estimated on the basis of standards taken every 10 minutes.

Solutions were perfused serially, from the lowest to the highest concentration. To avoid possible deterioration of the preparation with time, only four concentrations were perfused in any one animal. As a result, any one animal did not receive all concentrations studied. However, one or two concentrations (generally 8 mM and 96 mM) were used in all animals. An initial 10 minutes were allowed for adequate mixing and steady state absorption to be achieved. Steady state conditions were assumed by linear uptake over a 20 minute collection period (Figures 3.1a, 3.1b). For the sample shown, the Coefficient of Variation was 7.3% at 8 mM and 10.3% at 64 mM. 200 µl of each effluent aliquot was pipetted into 5 ml of scintillant (ACS, Amersham) and counted for one minute on an LKB beta counter. Mean counting error was 1.7% for the ^{3}H channel and 1.9% for the ^{14}C channel.

Uptake was calculated based on solute disappearance from the lumen. The uptake of D-glucose (Figure 3.2) and L-leucine (Figure 3.3) showed a significant non-linear component ($p<.01$). On the other hand, the uptake of L-glucose and D-leucine was consistant with a linear

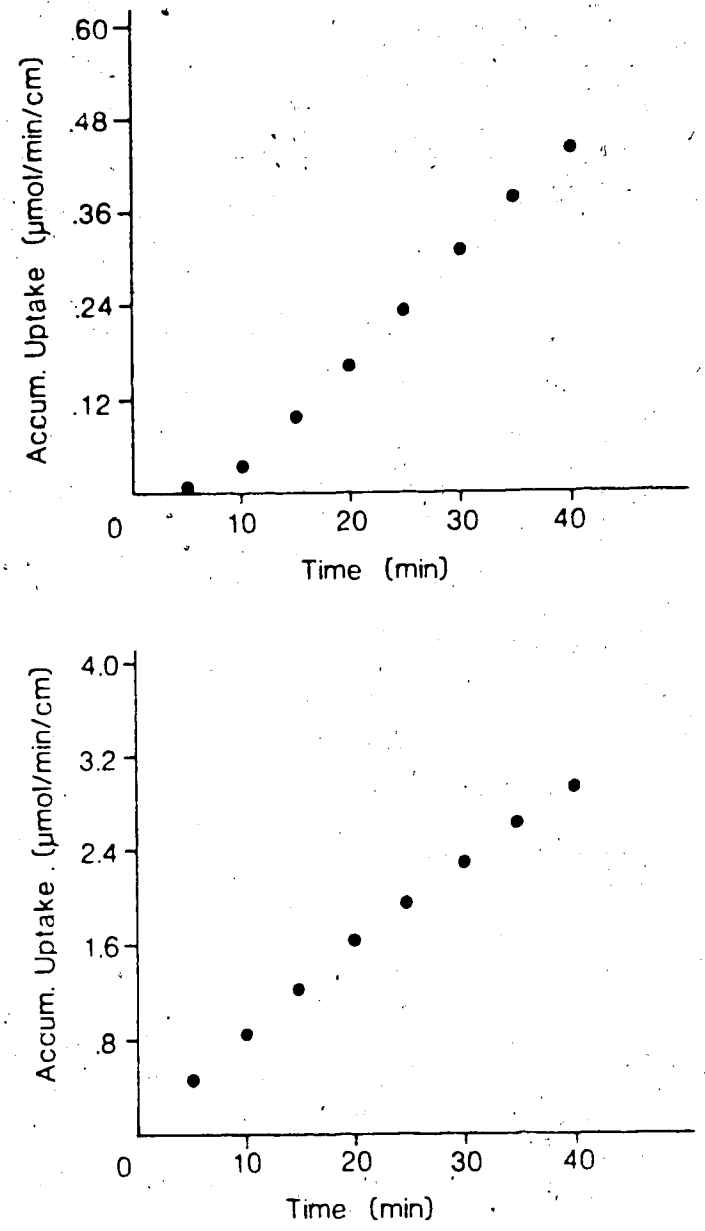

Figure 3.1a (Top) Accumulative uptake of D-glucose over time in a typical perfused animal. Substrate concentration, 8 mM.

Figure 3.1b (Bottom) Accumulative uptake of D-glucose over time in a typical perfused animal. Substrate concentration, 64 mM.

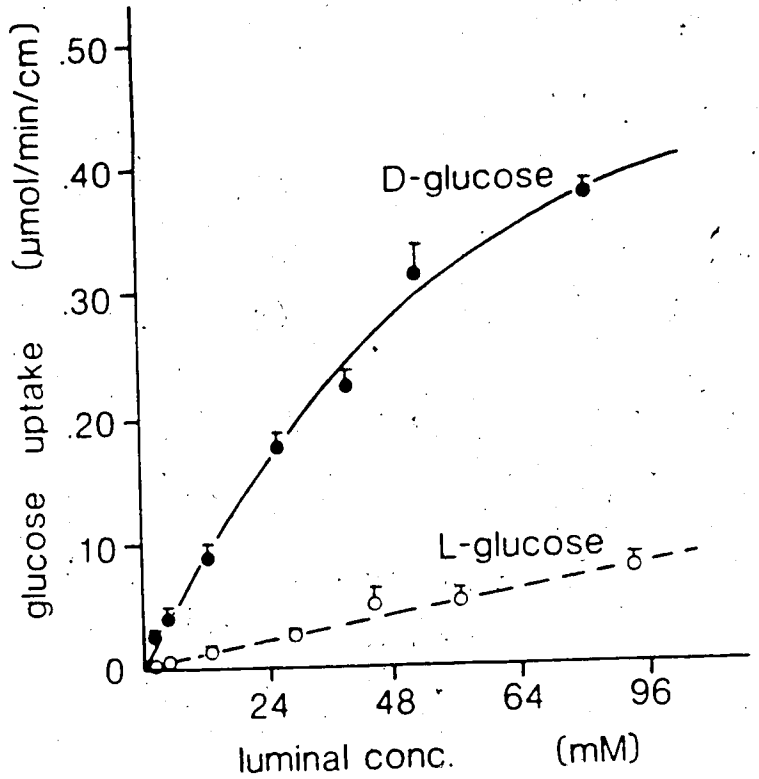

Figure 3.2 Total D- and L-glucose uptake *in vivo* in the upper jejunum of control rats. Points represent mean and standard error of the mean (SEM). Number of observations per point: D-glucose, 12-22; L-glucose 4-9.

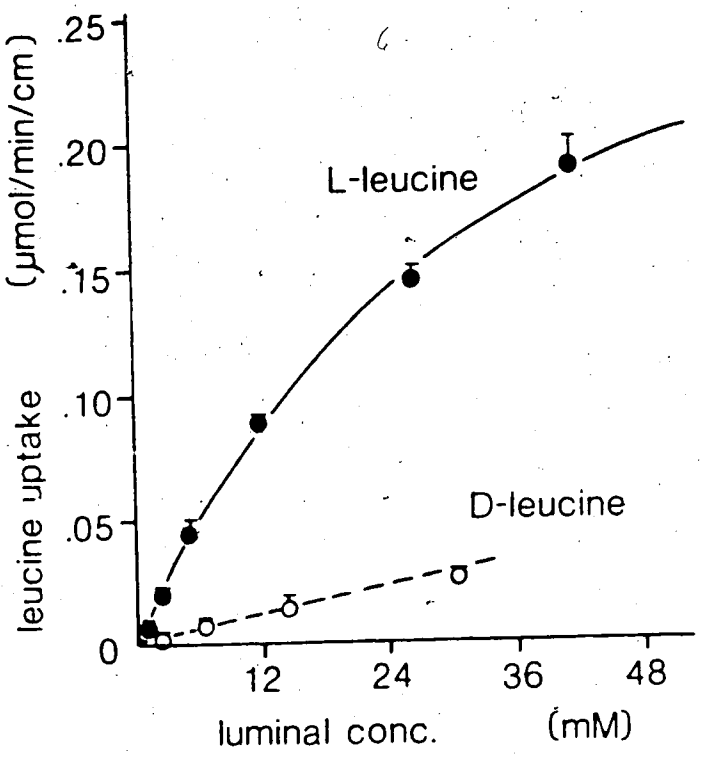

Figure 3.3 Total L- and D-leucine uptake *in vivo* in the upper jejunum of control rats. # of observations per point: L-leucine, 4-7; D-leucine, 4-6.

relationship. The slopes of the least squares regresssion lines (56) of the latter two compounds showed significance ($p<.01$), and no improvement in the mean square occurred by fitting the data to a higher order polynomial. In addition, the lines were not significantly different from a line through the origin. To estimate active (net) uptake, the contribution from passive diffusion was subtracted from each measurement of active uptake by the use of this calculated coefficient. At the end of the run, the perfused segment was removed, its stretched length measured, and weight determined after drying overnight at 70°C.

Phloridzin Sensitivity - Preliminary studies using the glucose transport inhibitor phloridzin to measure passive glucose absorption revealed substantial variation in glucose uptake in irradiated animals. To determine if these changes were due to a reduction in the ability of phloridzin to inhibit glucose uptake which were distinct from any possible changes in passive sugar uptake, a technique measuring potential difference (p.d.) *in vivo* was used (17).

Transepithelial p.d. was monitored by using salt bridges containing 1 M KCl leading to small beakers containing an identical solution into which had been placed silver electrodes which were connected to a high impedence electrometer. One salt bridge consisted of PE 90 tubing which also was filled with 3% agar. This was inserted into the proximal cannula entering the intestine, affording a luminal probe access. The other bridge consisted of a cotton

wick electrode which was held in a hollow glass tube and placed in contact with the peritoneal surface of the animal. The electrometer output was connected to a chart recorder for a permanent record. The jejunal segments were initially perfused with standard Krebs buffer also containing 64 mM mannitol and a steady state recording was taken of the p.d. generated by the hyperosmotic solution. This was taken as the baseline for subsequent measurements. The segments were then perfused serially with increasing concentrations of phloridzin ranging from 0 to 1.0 mM in standard Krebs buffer containing 64 mM D-glucose but no mannitol (final osmolarity, 374 mM).

Myeloperoxidase and ODC Assays Myeloperoxidase activity was measured as described in Chapter V by measuring the change in absorbance by guaiacol after the addition of H_2O_2. ODC activity was determined as described in Chapter VII by measuring $^{14}CO_2$ produced by the decarboxylation of ^{14}C labelled ornithine. As irradiation causes substantial changes in the protein content of enterocytes (11), both myeloperoxidase and ODC activity were expressed per unit DNA, using Burton's reagent as described in Chapter VII.

Irradiation Procedure - Animals were irradiated between 8:30 a.m. and 3:30 p.m. using a Picker ^{137}Cesium source at the Cross Cancer Institute, Edmonton, Alberta. Owing to source decay, the dose rate decreased from 1.42 to 1.31 Gy/min over the course of the study. This was compensated for by increasing the exposure time. Each animal

was anaesthetized with pentobarbital (60 mg/kg), and the abdomen (i.e. the area below the diaphragm) exposed to 3 Gy bilaterally (total dose = 6 Gy). This dose is below the $LD_{50/30}$ (the dose which produces death in half the animals by thirty days) of 9 Gy which has been reported for the rat (26). The large size of the applicator head (10 cm x 10 cm) minimized penumbra effects, but likely involved injury to other tissues such as testis and pancreas. Animals were placed beneath the applicator head (distance from head to bottom of animal, 4.5 cm). Feet were taped down to prevent obstruction of the radiation beam. Source to surface distance (SSD) was 15 cm.

Dosimetry was performed approximately every four months by Mr. Bert Meeker using a Victoreen dosimeter, and corrected for temperature and pressure. Uptake experiments were performed 3, 7, and 14 days after irradiation. Control animals were anaesthetized but not irradiated, and used three days later. The radiation unit was operated from a separate room, shielded by a concrete wall lined with steel plating. The operator wore a film badge, which indicated no detectable radiation exposure during the protocol.

Statistics - Analysis of covariance was used to detect significant differences in substrate uptake between treatment groups. Multiple analysis of covariance, using a second degree polynomial to fit the non-linear component (56) was employed where significant curvilinearity was present. It should be noted that this approximation is only

valid over the concentration range used and other forms of curve fitting must be used in extrapolating values, on the basis of some *a priori* evidence for the mathematical model chosen.

Km and Vmax values were estimated after subtraction of the passive component of uptake as measured by the stereoisomer. A BASIC programme employing an iterative, non-linear regression method described by Duggleby (19) was used to arrive at the final values. As the variance increased with the size of the y variable, a bisquare weighting ($1/y^2$) was used. Initial estimates of the Km and Vmax were made using an Eadie-Hofstee transformation. The programme then calculated the best fit of parameters by minimizing the residual mean square. Such a procedure reduces the error in the parameter estimates by avoiding additional error generated by methods relying solely on linear data transformations. This is of particular concern with transforms such as that of Lineweaver-Burk (36).

As the p.d. data was discontinuous, a nested design analysis of variance was used for comparisons using this technique. Elsewhere, unless noted, a t-test was used (56).

C. RESULTS

Uptake of L-glucose and D-leucine (Table 3.1a, 3.1b) showed no significant differences among the control and radiated groups. A significant reduction in the uptake of net D-glucose (Figure 3.4) was observed 3 days

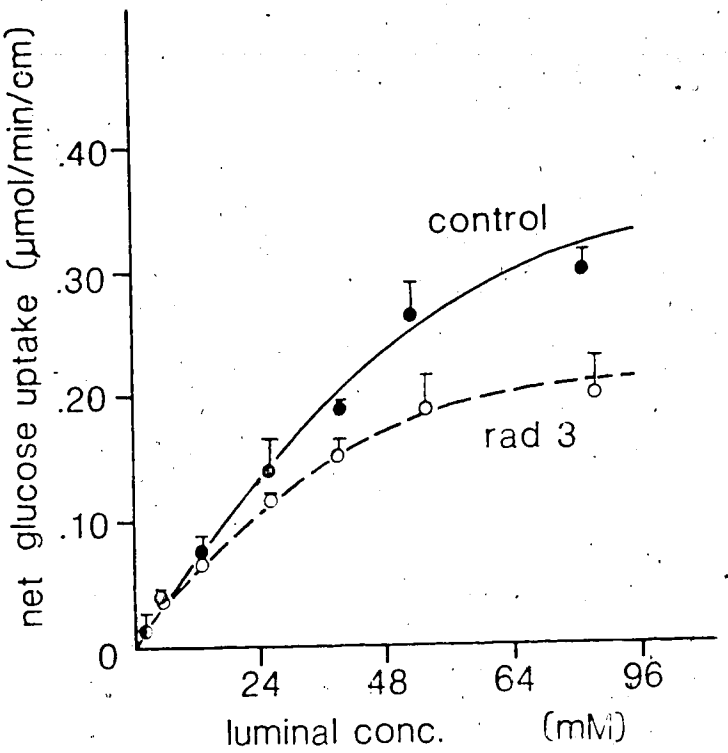

Figure 3.4 Net D-glucose uptake *in vivo* in the upper jejunum of control rats (12-22 observations per point) and in rats 3 days post-irradiation (7-12 observations). Curves were significantly different at $p<.05$. Uptake curves 7 and 14 days post-irradiation were not significantly different from the control curve.

post-irradiation ($p<.01$), but not at subsequent time points. This was reflected in a lower Km and Vmax for net D-glucose uptake at the 3 day point (Figure 3.5). Similar reductions in the net uptake of D-galactose, reflected in the reduction in kinetic parameters (Figure 3.6), and in net 3-O-methylglucose uptake (not shown), were also observed at the three day point. Net L-leucine uptake (Figure 3.7) was reduced at 3 days post-irradiation ($p<.005$) and after 7 days ($p<.05$), but had returned to control levels by 14 days after treatment. This was reflected in corresponding reductions in kinetic parameters (Figure 3.8).

The relation ln (Jd/D) showed an increase with increasing alcohol chain length up to a maximum attained with decanol (Figure 3.9). This was taken to indicate the limitation of diffusion by unstirred layers at chain lengths of 10 carbons or greater. Uptake of decanol could be increased by increasing the flow rate from 0.5 ml/min to 2.0 ml/min, but no significant differences in uptake were observed among control and irradiated animals (Figure 3.10).

Glucose-stimulated water uptake reached a maximum at approximately 48 mM (Figure 3.11), and water uptake at 96 mM was subsequently used as an extimate of maximal water absorption. Maximal water absorption declined substantially 3 days after irradiation (Figure 3.12), but returned to control levels by the 7 day time point. The dry weight/length ratio of the perfused segment was also

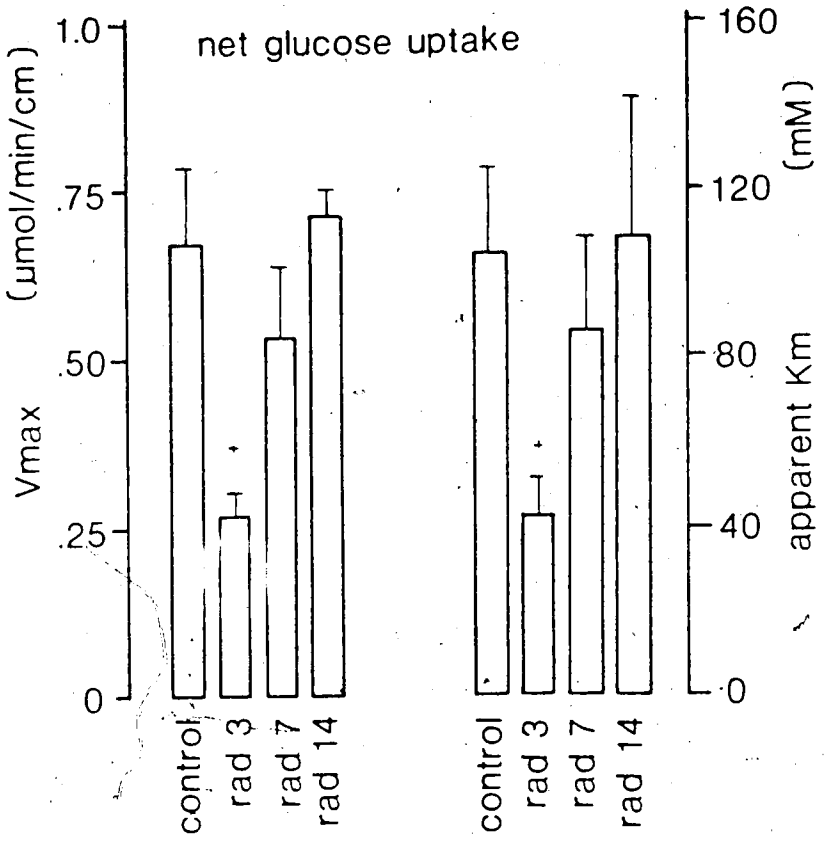

Figure 3.5 Apparent affinity constant (Km) and maximal transport rate (Vmax) of net D-glucose uptake *in vivo* in the upper jejunum of control and irradiated rats. + = $p<.005$ cf. control. # of animals used: control (22), rad 3 (12), rad 7 (11), rad 14 (14).

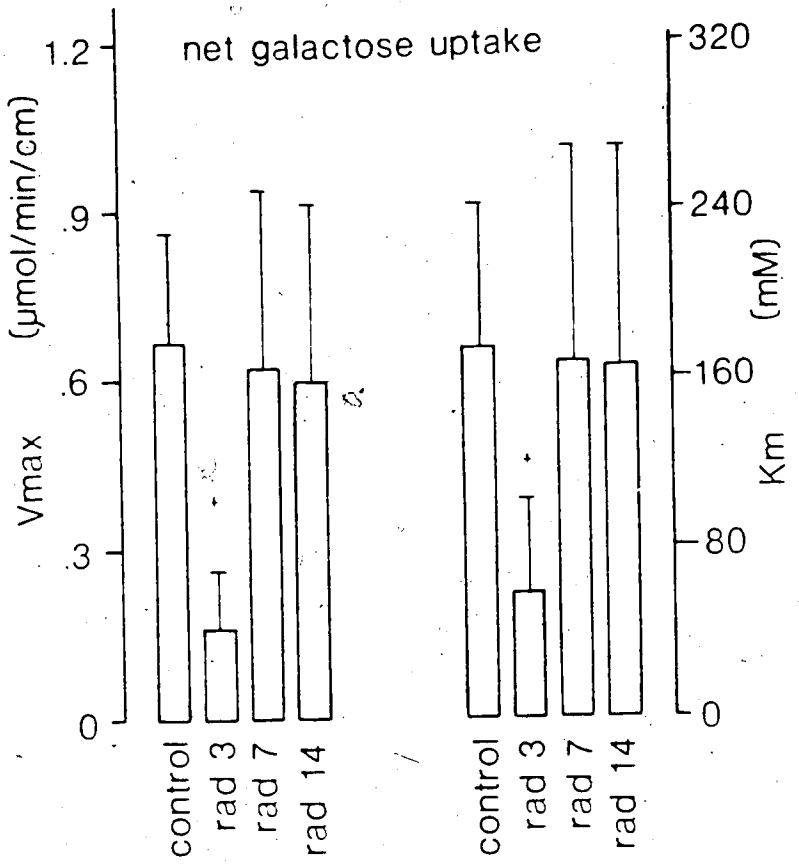

Figure 3.6 Km and Vmax of net D-galactose uptake *in vivo* in the upper jejunum of control and irradiated rats. * = $p<.05$ cf. control. # of animals used per estimation in all groups = 6.

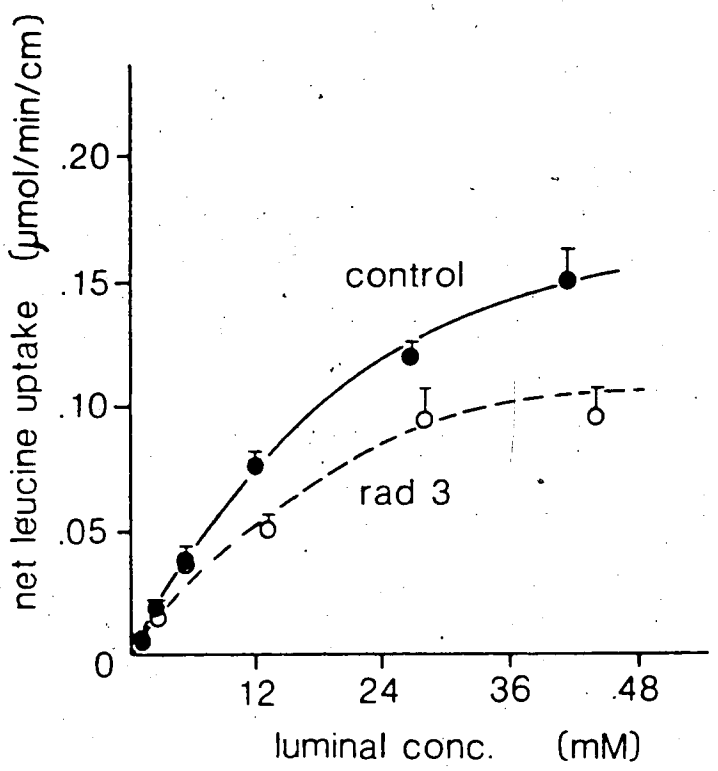

Figure 3.7 Net L-leucine uptake *in vivo* in the upper jejunum of control rats (4-7 observations per point) and rats 3 days post-irradiation (5-6 observations). Curves were significantly different at $p<.05$.

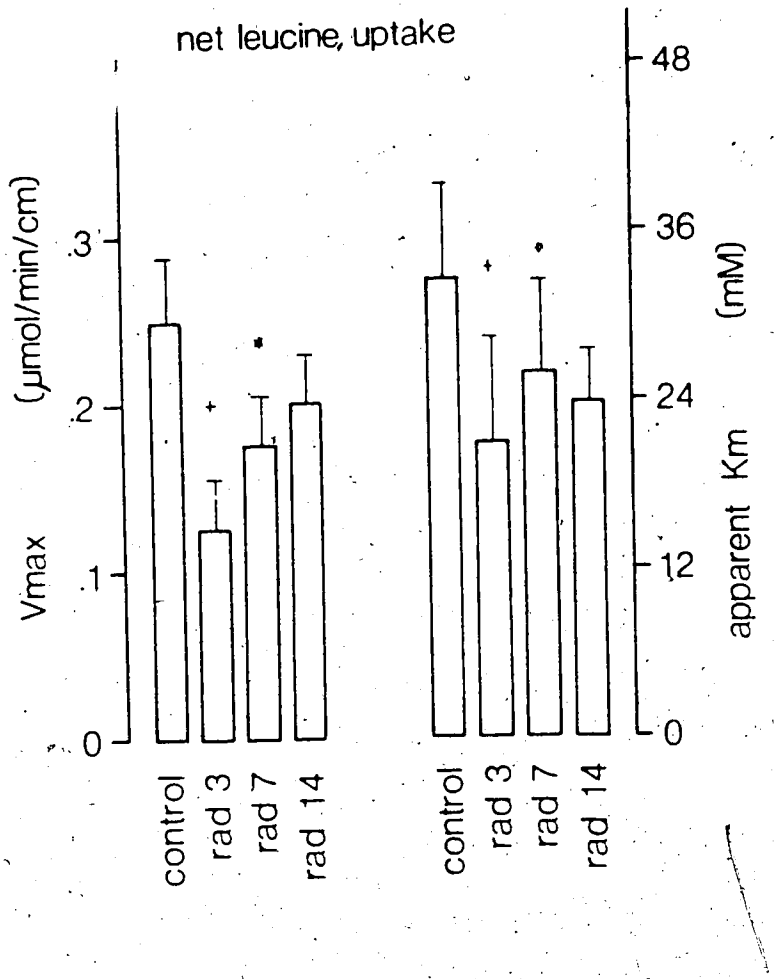

Figure 3.8 Apparent affinity constant (Km) and maximal transport rate (Vmax) of net L-leucine transport *in vivo* in the upper jejunum of control and irradiated rats. * = $p<.05$, + = $p<.005$ cf. control. # of animals used: control (7), rad 3 (6), rad 7 (7), rad 14 (4).

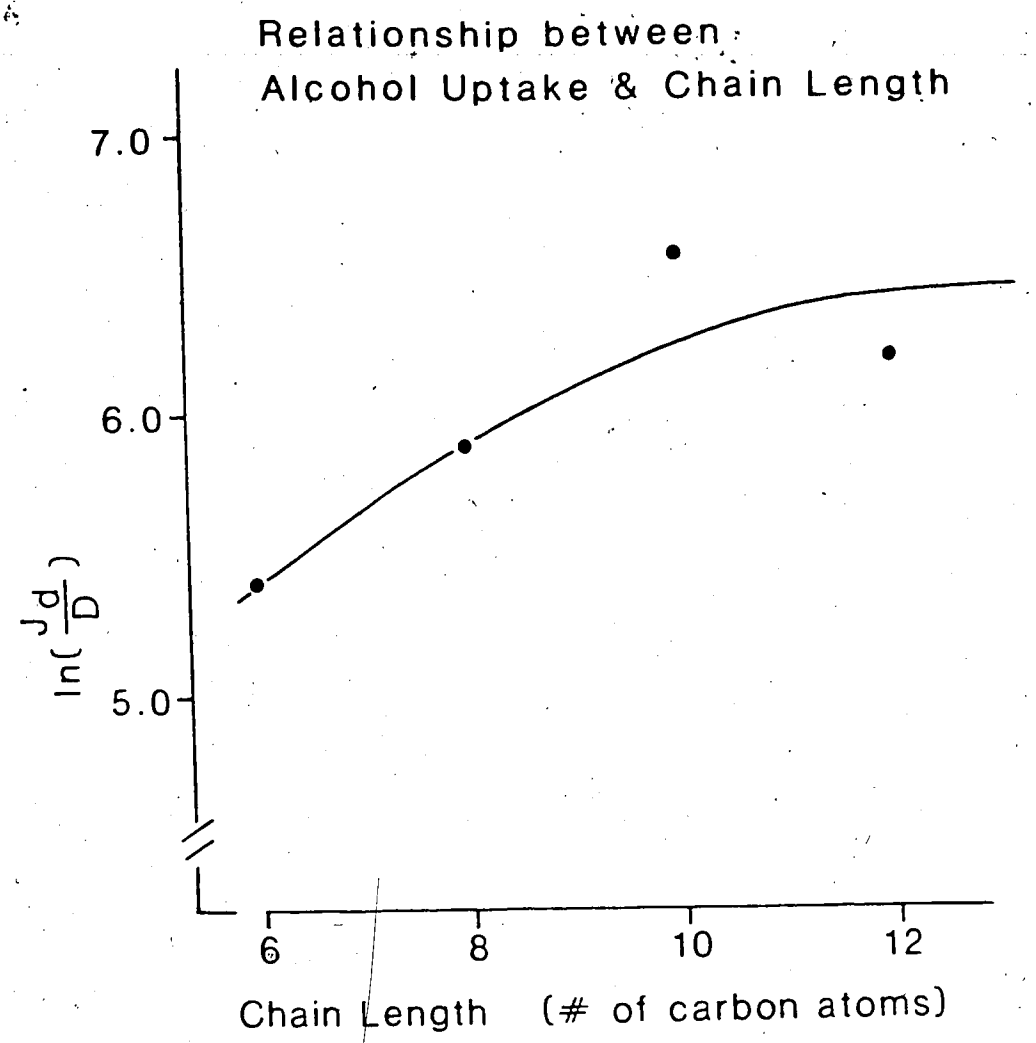

Figure 3.9 Natural logarithm of the ratio of the uptake rate (Jd) to the diffusion constant (D) of alcohols with increasing chain length.

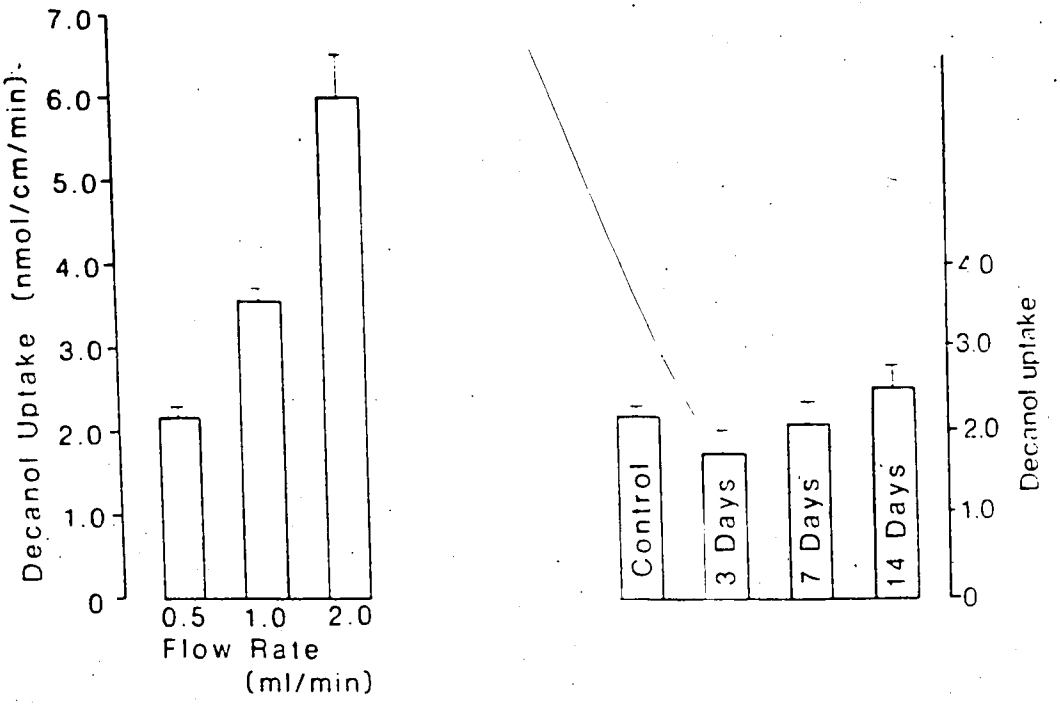

Figure 3.10a (Left) *In vivo* decanol uptake (normalized to 1 mM) in the upper jejunum of control rats, using increasing perfusion rates. # of animals used = 6 for all groups.

Figure 3.10b (Right) *In vivo* decanol uptake (normalized to .1 mM) in the upper jejunum of control and irradiated rats. Flow rate, .5 ml/min. No significant differences in uptake were observed. # of animals used = 6 for all groups.

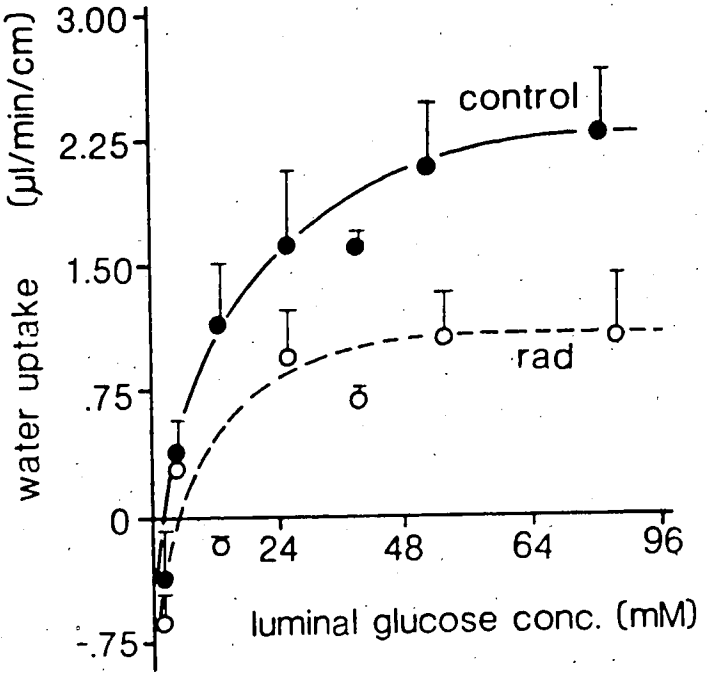

Figure 3.11 Glucose-stimulated water uptake *in vivo* in the upper jejunum of control rats (12-22 observations per point) and in rats 3 days post-irradiation (7-12 observations).

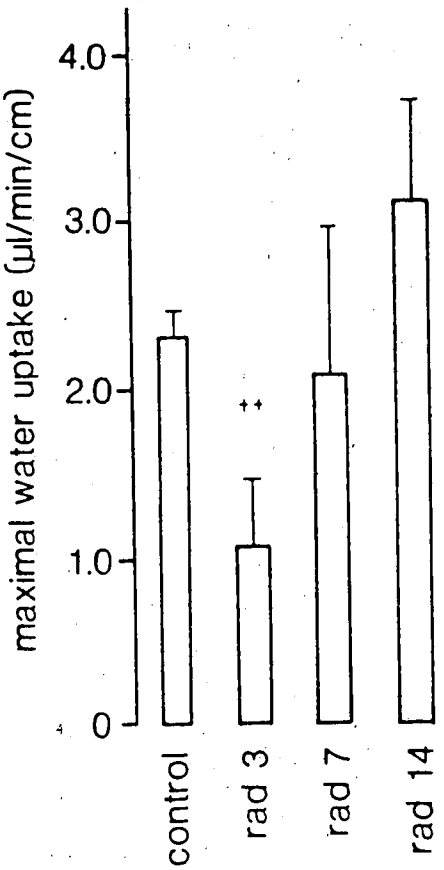

Figure 3.12 Glucose-stimulated water uptake *in vivo* in the upper jejunum of control and irradiated rats. # of animals used: control (8), rad 3 (7), rad 7 (5), rad 14 (6). Glucose concentration, 96 mM. + = $p<.005$

Table 3.1a
In vivo intestinal permeability coefficients of L-glucose in control and irradiated rats.

Treatment Group	Permeability Coefficient (μmol/min/cm/mM)
Control	$9.48 \pm 9.78 \times 10^{-4}$
3 days post-irradiation	$9.84 \pm .11 \times 10^{-4}$
7 days post-irradiation	$9.21 \pm .30 \times 10^{-4}$
14 days post-irradiation	$10.14 \pm .57 \times 10^{-4}$

Table 3.1b
In vivo intestinal permeability coefficients of D-leucine in control and irradiated rats.

Treatment Group	Permeability Coefficient (μmol/min/cm/mM)
Control	$8.8 \pm 1.6 \times 10^{-4}$
3 days post-irradiation	$10.4 \pm 1.2 \times 10^{-4}$
7 days post-irradiation	$11.8 \pm 1.8 \times 10^{-4}$
14 days post-irradiation	$14.3 \pm 1.1 \times 10^{-4}$

significantly reduced at 3 days, but normal by 7 days post-irradiation (Figure 3.13).

Phoridzin-inhibitable D-glucose uptake (Figure 3.14) revealed a marked suppression at 3 days, which was lower, but not significantly so at 7 days, and similar to control levels 14 days post-irradiation. The diffusion p.d. generated by hyperosmotic mannitol was slightly, but not significantly elevated at 3 days post-irradiation (Figure 3.15), significantly elevated at 7 days, and not different from controls at 14 days.

ODC levels were elevated 3 days after irradiation ($p<.005$), and declined gradually thereafter (Figure 3.16a),

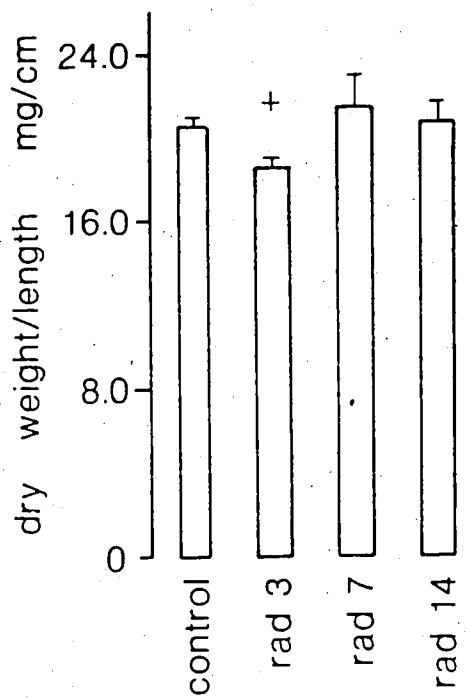

Figure 3.13 Dry weight/length ratios from jejuna of control and irradiated rats. # of determinations per estimate: control (31), rad 3 (18), rad 7 (16), rad 14 (22).+ = $p<.005$ cf. control.

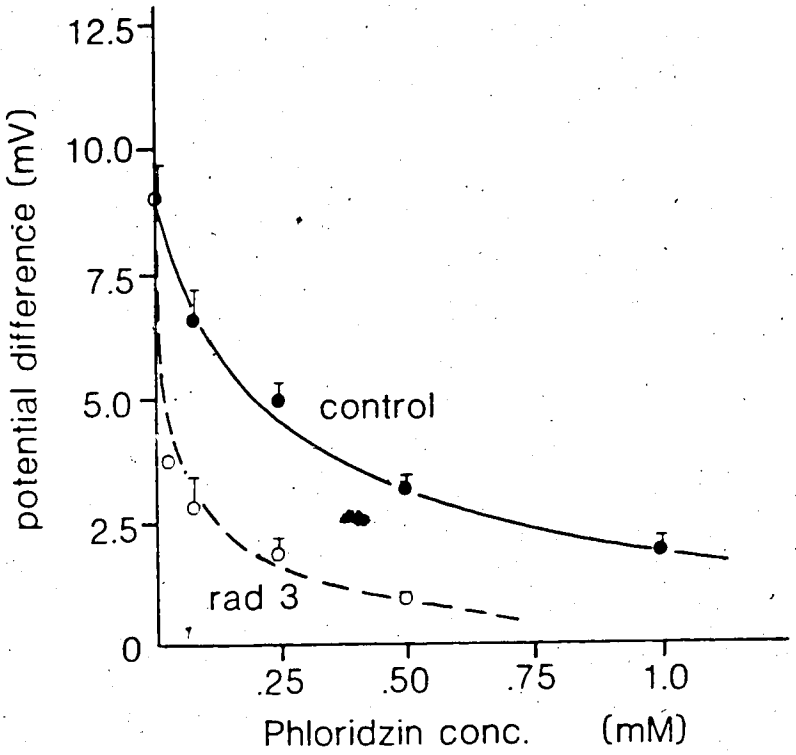

Figure 3.14 Phloridzin inhibition of D-glucose-generated potential difference (p.d.) *in vivo* in the jejunum of control rats (n=5 per point) and in rats 3 days post-irradiation (n=4). Curves were significantly different at $p<.01$. P.d.'s at 7 and 14 days were not significantly different from control.

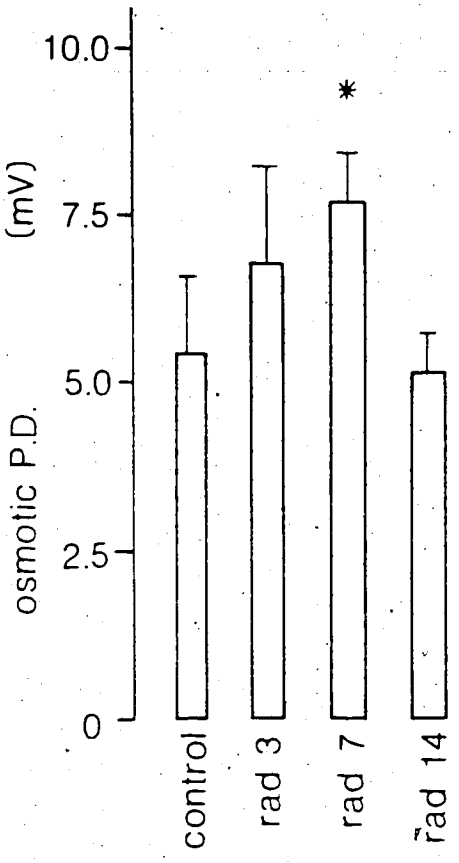

Figure 3.15 Osmotic potential difference (p.d.) *in vivo* in jejuna of control and irradiated rats. # of observations: control (5), rad 3 (8), rad 7 (6), rad 14 (5). * = $p<.05$ cf. control.

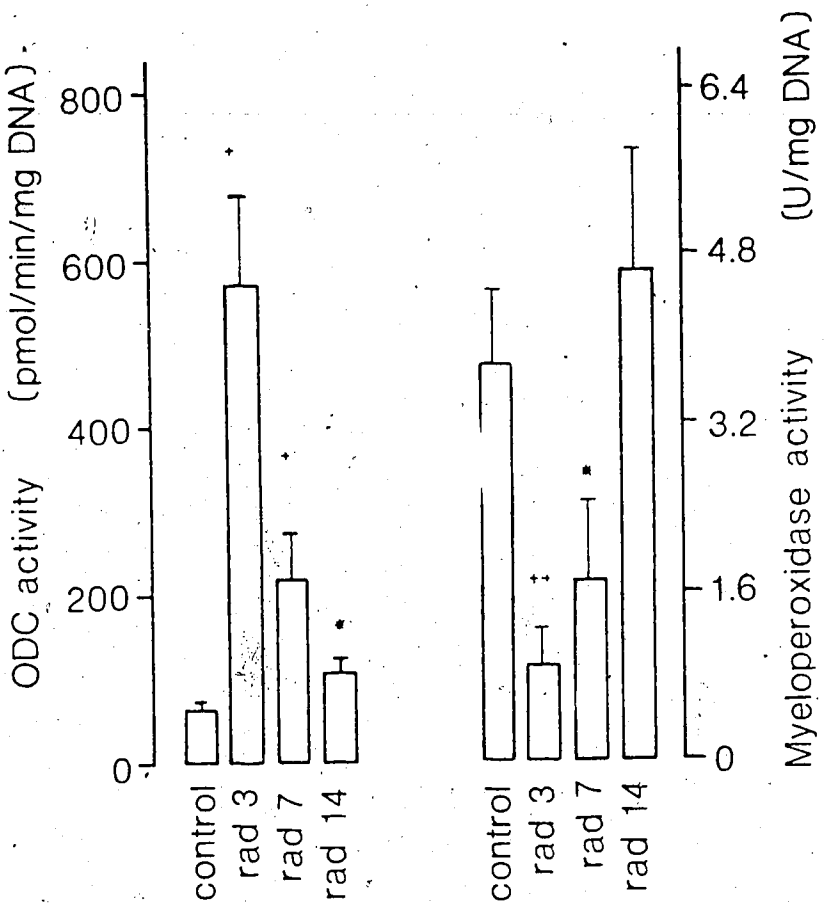

Figure 3.16a (Left) Intestinal ornithine decarboxylase (ODC) activity in control and irradiated rats. # of observations: control (7), rad 3 (10), rad 7 (7), rad 14 (6).

Figure 3.16b (Right) Intestinal myeloperoxidase activity in control and irradiated rats. # of observations: control (7), rad 3 (7), rad 7 (7), rad 14 (8). * = $p<.05$, + = $p<.005$, ++ = $p<.001$ cf. control.

although a small elevation was still evident at the 14 day point. Myeloperoxidase levels were depressed after radiation treatment and remained so until 14 days later, when activity levels had returned to control levels (Figure 3.16b).

D. DISCUSSION

The results clearly indicate that the process of recovery after radiation damage to the intestine is not a uniform one, but rather depends on the enzyme system studied. Active transport of the various substrates studies here is reduced three days after the radiation insult, but is essentially normal by the seventh day. By comparison, ornithine decarboxylase activity and mucosal permeability (assessed by the diffusion p.d.) are still elevated at 7 days. In addition, decreased myeloperoxidase content indicates that leukopenia is still present at this time.

Many difficulties exist in the interpretation of transport data obtained *in vivo*. The presence of large unstirred layers distorts the values obtained for the kinetic parameters, and it remains a matter of considerable controversy whether the uptake kinetics are characteristic of one or more transporters, or an active transporter and a passive uptake component (1, 17). It must be stressed that the data obtained here cannot shed light on any particular model of transport function. The parameters reported here must therefore be considered to express composite values and are valid only in treatment comparisons where the data have

been collected in a comparable manner. The purpose of the transport studies reported here, however, was only to establish whether changes in absorption provided a good indicator of tissue recovery.

Passive uptake of D-glucose and L-leucine as assessed by the uptake of their stereoisomers appears either unaffected by radiation damage or recovers in less than 72 hours. The dependence of the uptake of the stereoisomers on simple diffusion has been assumed by its linear, apparantly non-saturable nature and the inability to accumulate the substrate against a concentration gradient (12, 66). However, linearity could also be achieved by a high capacity carrier of low affinity. Caspary (9) and Bihler (8) found sodium dependence and phloridzin inhibition of L-glucose transport at low concentrations (<1 mM). The use of higher concentrations in the present studies, as well as the presence of significant unstirred layers, likely masks all but the passive component of uptake of this substrate. Neale (45) found that L-glucose could accumulate against a concentration gradient *in vitro* in everted sacs from starved animals. Whether this finding is relevant in the present studies is debatable, however, as evidence from the following chapter indicates that under similar conditions, irradiated animals continue to feed.

Other measures of passive uptake have relied on the use of phloridzin to inhibit active uptake (17, 46). The finding that phloridzin sensitivity is substantially

increased 3 days post-irradiation (Figure 3.13) demonstrates that when this inhibitor is used to determine passive D-glucose transport, care must be taken in the selection of the concentration used, especially where adaptive changes are likely to have occurred. Even within the gut, differences in phloridzin sensitivity have been reported, with more phloridzin required in the jejunum than in the ileum for equivalent inhibition of galactose uptake (3, 4).

Several reports have described the presence of a phloridzin hydrolase in the intestine, which is either identical to or closely linked with lactase, as indicated by biochemical co-extraction (27, 63). Further, patients with a lactase deficiency have also been shown to lack phloridzin hydrolase (42). The result of decreased phoridzin hydrolase activity would be to increase the effective phloridzin concentration in the lumen, as less is hydrolyzed, thereby inhibiting glucose transport to a proportionately greater extent. Other disaccharidases are also affected by radiation injury. In biopsy samples from human patients undergoing radiotherapy, decreased maltase, sucrase, and lactase activity have been reported (58). In general, some disaccharidase activities may be either more susceptible to damage, or require a longer period to recover (28). The time lag between injury and assessment may be a factor in this apparent differential sensitivity to injury. Becciolini (6) has reported that maltase and invertase activities declined faster, but recovered faster, than either lactase or leucine

aminopeptidase activities in rats receiving 6.5 or 12 Gy of X-rays.

Earlier studies employing single concentrations of substrate also reported the depression of hexose transport *in vivo* (47, 57). The present study extends these findings by the use of a larger concentration range, and by the observation that passive transport is not appreciably altered under these conditions. While reductions in both Km and Vmax were observed, the Km change is likely to be an artifact produced by the presence of unstirred layers of appreciable size. In the presence of unstirred layers, the "apparent" Km is reduced in proportion to reductions in Vmax (36).

The inability to demonstrate changes in the uptake of substrates such as decanol whose uptake is limited by unstirred layers (29, 59) indicates that the resistance of this barrier is unchanged by the conditions and treatments employed here and cannot therefore account for the absorption changes seen. This appears to be analagous to other *in vivo* studies in the literature in which decreased absorption has been observed without a measurable change in the dimensions of the unstirred layer. Debnam and Levin (18) found decreased active glucose uptake in the starved rat *in vivo*, without a change in the unstirred layer. Similarly, Hollander (29) found the dimensions of the unstirred layer *in vivo* were unchanged in aging rats despite the finding of increased glucose absorption *in vitro*.

Decreased intracellular metabolism of actively transported substrate, as observed during starvation (35), could decrease the chemical gradient of the substrate across the membrane, contributing to decreased uptake. However, 3-O-methylglucose and galactose, sugars which share the glucose carrier but are minimally metabolized within the enterocyte (2, 15), also show similar reductions in uptake 3 days after irradiation. Alterations in the chemical gradient across the cell are therefore unlikely to be a factor in the decreased absorption *in vivo* under these circumstances.

Keelan *et al.* (32) have shown that total mucosal surface area is reduced 3 days after abdominal irradiation. This suggests that the decrease in both transport and water uptake could be a result of reduced surface area. Correlating function with histological parameters, however, is complicated by the possibility that the villus tip cells are the major site of absorption, as indicated by autoradiographic studies showing the uptake of labelled substrate concentrated in area of the villus tips (33, 55). The lack of correlation between function and morphology is also suggested by the finding that changes in villus height are often not observed until 1 to 2 days after radiation treatment, but decreases in water and sodium absorption (as determined by *in vivo* perfusion), have been found to occur as early as 6 hours after treatment (14).

In spite of the reduction in surface area 3 and 7 days after irradiation (32), the enterocyte appears capable of

adapting to the injury by increasing the uptake of D-glucose per cell (11) at these time points. Morphological changes retain some importance in the pathophysiology of intestinal radiation damage, however. For instance, the reduction in surface area at 7 days is due mainly to a reduction in the number of villi per unit surface area, although villus height has returned to normal. Even if most transport occurs at the villus tip, fewer tips are present, and less absorption would be expected were it not for the increase in function per enterocyte. Such a combination of events would therefore tend to minimize the changes seen *in vivo*.

Changes in blood flow to the mucosa following intestinal trauma are likely to affect absorption of both fluid and substrate (66, 68). Indeed, it has been suggested in the literature that such long term changes result from vascular lesions (24). Since endothelial cells reproduce sporadically, and over a longer time scale than do enterocytes, (25) endothelial reproductive cell death would not likely appear for months. Such a hypothesis, however, awaits more rigorous testing, as actual experimental evidence is scant.

Microangiographs in rats (22) indicate substantial intestinal arteriole occlusion and vascular congestion following irradiation. While the dose used (1450R) was sufficient to cause acute mortality, blood flow effects may only differ in degree and duration at lower doses. Eriksson (24) found in the cat, that the capillary filtration

coefficient (a measure of the capillary bed open to the circulation) was reduced by X-irradiation, but returned to normal more rapidly with lower radiation doses.

Cobden (13) found that methotrexate, a cytotoxic drug, resulted in a decrease in mucosal permeability, when histological evidence of damage was greatest. On the other hand, permeability was increased when the detergent cetrimide was used, coinciding with damage primarily to the villus tips. In the present studies, permeability was unchanged at 3 days and increased at 7 days post-irradiation. A decrease in the passive diffusion of water, which is limited by the surface area available (37), could have been masked by a concomitant increase in mucosal permeability at the villus tip on the third day. Some damage to the villus tip could have persisted through to the seventh day to produce the increase in permeability. As noted above, this occurs at a time when the functional capacity, and possibly the workload of the tip cells are greatest.

The sensitivity of intestinal leukocytes to radiation injury is reflected in the extended depression of myeloperoxidase activity in the irradiated rats. Up to two weeks were required for myeloperoxidase levels to return to pre-irradiation levels, in spite of the prior return of villus height and *in vivo* transport activity to normal. Thus, the diffuse inflammation which is such a prominent part of chronic radiation enteritis is a latent response

which may not be directly associated with the initial mucosal damage observed here. On the other hand, a combination of leukopenia and increased mucosal permeability is likely to make the radiated bowel more susceptible to bacterial invasion. Radiated rats succumb more rapidly to Salmonella enterotoxin, and show extensive disruption of tight junctions (62).

Ever since Dykstra (20) demonstrated parallel increases in polyamine production and RNA synthesis following partial hepatectomy, the requirement for ornithine decarboxylase activity has been recognized in a wide variety of circumstances involving tissue regeneration. The elevation in ODC activity and coincident mucosal hypertrophy seen in such diverse adaptive responses as lactation (70), jejunectomy (42), or injury with ARA-C (41), can be abolished by administering α-difluoromethylornithine, an irreversible inhibitor of ODC.

The exact localization of the increase in ODC activity observed in the present experiments could not be determined by the techniques employed here. Baylin (5) and Shakir (53) found higher activity of both ODC and diamine oxidase, which is involved in the catabolism of polyamines, in the villus tips than in the crypts. By comparison, spermidine and spermine and the enzyme required for their synthesis, S-adenosylmethionine decarboxylase (SAMD), are found preferentially in the crypts (50), where cell proliferative activity predominates. This could reflect specialized

functions for polyamines dependent on their location. Polyamines have been shown to stimulate glucose transport in renal brush border vesicles (23) and in fat cells (38), an effect which may be mediated through an intermediate produced by diamine oxidase (39). An enzyme which acts both to stimulate cell regeneration as well as transport function would have obvious advantages in the return of normal function to the damaged intestine.

The addition of radiation damage to the list of trophic agents capable of stimulating ODC activity may make it a useful enzyme for monitoring the recovery process after intestinal damage. The continued elevation of the activity of this enzyme when some parameters of intestinal function have returned to pre-irradiation levels suggests that some prolonged stimulus remains well after the initial injury. Whether the stimulus for the continued increase in polyamine production could be protracted cell loss remains to be confirmed. It is clear, however, that some systems, such as active transport, are capable of more rapid compensation in the face of injury, while others, including mucosal permeability and leukocyte populations, require more time to return to normal. Increased ODC activity can also be correlated with the recovery period, but whether ODC activity is obligatory for post-injury repair also awaits more definitive studies.

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IV. Experiment 2: The Effect of Abdominal Irradiation on Enterocyte Migration Rate

A. INTRODUCTION

The small intestine shows a high rate of proliferative activity, largely as a consequence of the short lifespan (36 to 48 hours in the rat) of the average enterocyte (37, 15). It has been recognized for some time that differentiated mucosal cells are generated from precursor cells in the base of the intestinal crypts and migrate up the villus to be exfoliated into the lumen upon reaching the villus tip (21).

Moreover, there also appears to be a maturation in cell function as the enterocyte progresses to the top of the villus. Enzymatic studies with disaccharidases (10) and alkaline phosphatase (44), and histochemical studies with enzymes such as succinic dehydrogenase (29) have revealed that the activity of several enzymes associated with the differentiated function of the enterocyte increases as cells approach the villus tip. On the other hand, enzymes such as thymidine kinase, which are associated with cell replication, are found primarily in the crypt, and their cellular concentration decreases as enterocytes migrate upward (18). Autoradiographic studies suggest that a gradient also exists for glucose (19) and amino acid transport (1, 36), as determined by the ability of the tip cells to preferentially take up labelled substrate.

Another activity gradient occurs longitudinally along the small intestine, whereby D-glucose uptake is considerably greater in the proximal intestine than in the ileum (3, 32), although this may not be true of other substrates. Coincidentally, average villus height is less in the ileum, and the enterocyte transit time from crypt to villus tip is shorter (6, 22, 28). This results in a shorter cell lifespan in the distal intestine. The possibility therefore exists that the age of the enterocyte may be connected with the transport capacity of D-glucose.

Abdominal irradiation results in a temporary cessation of DNA synthesis which lasts between 6 and 12 hours (5, 14, 27, 36). By the second day after radiation treatment, villi are demonstrably shorter (31, 41). This generally coincides with a depression in transport function (11, 30). Mitotic activity subsequently increases, as determined by increased mitotic figures and greater 3H-thymidine incorporation (15, 16), and the villi are repopulated. Depending on the exposure dose (2, 20, 36), it takes several days for villus height to return to pre-irradiation values, however. Unless the migration rate has been retarded, the cells repopulating the villus are therefore likely to reach the top of the shorter villi sooner, possibly resulting in a less mature cell population, and compromising intestinal function.

The following experiments were therefore designed to determine the effect of moderate doses of radiation on cell migration rates in the intestine. If a change occurred in

the age composition of the enterocyte population, this could be a factor in the development of depressed transport function that follows radiation treatment.

B. METHODS

Pentobarbital anesthetized male Sprague Dawley rats received 6 Gy directed at the abdomen, as described in Chapter III. 3, 7, or 14 days later, each animal was injected i.p. at 5 p.m. with 90 μCi. ^{3}H-thymidine (sp. act. 72 Ci/mmole) in 0.2 ml of 0.9% saline. No carrier was added to the thymidine. Animals were sacrificed by an overdose of pentobarbital 5, 20, 30, or 40 hours later. The entire intestine was then removed, and washed with phosphate buffer as described in Chapter VII. Mucosal rings were taken 2 cm. below the ligament of Trietz (proximal jejunum), at half the total length (mid-gut) and 2 cm. above the ileo-cecal valve (distal ileum). The segments were everted for better histological viewing, and preserved in 10% phosphate buffered formaldehyde (Fisher).

Specimens were subsequently dehydrated through a series of alcohols (ethanol 50% to 90%, followed by 100% methanol) and fixed in methacrylate as previously described (23). Methacrylate embedding medium was made by mixing 80 ml of 2-hydroxyethyl methacrylate, 15 ml of 2-butoxythanol and 0.3 g of benzoyl peroxide (Solution A) with 10 ml of polyethylene glycol (MW = 400) and 1 ml of N,N'-dimethylaniline (Solution B) in a 50:1 ratio. Tissue

sections 2 μm thick were floated onto a microscope slide which, after drying, was dipped in Kodak NTB2 emulsion, and stored in the dark for 12 weeks. After developing in Kodak D-19 developer (5 minutes) and fixing in Kodak fixer (diluted 1:4) for 5 minutes, the slides were rinsed in running water (all solutions at 15° C) for 5 minutes, and air dried. Measurements were taken of crypt depth, villus height, and distance from the crypt-villus junction travelled by the grain front, using an eyepiece micrometer.

Statistics - Mean migration rate was estimated from the slope of the least squares regression line through the points obtained from the advance of the grain front. The slope of this line was in all cases significant at $p<.001$. Due to the limited number of time points, however, variations in the migration rate could take place without detection, and fitting the data to a linear function must therefore be considered a matter of convenience.

Differences in migration rates between treatment groups was determined by analysis of covariance. In other comparisons, a t-test was used.

C. RESULTS

Three days after radiation treatment, villus height was reduced in all segments by up to 40% (Figure 4.1). Crypt depth was somewhat increased, but this only reached significance in the ileum. Villus height had returned to control levels or above by the 7 day time point, but crypt

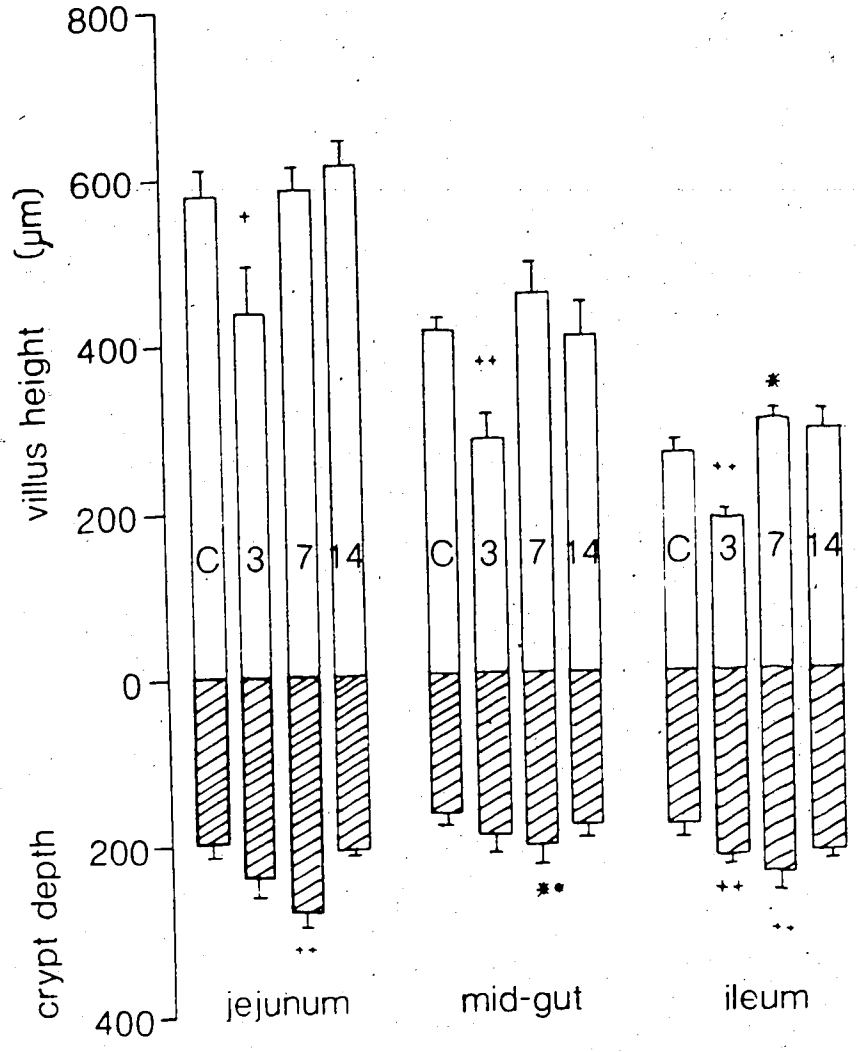

Figure 4.1 Villus height and crypt depth in the proximal jejunum, mid-gut and terminal ileum of control and irradiated rats. * = p<.05, ** = p<.01, + = p<.005, ++ = p<.001 cf. control.

depth was significantly increased compared to controls in all segments. Both villus height and crypt depth were normal by 14 days after treatment.

In the proximal jejunum, the grain front appeared on the villus markedly earlier in animals three days post-irradiation compared to controls (Figure 4.2), although the mean migration rate was not significantly different from controls. At 7 days the mean migration rate was increased, whereas no difference in mean migration rate could be seen at 14 days post-irradiation. This profile of migration rates was also present in the mid-gut (Figure 4.3), but changes did not reach significance in the terminal ileum. Mean migration rate was not uniform along the length of the intestine, and in general was most rapid in the proximal jejunum, but similar between the mid-gut and terminal ileum.

Photographs of villi and crypts from control and irradiated animals are shown in Figures 4.4 through 4.7. Although a higher magnification was routinely used to plot the grain front in these autoradiographs, the grain front is still faintly visible in most of the Figures. A higher power magnification of a villus showing a clearer picture of the grain front is shown in Figure 4.8.

Values for cell age of villus tip cells were estimated from the linear regression equation. In the duodenum, these corresponded to 40.5, 29.7, 27.6, and 45.2 hours for control rats and rats 3, 7 and 14 days post-irradiation respectively, indicating substantually younger cells at the

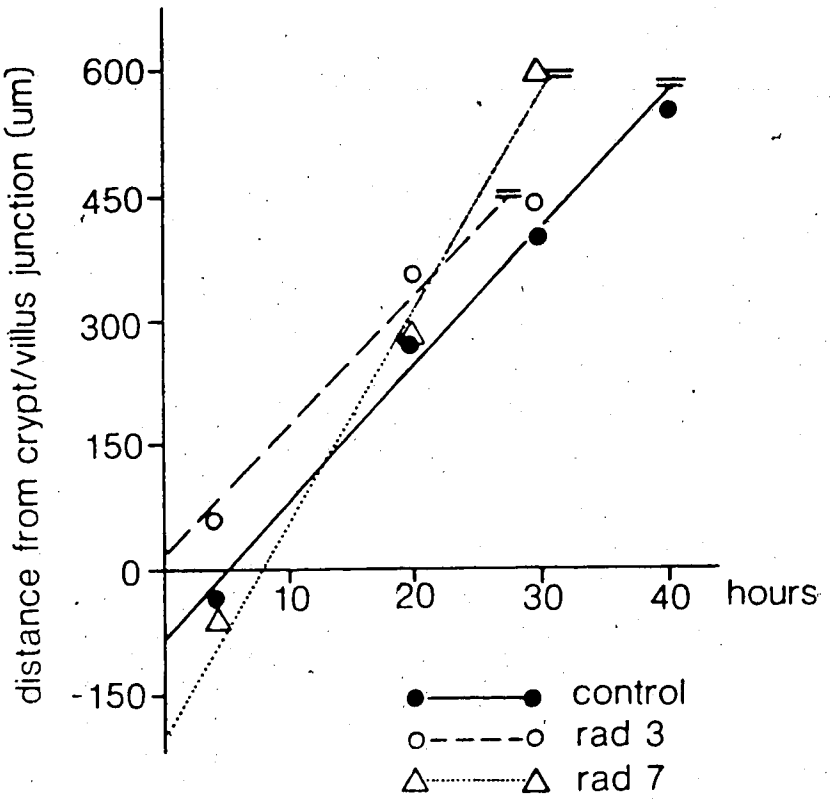

Figure 4.2 Enterocyte migration in the proximal jejunum of control and irradiated rats. Horizontal parallel lines indicate height at the villus tip. Data obtained at 14 days post-irradiation (not shown) was not significantly different from controls. Slope at 7 days post-irradiation was significantly greater than in controls ($p<.05$). Each point represents mean of 3 animals.

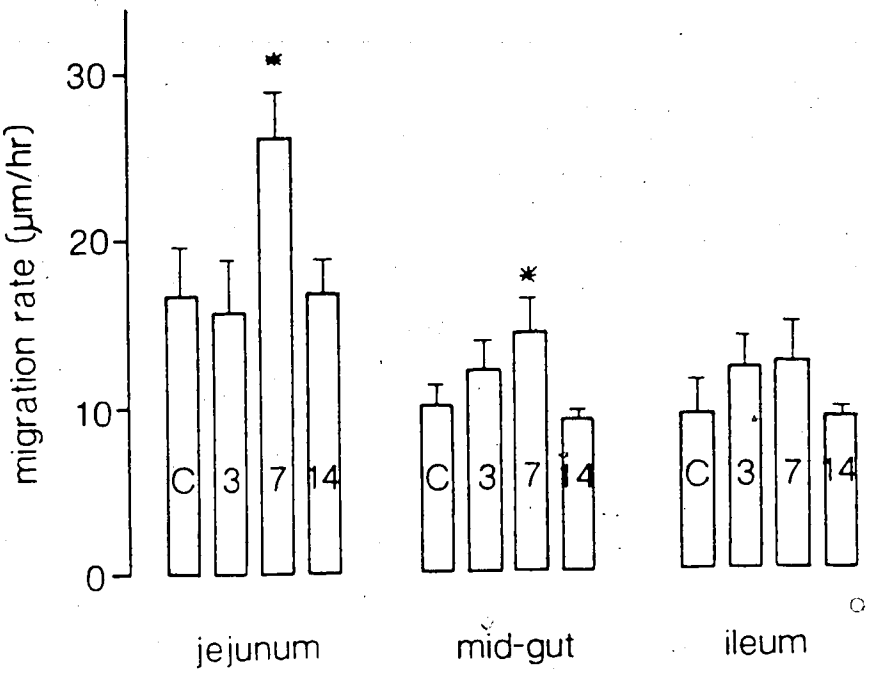

Figure 4.3 Mean enterocyte migration rates in the proximal jejunum, mid-gut, and terminal ileum of control and irradiated rats. # of animals per estimation: control (12), rad 3 (9), rad 7 (9), rad 14 (12). * = $p<.05$ cf. control rate.

Figure 4.8 Higher power magnification of villus from proximal jejunum 3 days post-irradiation showing grain front. Magnification, 750 X.

villus tip at the 3 and 7 day points.

D. DISCUSSION

As early as 1960, Sherman (36) demonstrated that cell migration continued in the intestine despite the lack of labelled thymidine uptake into DNA which prevailed for up to 12 hours after radiation treatment. As a result, labelled enterocytes appeared on the villus sooner than in controls. It would be expected that this would lead to eventual cell depletion in the crypts. Under normal circumstances, the crypt consists of a proliferative zone, where mitosis occurs, and a maturation zone, where mitosis does not generally procede, as assessed by the lack of mitotic figures or labelled thymidine incorporation (21). After irradiation however, the maturation zone disappears, and mitotic figures are found throughout the crypt (4, 15, 24).

In this regard, the sequelae radiation-induced damage resemble those produced by a variety of other intestinal injury syndromes. The elimination of the maturation zone, suggestive of crypt cell depletion, has been observed experimentally in the rat, following ischemia (34) and four days after infection by the nematode Nippostrogylus brasiliensis (38). In humans, mitotic figures can be observed on the villi in celiac disease (29).

The present studies indicate that earlier appearance of labelled enterocytes on the villus still occurs three days after radiation treatment, when villus height is

reduced. In addition, there is no change in the mean migration rate at this time point in any segment of the intestine. This again resembles other situations produced by different modes of injury. Disruption of DNA synthesis by colchicine in the rat intestine fails to alter the migration rate (17), nor does giardia infection in mice (25).

Changes in villus height are often not observed until 48 to 72 hours after radiation injury (9, 27). It would appear therefore, that a lag period exists during which villus height is maintained due to continued recruitment of cells from the crypt and an unaltered migration rate. Given this lack of change in migration, the reduction in villus height three days post-irradiation may principally be a function of more rapid exfoliation of tip cells, rather than the lack of supply of new cells or mitotic activity in previously non-proliferating cells. The net result of the unchanged migration rate, earlier appearance on the villus, and more rapid exfoliation, is an effective reduction in the average lifespan of the enterocyte from about 42 hours to half that time. Thus cells at the villus tip are considerably younger 3 days after radiation exposure.

Cell proliferation resumes within a matter of hours after radiation treatment (36), and often excedes proliferation rates in controls (6, 16), so that villus height eventually returns to normal. However, seven days after radiation treatment, when villus height has returned to control levels, the mean migration rates in the duodenum

and mid-gut are significantly elevated. This would suggest the continuation of some damage process whereby cell death and exfoliation occur at a more rapid pace, but villus height is unchanged due to increased cell proliferation and an increased migration rate.

Such an adaptation again bears some similarity to other intestinal injury situations. Cell proliferation can be increased in the presence of mucosal injury such as ulcerative colitis (35), or celiac disease (45, 43) and in the latter case, this occurs despite the presence of blunted villi. DNA loss into the lumen is increased in celiac disease (8), suggesting that the rate of cell loss can outstrip the rate of cell production, even if the proliferative index is increased. Experimentally, an increase in crypt depth and cell migration rate in the duodenum occurs 96 hours after methotrexate administration (40) and in the mouse graft-versus-host intestinal rejection model described by Ferguson (13). Ferguson speculated that this hyperplastic response was mediated by immune cell products. The fact that increased crypt depth and a faster migration rate occur after radiation treatment despite the presence of leukopenia (42) indicates that the mediation of cell-borne immune elements is not obligatory for this process to occur.

Although significant increases in the mean migration rate were observed in the duodenum and mid-gut 7 days post-irradiation, the rate in the terminal ileum was not

significantly different from control levels. As ileal villus height was normal or even greater than normal at this time, this suggests that cell loss had returned to pre-irradiation levels. This is consistent with previous reports that the distal intestine recovers from damage more rapidly than the upper gut (2, 30). The means by which this segment of the gut recovers more quickly is uncertain, but it would be interesting to investigate whether it was connected with a lesser metabolic work load compared to the upper gut, where absorption of most substrates takes place.

A number of limitations to the technique used should be borne in mind. First, the rate of advance of the grain front up the villus represents the fastest rate of the cells in the villus population. Many cells in the lower villus and throughout the crypt remain labelled even after the front has reached the villus tip and the first cells have been exfoliated. Some of this labelling can be attributed to other cell types such as Paneth cells or progenitor cells which are known to migrate at a slower rate that differentiated enterocytes or do not migrate at all (12). Thymidine is metabolized rapidly in vivo so its biological availability is limited to a few minutes (26). However, the label may be incorporated at any point during the DNA synthesis (S) phase of the cell cycle, which lasts about 10 hours (12), so staggered mitosis may account for much of the residual labelling.

Secondly, the possibility that the incorporation of a radioactive marker into the DNA of the enterocyte may itself alter nucleic acid synthesis must be considered (26). In these experiments, however, lower specific activity was used in the thymidine marker than usually described to reduce the possibility of such effects. This was compensated for by exposing the slides for longer periods.

Third, owing to the taper of the villus towards its tip, the migration speed may increase as the underlying surface area diminishes. This provides a problem, however, only if there is heavy weighting on labelling in the tip area. Measuring grain advancement at several timepoints in the present experiments was intended to reduce this problem. Finally, no data can be directly derived concerning the rate of cell production. The purpose of the current experiments, however, was to determine the average age of enterocytes as they reached the villus tip and were subjected to their greatest functional workload. The methods described here provide an adequate assessment of tip cell age.

Evidence is accumulating that cell age and location are important factors in the expression of cell function. Non-specific esterase, which is stained heavily in the crypt as well as on the villi, is reduced after 2.9 Gy. (33), coinciding with the loss of the crypt maturation zone. Similar decreases in non-specific esterase and neutral glucosidase have been correlated with an increased proliferation rate after ischemic damage (34). In rats

parasitized by the nematode Nippostrongylus brasiliensis, the increase in cell proliferation seen 10 days after infestation coincided with a decrease in the brush border enzymes maltase and alkaline phosphatase, as well as the cytosolic enzyme succinic dehydrogenase (39).

It has also been suggested that cell age is important in the pattern of L-valine uptake in the rat jejunum. Autoradiographic measurement of valine uptake has indicated that cells take up valine much more rapidly once they pass the 40 hour mark (37). This biphasic uptake characteristic is not seen in follicle-associated enterocytes which are of similar age and distance from the crypt-villus junction. In this case, then, the increased uptake is caused by a cellular processs rather than by greater diffusional barriers to cells lower on the villus. It is therefore possible that the reduction in D-glucose and L-leucine uptake *in vivo* after radiation treatment could be a consequence the presence of younger, more immature cells at the villus tip.

Previous work by Sherman (36) dealt exclusively with the rate of cell migration within the first few hours after irradiation. The present study extends those findings by showing that the rate of migration changes as the repair process proceeds. Such changes may result in cells arriving at the villus tip at a younger age despite a normal histological appearance of the villus.

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V. Experiment 3: Inhibition of Intestinal Ornithine Decarboxylase Activity by Starvation or Difluoromethylornithine (DFMO) After Abdominal Irradiation

A. INTRODUCTION

Interpretation of studies dealing with intestinal damage are often complicated by a concomitant decline in food intake. It has long been recognized that starvation and food restriction may themselves have profound effects on the functional and morphological appearance of the intestine. In 1927, for instance, Sun noted that starvation resulted in reduced villus height in the small intestine of the albino mouse (44). In general, there is a depression in cell migration rate and cell production (1, 3, 5, 20) decreased villus height and reduced uptake of hexoses and amino acids (8, 15, 38, 45). This phenomenon is not due simply to malnutrition, as similar changes to the intestine are also observed with parenteral nutrition despite an adequate nutrient intake in animals (12, 16, 48, 49) and in man (11).

Reduced villus height (34, 47) and decreased hexose (31, 32) and amino acid uptake (26) are common symptoms of acute intestinal radiation damage in experimental animals and in human patients (7). Reduction in food intake and weight loss (39) may occur in conjunction with radiation damage, making it difficult to dissociate intestinal changes due to the initial injury from those arising from reduced food intake.

Replacement of damaged cells likely plays an important role in the recovery of the intestinal epithelium from injury. The actions of ornithine decarboxylase are probably central to this process. This enzyme is the rate limiting step in the production of polyamines putrescine, spermidine and spermine (36) and is closely linked with the regulation of DNA and RNA synthesis and cell growth (37). Recently, the importance of ODC in the intestine has been demonstrated by Luk and his colleagues, who showed that ODC activity is elevated by the administration of the cytotoxic drug ARA-C (21). Further, most of the adaptive responses, including the recovery or elevation in villus height and disaccharidase activity, could be inhibited by the irreversible ODC inhibitor DFMO.

Villus atrophy occurs in otherwise intact animals after DFMO administration (51). Owing to the role of ODC in regulating DNA synthesis, inhibition of this enzyme may bear some similarity to the intestinal effects of food restriction. The purpose of the present study, therefore, was to compare the effects of DFMO and starvation on mucosal function and morphology and to determine how both treatments affect intestinal recovery after radiation injury.

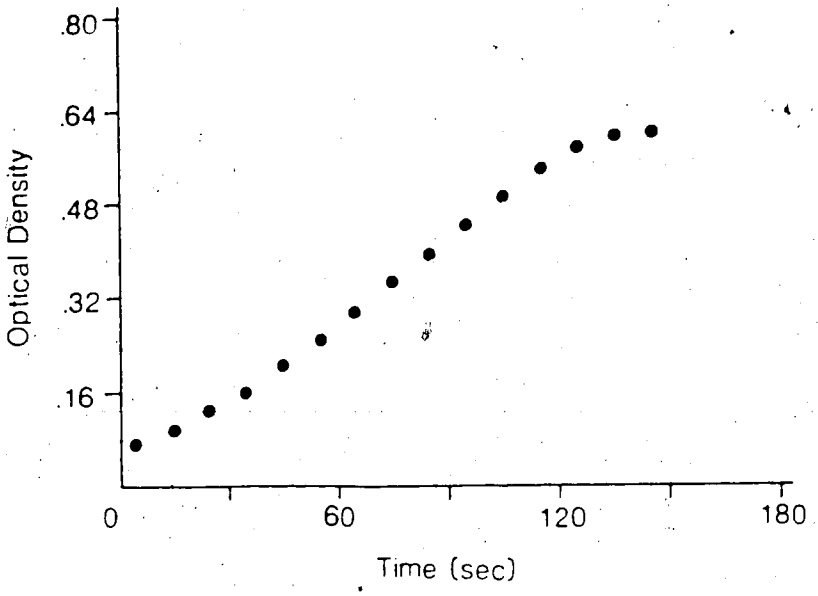
B. METHODS

Assays - *In vivo* transport of glucose or galactose was measured in anesthetized animals using ^{14}C-labelled substrate with ^{3}H-inulin used to correct for fluid movement

as described in Chapter III. Ornithine decarboxylase activity was determined in mucosal homogenate supernatant by measuring the evolution of CO_2 from ^{14}C-labelled ornithine, as described in Chapter VII.

Myeloperoxidase activity was measured according to Maehly and Chance (24) as modified by Sm[illegible]astro (40). The distal 2/3 of the intestine was rem[illegible] washed with ice cold phosphate saline buffer described in Chapter VII, with the mucosa then being scraped off with a glass slide, and homogenized on ice (1:8 vol/vol) in 10 mM sodium phosphate buffer, pH 6.0. 100 µl of whole homogenate was added to 500 µl of the cold sodium phosphate buffer, 250 µl of 20 mM aqueous guaiacol and 20 µl of 40 mM H_2O_2 (final concentration, 0.9 µmol/ml). The increase in absorption at 470 nm was measured over 30 seconds in a Phillips spectrophotometer. Four to five estimates were taken for each sample, with a mean Coefficient of Variation of 9.3%.

The rate of change in absorbance was found to be linear over time between 30 and 120 seconds after the start of the reaction (Figure 5.1), and readings were therefore taken between these times. By diluting down homogenate from an untreated animal, myeloperoxidase activity was shown to be linear over a range of DNA concentrations (Figure 5.2), and the line passed through the origin. The concentration of H_2O_2 was not rate limiting above 10 mM (final concentration, 0.2 µmol/ml). The effect of H_2O_2 concentration on myeloperoxidase activity in mucosal homogenate from a



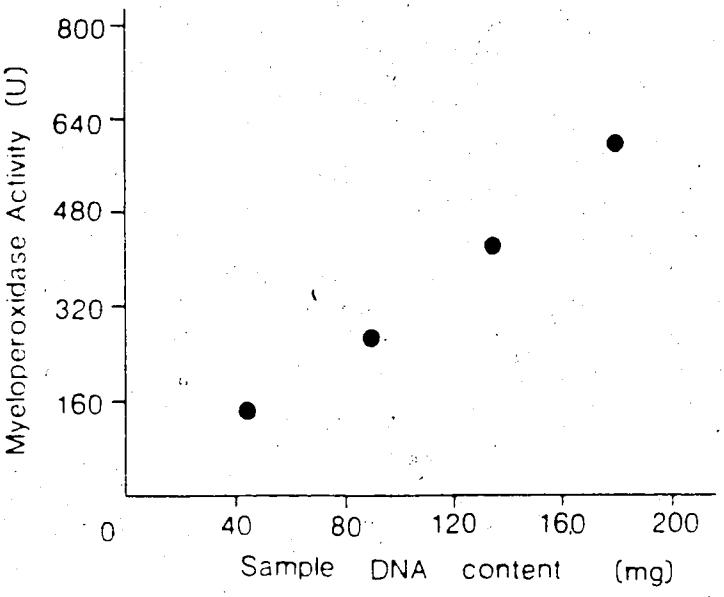


Figure 5.1 (Top) Change in optical density over time of myeloperoxidase sample from a typical animal.

Figure 5.2 (Bottom) Change in myeloperoxidase activity with increasing DNA content. Results obtained by serial dilution of homogenate in a typical control animal.

typical, untreated animal are shown in Figure 5.3. As the reaction proceeds until all peroxide is consumed (24), lower peroxide concentrations were seen to result in a shorter period of linearity of the reaction with respect to time.

One unit of myeloperoxidase was defined as that amount catalyzing the breakdown of 1 μmol of H_2O_2 per minute. Molar estimations of peroxide were made on the basis of 4 moles $H_2O\mu$ consumed per mole of tetraguaiacol formed (ϵ = 26.6 mM^{-1}) (22). Results were expressed per unit DNA as described in Chapter VII.

Animals and Treatments - Male Sprague-Dawley rats weighing approximately 250-300 g. were used in all studies. Irradiated animals which were anesthetized with pentobarbital received 6 Gy. directed at the abdomen using a Picker ^{137}Cesium source as described in Chapter III. Animals were fed *ad lib.* until the morning of the radiation treatment. Food was then removed for three days until use, but free access to water was allowed. Non-irradiated animals had their chow removed for a similar period. Body weights and food intake not measured, although Smith (39) has documented that food intake and body weight are both reduced by irradiation.

ODC Inhibition - DFMO, kindly supplied by Dr. Peter McCann, Merrell Dow Research Center, Cincinnati, Ohio, was administered as a 2% solution in the drinking water of both irradiated and control animals and continued for seven days. In the case of the irradiated animals, DFMO administration

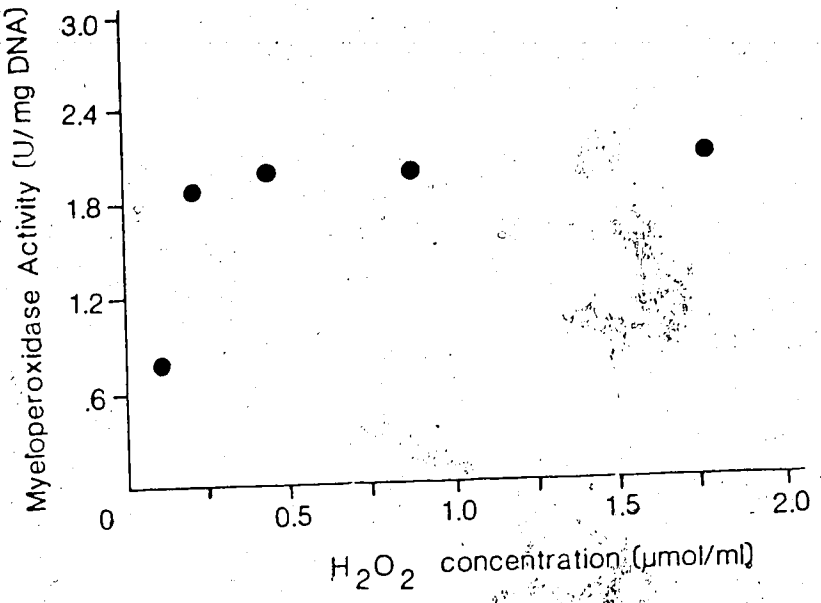


Figure 5.3 Change in myeloperoxidase activity with increasing H_2O_2 concentration in medium. Results obtained in a typical control animal.

commenced immediated after the irradiation procedure. All animals continued to eat, but often developed diarrhea within two days of the onset of DFMO administration.

Statistics - Differences in uptake between treatment groups were tested using multiple analysis of covariance (41). Unless otherwise noted, other comparisons used t-tests.

C. RESULTS

Starvation - Starvation alone reduced the uptake of both D-glucose (Figure 5.4) and D-galactose (Figure 5.5) compared to that in control animals. Irradiation did not result in any further reduction in uptake of these substrates. Glucose-stimulated water uptake was also reduced by starvation (Figure 5.6), but again was not further reduced by combined starvation and irradiation.

No change in ODC activity was observed after starvation alone (Figure 5.7). The expected increase in the activity of this enzyme following irradiation was abolished by starvation. Myeloperoxidase activity was increased following three days of starvation (Figure 5.8). This was significantly reduced in rats which were both starved and irradiated. Compared to rats receiving irradiation treatment only, however, myeloperoxidase activity was significantly elevated in starved irradiated rats ($p<.05$).

Starvation reduced villus height, although this did not reach significance in the ileum (Figure 5.9). No further

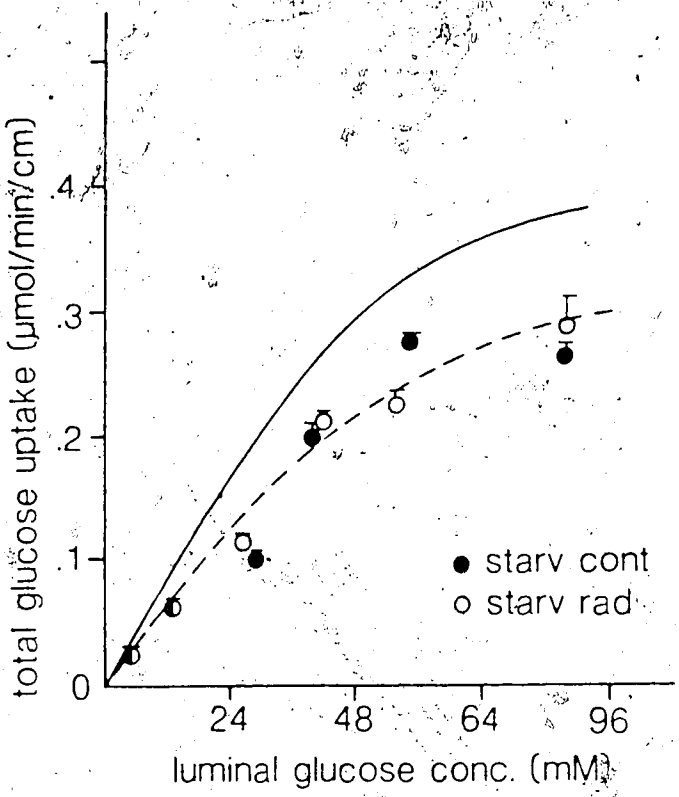


Figure 5.4 Total glucose uptake *in vivo* in the upper jejunum of control (4-6 observations per point) and irradiated rats (4-6 observations) after starvation for 3 days (STARV). For reference, solid line represents uptake in fed non-irradiated rats; broken line, uptake in fed irradiated rats. Overall uptake was significantly lower in starved vs. fed non-irradiated rats ($p<.005$). Uptake in starved groups was not significantly different.

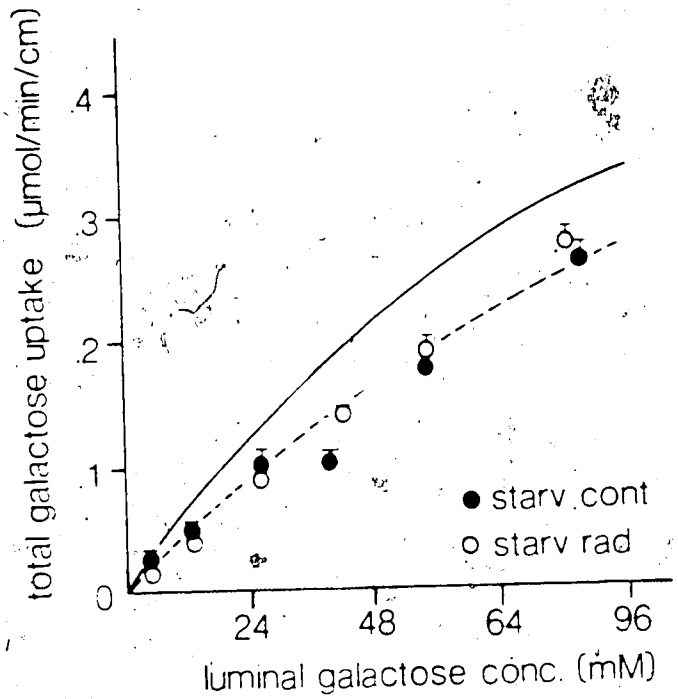


Figure 5.5 Total galactose uptake *in vivo* in the upper jejunum of control (4-6 observations per point) and irradiated rats (4-6 observations) after starvation for 3 days (STARV). Solid line, uptake in fed non-irradiated rats; broken line, uptake in fed irradiated rats. There was no significant difference in uptake between the two irradiated groups.

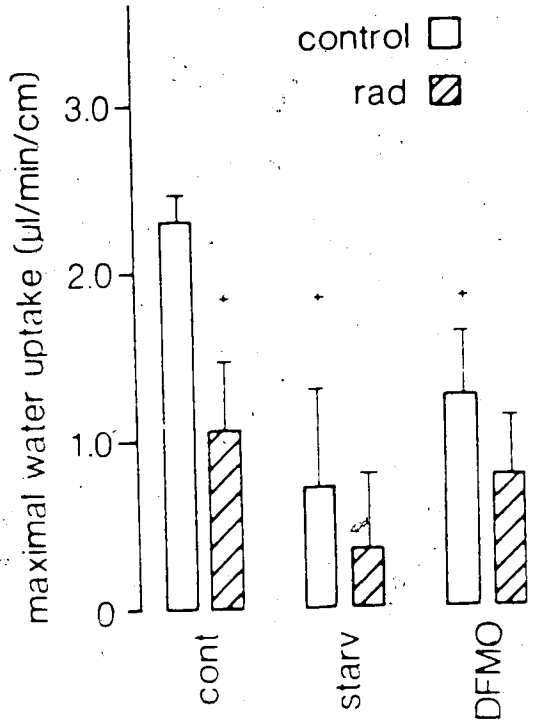


Figure 5.6 Glucose-stimulated water uptake *in vivo* in the upper jejunum of control and irradiated rats after no further treatment (n=8 & 7 respectively), starvation for 3 days (STARV, n=6,4), or difluoromethylornithine (DFMO) administration for 7 days (n=6,6). Glucose concentration, 96 mM. * = $p<.005$. Comparisons made between control (at far left) and non-irradiated treatment groups, and between each non-irradiated and irradiated treatment group.

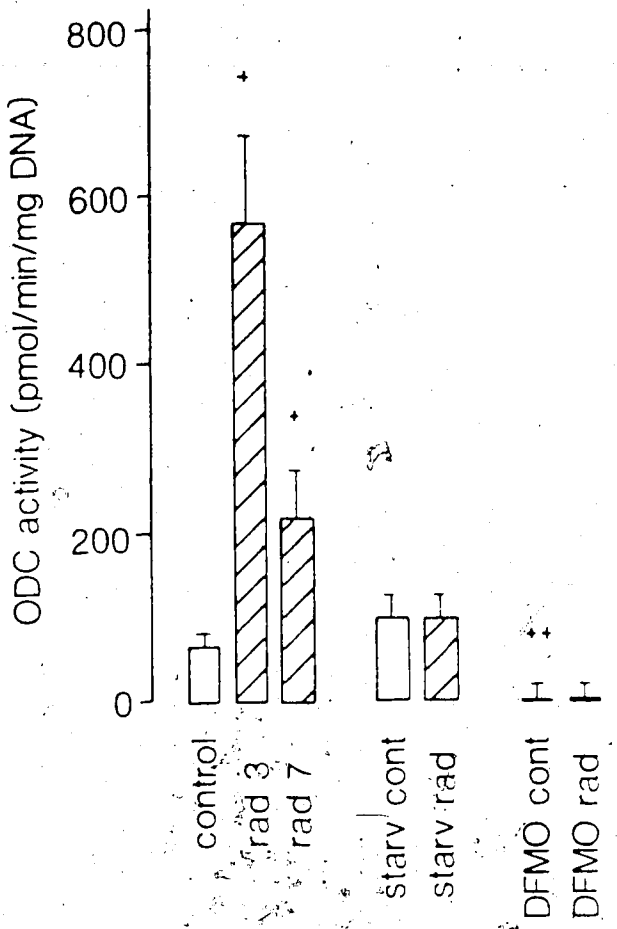


Figure 5.7 Intestinal ornithine decarboxylase (ODC) activity in control and irradiated rats after no further treatment (control, n=7; rad 3, n=10; rad 7, n=7), starvation for 3 days (starv, n=5,6) or administration of difluoromethylornithine (DFMO) for 7 days (n=4,4). + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

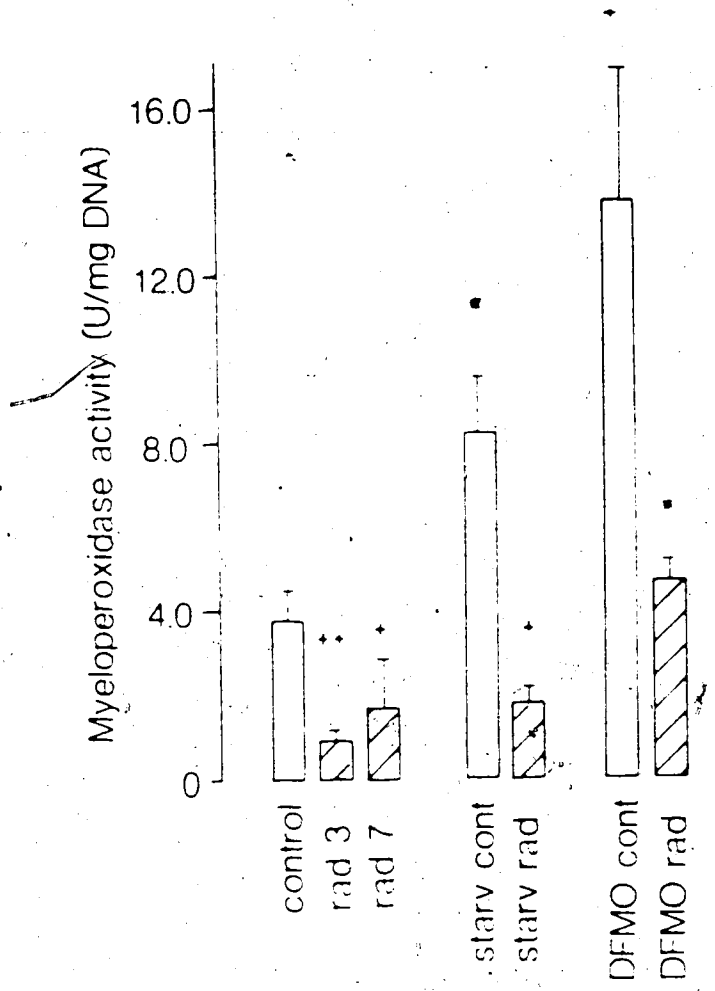


Figure 5.8 Intestinal myeloperoxidase activity in control and irradiated rats after no further treatment (control, rad 3, rad 7; n=7), starvation for 3 days (STARV, n=8,4) or administration of difluoromethylornithine (DFMO) for 7 days (n=4,4). * = p<.05, + = p<.005, ++ = p<.001. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

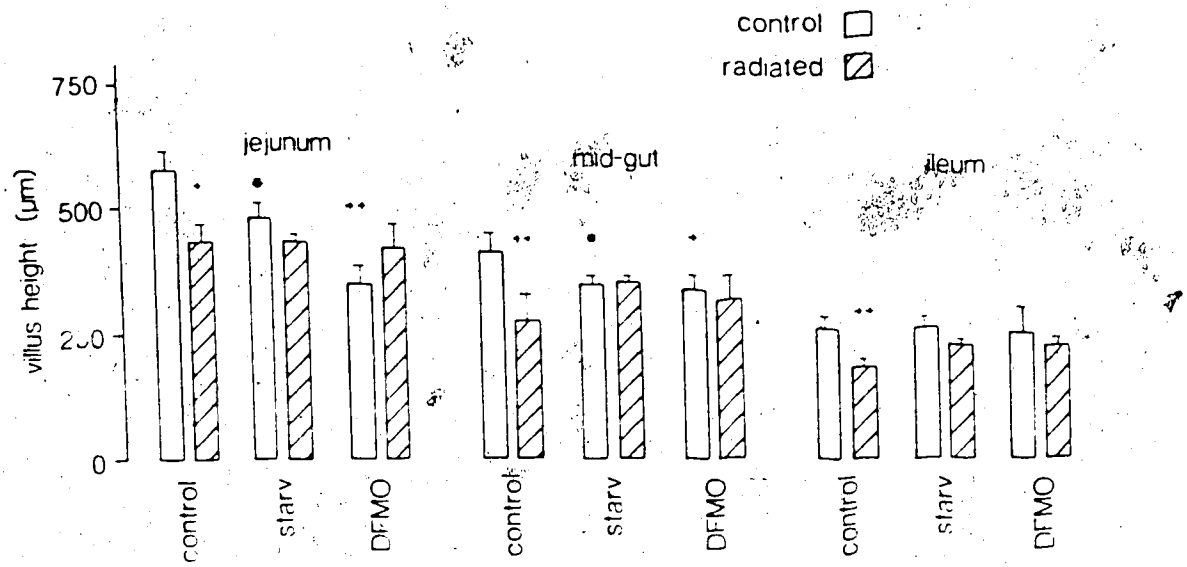


Figure 5.9 Villus heights in control and irradiated rats after no further treatment (n=12,9), starvation for 3 days (STARV, n=4,4), or administration of difluoromethylornithine (DFMO) for 7 days (n=6,4). * = $p<.05$, + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

changes were observed in villus height in starved rats which were also irradiated. The jejunal dry weight/length ratio was significantly reduced by starvation, although no further reduction occurred in the ratio in jejuna from starved irradiated animals (Figure 5.10).

DFMO Administration - Preliminary data from irradiated rats administered DFMO for three days indicated no change in D-glucose uptake *in vivo* compared to irradiated controls. DFMO administration was therefore extended to seven days to determine if continued administration would prevent the recovery from radiation-induced intestinal injury usually evident by seven days post-irradiation (Chapter III). After 7 days of DFMO in their drinking water, non-irradiated rats had decreased D-glucose uptake (Figure 5.11) and water absorption (Figure 5.6) compared to controls. Irradiated rats receiving DFMO showed no further change in D-glucose or water transport. ODC activity was negligible in both groups receiving DFMO (Figure 5.7). Even if animals consumed less drug following irradiated due to decreased water intake, drug intake appeared to be adequate to suppress ODC activity.

Myeloperoxidase activity was substantially elevated in non-irradiated rats receiving DFMO for seven days (Figure 5.8). Irradiation decreased the mucosal content of this enzyme, but activity was still higher ($p<.025$) than in rats 7 days post-irradiation which had not received DFMO.

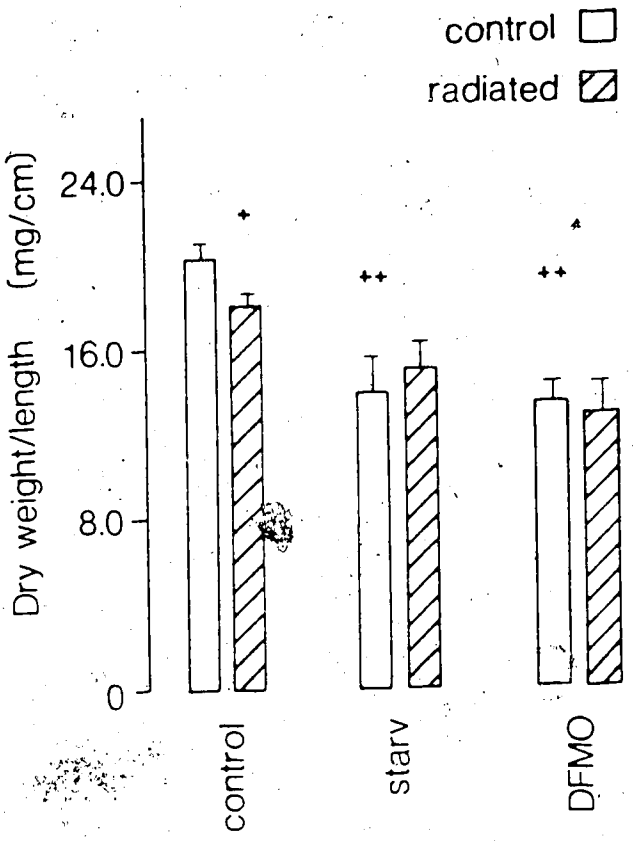


Figure 5.10 Dry weight/length ratios of control and irradiated rat jejuna after no further treatment (n=40,22), starvation for 3 days (STARV, n=6,4), or administration of difluoromethylornithine (DFMO) for 7 days (n=6,6). + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with control (far left); irradiated rats compared with non-irradiated control to immediate left.

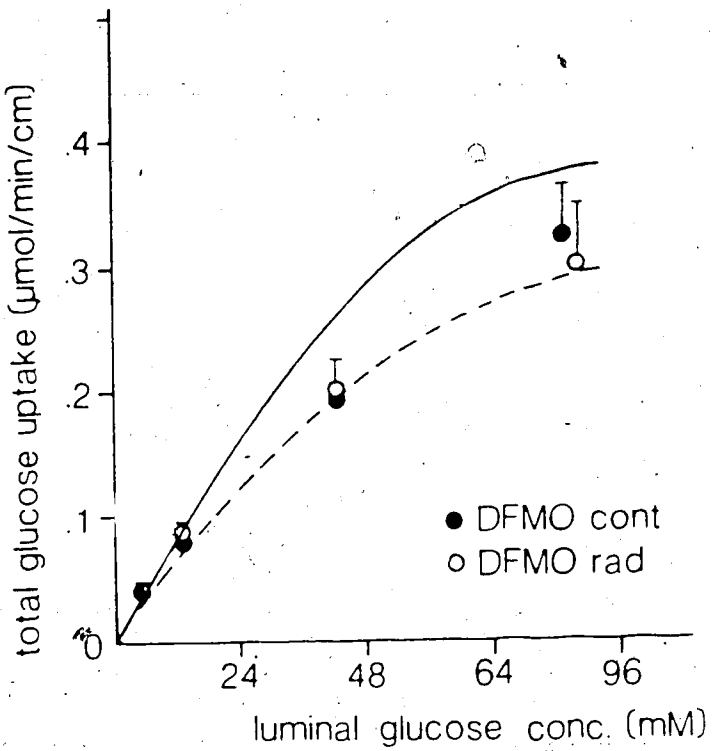


Figure 5.11 Total glucose uptake *in vivo* the upper jejunum of control and irradiated rats after difluoromethylornithine (DFMO) administration for 7 days. Solid line, uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. Overall uptake was significantly lower in DFMO-treated vs. untreated non-irradiated rats ($p<.005$). Uptake in DFMO-treated groups was not significantly different.

DFMO decreased villus height in the proximal jejunum and mid-gut but not in the terminal ileum of both groups receiving the drug (Figure 5.9). The jejunal dry weight/length ratio was also decreased compared to controls (Figure 5.10). There were no significant differences between the DFMO groups in either villus height or mucosal weight.

D. DISCUSSION

Several lines of evidence indicate that the morphological and functional changes that occur after abdominal irradiation do not follow the pattern observed with starvation. Differences in weight/length ratios, capacity for ODC stimulation, and myeloperoxidase activity exist between starved and irradiated animals which cannot be accounted for by the curtailment of food intake. In addition, many of the changes that result from starvation can be duplicated by DFMO administration.

After irradiation, the ratio of dry weight to length in the upper jejunum is only reduced by 10 per cent compared to 31% after three days of starvation (Figure 5.9). Villus height, however, is reduced to a similar extent by either irradiation (24% in the jejunum) or starvation (17%). This discrepancy between the size of the change in mucosal weight compared to the change in villus height after irradiation probably reflects differential effects of irradiation on the epithelial and muscular elements of the intestine. This is consistent with previous reports that muscle weight is

unchanged following radiation treatment (13, 32). The greater changes in mucosal weight occurring with starvation may therefore reflect additional atrophy in the muscle layer. That decreases in mucosal weight and villus height that occur after DFMO are similar to those seen with starvation suggests that a similar mechanism is a work with these two treatments.

Cell proliferation and cell migration rate are both decreased during starvation (1, 20). By comparison, results from experiments in Chapter IV show that the enterocyte migration rate is either unchanged or increased after radiation treatment. Thus, changes in morphological parameters that occur with irradiation are not consistent with those seen after starvation or food restriction.

The increase in ODC activity that occurs three days following radiation treatment is abolished by starvation (Figure 5.7). Irradiated animals that have continued access to food must therefore still be eating, in order for this increase in enzyme activity to be sustained. Since intestinal ODC activity is elevated for more than seven days after abdominal irradiation, at a time when glucose transport and villus height have returned to control levels (Chapter III), continued feeding is required for the stimulation of this enzyme even when other parameters of intestinal function appear normal. Indeed, the ability of DFMO to suppress glucose transport and to decrease villus height and gut weight in the non-irradiated animals

demonstrates that ODC is required for the normal functioning of the intestine. That starvation did not lower ODC activity in the intestine may be due to the habits of the rat, which generally feeds at night. ODC activity is elevated by feeding, but this increase is transient (25), and declines after a few hours to levels probably comparable to those seen during food deprivation.

Since glucose metabolism is reduced in intestinal tissue of starved animals (19), decreased intracellular removal of this substrate could result in a decreased chemical gradient for glucose movement across the membrane, resulting in decreased glucose uptake. However, the uptake of galactose, which is poorly metabolized by the enterocyte (4) is reduced to a similar degree to that of glucose after irradiation, starvation, or a combination of both.

Other evidence suggests that possible changes in the chemical gradient that result from starvation are likely small in magnitude and do not contribute significantly to changes in uptake. Using the vascularly perfused rat, Windmueller (50) demonstrated that more than 95% of absorbed [illegible]elled glucose appeared in the vascular bed unchanged. [illegible]r, tracer studies by Radziuk (35) indicate that 90% of [illegible]gested glucose load appears in the general circulation, and at least part of the remaining 10% must be accounted for in hepatic uptake from the portal circulation. Thus, the proportion of luminal glucose metabolized by the enterocyte, which normally uses glutamate as its major fuel (50), is

normally small. A further reduction in this proportion is unlikely to be detectable in the system employed here.

It is of interest that villus height reductions were observed only in the upper gut of the DFMO treated rats, with minimal change in the lower ileum. DFMO is sufficiently well absorbed by the intestine to inhibit growth in extra-intestinal tissue such as the prostate, skin, and uterus (15). Since intravenous DFMO can produce atrophic changes to the intestine (51), there would appear to be sufficient drug available to the ileum for ODC inhibition. The lesser effect in the ileum is therefore not due to insufficient drug delivery as a consequence of proximal absorption of the compound.

Preliminary studies indicate that ileal glucose uptake *in vivo* is also minimally affected by the drug. It is unlikely, therefore, that products of ODC have much influence on ileal morphology or function except when ODC levels are subject to stimulation in conditions such as jejunectomy when hypertrophy occurs (22). Although no evidence is available concerning DFMO interference with the glucose transporter, the drug does not inhibit amino acid transport (9). It is more likely, therefore, that the transport effects observed are secondary to developmental changes in the intestine.

Polyamines appear to play a role in the control of inflammatory processes. Spermine reduces serotonin-induced inflammation in the rat paw edema model (28) and also

inhibits serotonin release (43). The role of polyamines as membrane stabilizers may be central to this property (46). the presence of complement, polyamine antibodies lyse cells in culture, and this effect can be overcome by exogenous polyamine (33). Polyamines also inhibit leukocyte aggregation (6), although this phenomenon may be the result of polyamine metabolites produced by polyamine oxidase and diamine oxidase (2, 27).

The elevation of myeloperoxidase activity in starved and DFMO treated animals compared to their respective controls, with or without irradiation (Figure 5.8), would appear to be consistent with this anti-inflammatory property of polyamines or their metabolites. Negligible levels of ODC correlate with increased myeloperoxidase activity, and by implication, leukocyte infiltration, as the activity of this enzyme is proportional to leukocyte numbers in the tissue (10, 17, 40). While the decline in the peripheral leukocyte count that occurs after radiation exposure (18) undoubtedly decreases the size of the pool available for re-infiltration of the intestine, the data from the starved and DFMO treated animals suggests other factors may also control granulocyte populations in the gut. Ten days starvation in man (28) produces no change in the peripheral leukocyte count, and DFMO administration to rats actually decreases the number of peripheral leukocytes (23). In contrast, both these circumstances coincide with increased intestinal myeloperoxidase content, which can only be partially

diminished by radiation treatment.

Other investigators have found that exclusion of nutrients or pathogenic organisms from the gut decreases intestinal leukocyte populations. Germ-free rats (40) and rats on intravenous feeding for 10 days or more (12) have decreased intestinal myeloperoxidase content. One might therefore expect lower myeloperoxidase levels in the starved animals as well. The increase seen, however, may only be transient, due to residual bacteriological content, which may trigger leukocyte accumulation in the absence of the control normally exerted by the action of polyamines. Fasting for periods longer than the 3 day period used here may be necessary to reduce bacteria populations in the gut. Unfortunately, extended fasting presents technical (and ethical) concerns about the viability of the animal.

It should be emphasized that the data presented here indicate that starvation or DFMO only delays recovery of normal morphology and function after radiation injury. There are no indications that recovery could be harmed by these procedures. After starvation, ornithine decarboxylase activity can be stimulated by refeeding, and preliminary results (not shown) show that comparable post-starvation induction occurs if the animals had also been irradiated. This demonstrates that the response remains intact after damage providing that the proper combination of signals is present. In fact, Stanley (42) has found in the rat colon that sodium deoxycholate, an agent capable of stimulating

ODC activity, acted synergistically with starvation refeeding to produce an increase in ODC levels several fold greater than that produced by the individual stimuli.

In summary, the present results emphasize that starvation serves as an important stimulus to ODC activity in the gut and that starvation or administration of ODC inhibitors can postpone the recovery of the intestine from damage. Whether the postponement of the recovery process is beneficial or detrimental to the long term prognosis of intestinal radiation damage awaits further study.

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VI. Experiment 4: Modification of Acute Intestinal Radiation Damage by Inhibitors of Prostaglandin Synthesis and Xanthine Oxidase

A. INTRODUCTION

Attempts to influence the course of recovery from intestinal injury are generally based on the premise of modifying an inflammatory reaction (62, 63, 32). Acute radiation damage to the intestine, however, does not involve mucosal infiltration by immune elements, and the leukocyte content of the intestine is actually reduced for up to two weeks after radiation treatment (Chapter III). The amelioration of the acute effects of abdominal irradiation must therefore have a different basis. The present experiments were designed to study agents which could influence the course of recovery from intestinal radiation damage. Several tests were used to monitor recovery from intestinal injury, including myeloperoxidase activity (to measure leukocyte infiltration) (64), glucose transport *in vivo*, and ornithine decarboxylase activity.

Ornithine decarboxylase (ODC) plays an important role in the recovery of the intestinal epithelium from injury. In the rat, intestinal ODC levels are elevated for more than a week after the administration of the cytotoxic drug ARA-C (38), or after abdominal radiation treatment (Chapter III). After intestinal damage, both ODC induction as well as morphological and functional signs of recovery can be

blocked by DFMO (Chapter V), an irreversible inhibitor of ODC (38). ODC has been linked with the regulation of DNA synthesis and cell proliferation (54, 55), and its short half life (approximately 11 minutes) (58), facilitates rapid induction by stimuli promoting cell growth.

Prostaglandin and prostacyclin release has been reported to follow irradiation of the small bowel (7), as well as several other tissues (17, 18, 26). Increased prostaglandin levels in the gut stimulate intestinal fluid secretion (9) and could aggravate the initial injury. Some success has been reported with the use of aspirin to control intestinal complications of radiation treatment (42). Two commonly used prostaglandin synthesis inhibitors, indomethacin and aspirin (acetylsalicylic acid) were used to study how recovery was affected by the inhibition of prostaglandin release. Since aspirin is also a weak acid, which may affect fluid secretion by interfering with Na^+/H^+ exchange (21), a structurally similar weak acid with no antiprostaglandin activity, p-aminobenzoic acid (PABA), was also tested.

Steroidal anti-inflammatory drugs such as cortisone also indirectly decrease prostaglandin production, by the inhibition of phospholipase A2 (PLA_2), which provides the precursor of prostaglandin synthesis, arachidonic acid (19). In addition to its anti-inflammatory properties, cortisone is known to increase glucose and electrolyte transport in normal animals (5, 60). Prednisolone, the active form of the

synthetic steroid prednisone, was chosen to determine whether this drug would also be able to stimulate transport under circumstances where intestinal damage does not involve an immune reaction.

The irradiation of living tissue results in the generation of oxygen free radicals such the hydroxyl radical ($OH\cdot$) (11), which can be toxic to biological tissues by virtue of their extreme reactivity. Recently, it has been suggested in another damage syndrome, intestinal ischemia, that generation of free radicals may occur secondary to the initial injury (52), as a byproduct of the degradation of ATP by xanthine oxidase. Oxygen free radical production by xanthine oxidase can be inhibited by allopurinol, and this drug was therefore used in these experiments to determine if secondary free radical production could contribute to radiation damage in the gut.

B. METHODS

Assays - Ornithine decarboxylase activity was determined in mucosal homogenate supernatant by measuring the evolution of CO_2 from ^{14}C-labelled ornithine, as described in Chapter VII. Myeloperoxidase activity, which correlates quantitatively with number of leukocytes present in the tissue, was determined spectrophotometrically using colour development due to tetraguaiacol formation in the presence of exogenous H_2O_2, as previously in Chapter V. *In vivo* transport of glucose was measured in anesthetized

animals using ^{14}C-labelled substrate with ^{3}H-inulin used to correct for fluid movement as described in Chapter III.

Animals and Treatments - Male Sprague-Dawley rats weighing approximately 250 to 300 g. were used in all studies. Irradiated animals were anesthetized with pentobarbital, and received 6 Gy. directed at the abdomen as described in Chapter III. Drugs were injected intraperitoneally to irradiated animals daily for four days, beginning one hour prior to irradiation, and non-irradiated animals received the drug for four days. Unless noted, all drugs came from Sigma Chemical Co.

Prostaglandin Synthesis Inhibition Indomethacin (2.5mg/ml, 2.5 mg/kg), aspirin (BDH, 60 mg/ml, 50 mg/kg), or PABA (sodium salt, 50 mg/ml, 36 mg/kg, equimolar with aspirin), were administered in .25 ml propylene glycol. The dose of aspirin was based on the lower effectiveness of aspirin in inhibiting cyclo-oxygenase compared to indomethacin (44). The doses of drugs used here were chosen for their ability to inhibit prostaglandin synthesis *in vivo* (2, 44) without producing ulceration (57).

Xanthine Oxidase Inhibition - Allopurinol (60 mg/ml, 30 mg/kg) was dissolved in 0.9% saline, pH 10.5 to facilitate solubility, with 0.25 ml injected i.p. daily beginning two days prior to irradiation, and continued for a subsequent 3 days.

Glucocorticoids - Prednisolone (1.2 mg/ml, 1 mg/kg), was dissolved in 0.9% saline, and injected as described

above.

C. RESULTS

Aspirin - Aspirin treatment reduced glucose transport compared with untreated controls (Figure 6.1), but glucose stimulated water transport remained unchanged (Figure 6.2). Radiation treatment caused no further significant change in either parameter. ODC activity was increased by aspirin treatment alone (Figure 6.3) and again no further changed occurred if rats receiving this drug were also irradiated. On autopsy, diffuse adhesions were noted in the aspirin treated irradiated group. This was not observed in any other group.

PABA - PABA caused no significant change in glucose or water transport (Figures 6.4, 6.2). Both these parameters were reduced by the addition of radiation treatment, and in turn were not different from irradiated animals not treated with PABA. ODC levels were not significantly changed by PABA (Figure 6.3) and remained unchanged in the drug group exposed to the radiation procedure. As a result, ODC levels in the latter group were significantly lower ($p<.01$) than in the non-drug irradiated group. Myeloperoxidase activity was unchanged by PABA administration (Figure 6.5), or by combined drug and irradiation procedures. As a result, myeloperoxidase levels in the irradiated/PABA group were significantly higher ($p<.05$) than in irradiated animals not receiving PABA.

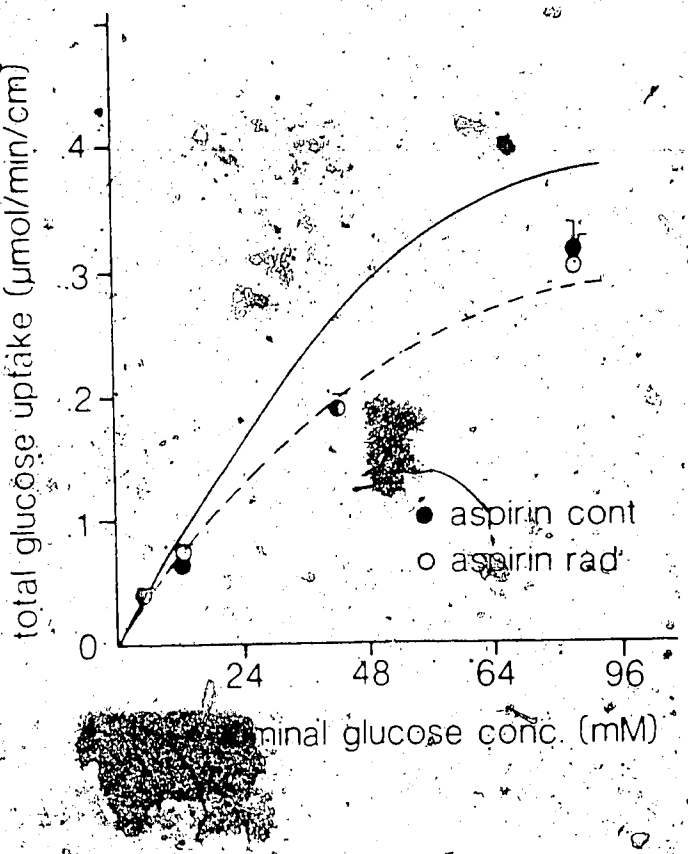

Figure 6.1 Total glucose uptake *in vivo* in control and irradiated rats after administration of aspirin for 3 days. # of observations per point: aspirin control (6), aspirin rad (8). For reference, the solid line represents uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. Uptake in both aspirin treated groups was significantly lower than in control non-irradiated rats ($p<.05$) but not different than control irradiated rats.

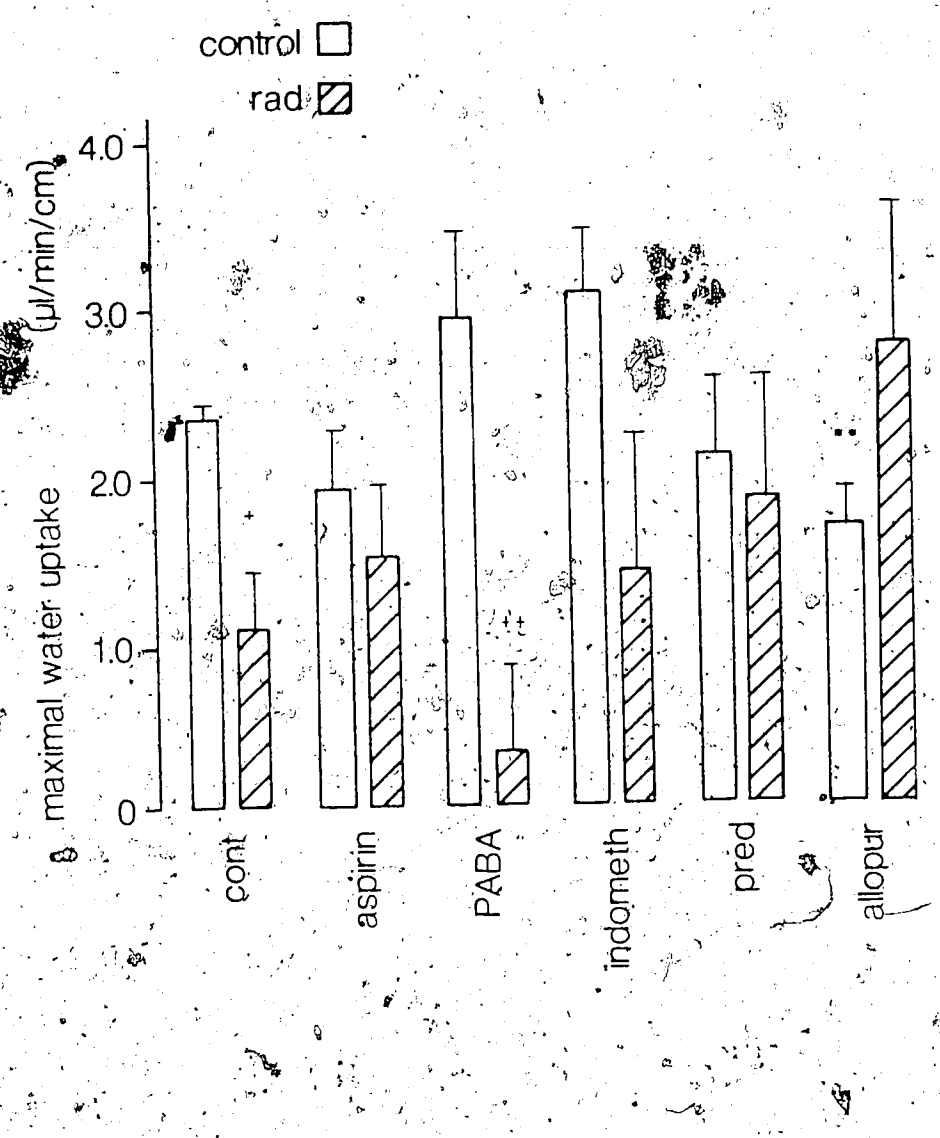

Figure 6.2 Glucose-stimulated water uptake in the proximal jejuna of control and irradiated rats after no further treatment (n=8 & 7 respectively), or aspirin (n=6,6), PABA (n=6,7), indomethacin (n=5,8), prednisolone (n=6,6), or allopurinol (n=4,5) administration. Glucose concentration, 96 mM. ** = $p<.01$, + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

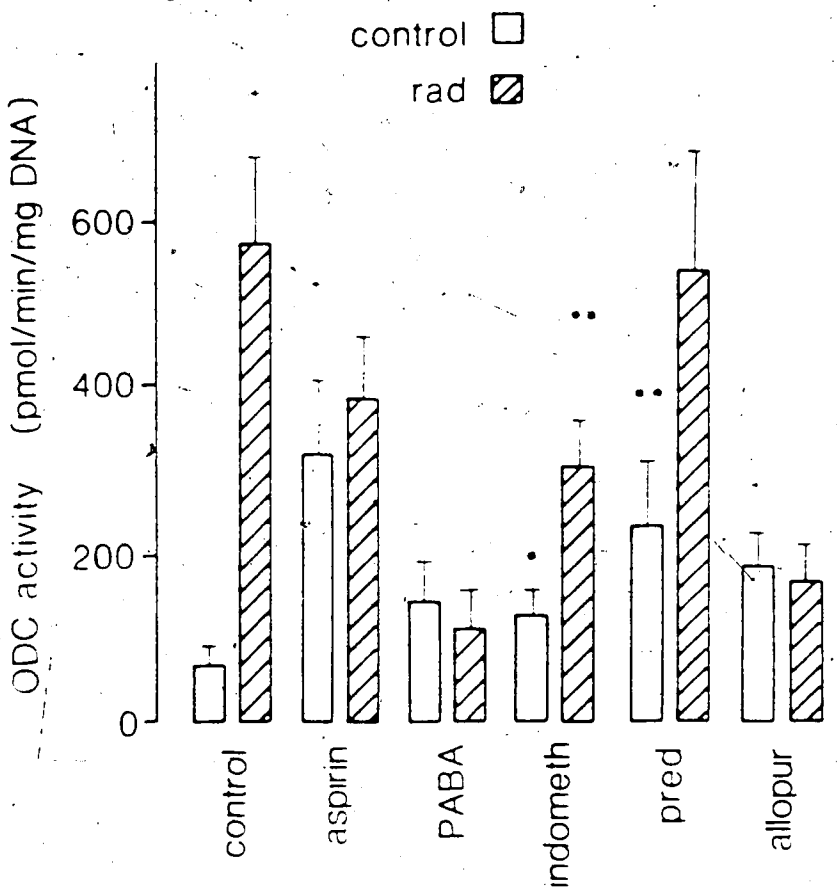

Figure 6.3 Intestinal ornithine decarboxylase (ODC) activity in control and irradiated rats after no further treatment (n=7,10), or aspirin (n=6,6), PABA (n=6,7), indomethacin (n=7,8), prednisolone (n=4,5), or allopurinol (n=6,7) administration. * = $p<.05$, + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

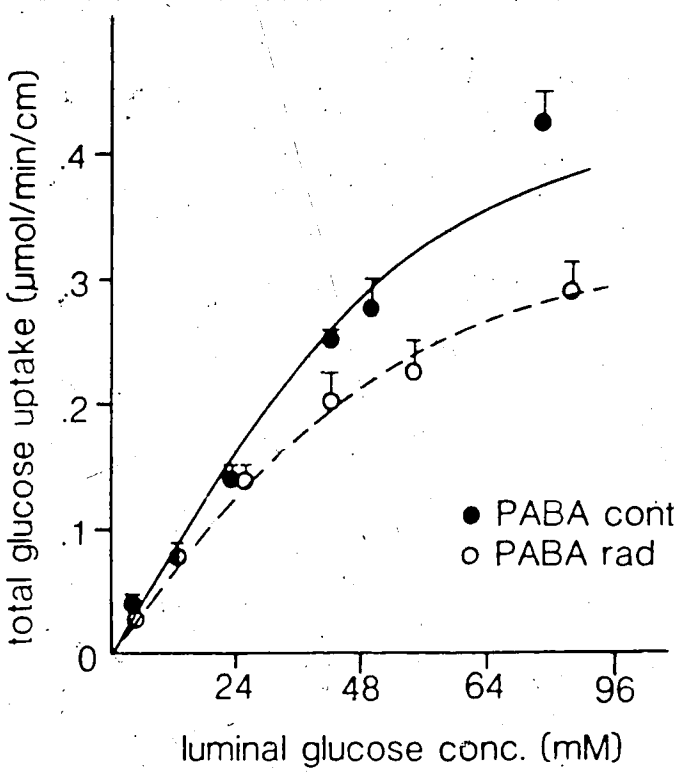

Figure 6.4 Total glucose uptake in control and irradiated rats after administration of p-aminobenzoic acid (PABA) for 3 days. # of observations per point: PABA control (4-6), PABA rad (4-6). Solid line, uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. No significant differences were observed between corresponding groups.

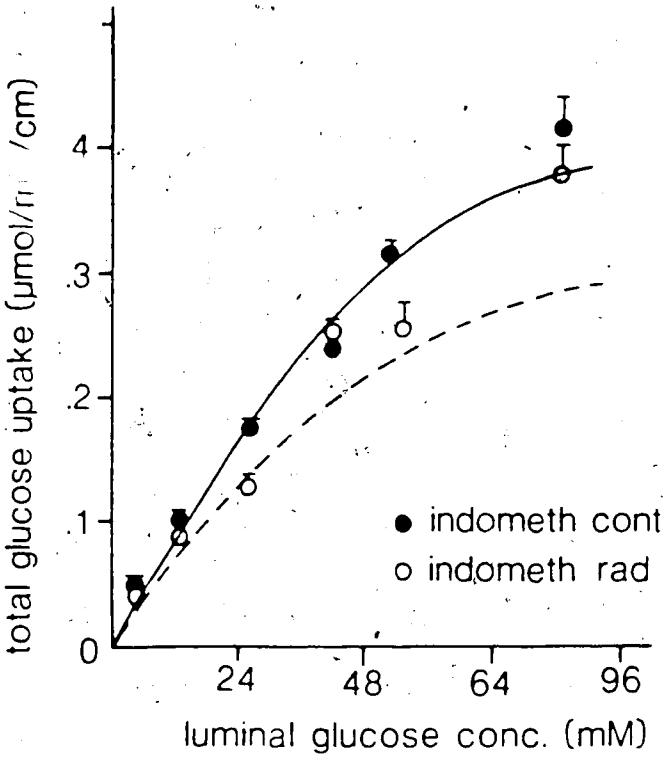

Figure 6.5 Total glucose uptake in control and irradiated rats after administration of indomethacin (INDOMETH) for 3 days. # of observations per point: INDOMETH control (6-8); INDOMETH rad (6-8). Solid line, uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. Uptake in both indomethacin treated groups did not differ significantly from that in control non-irradiated rats.

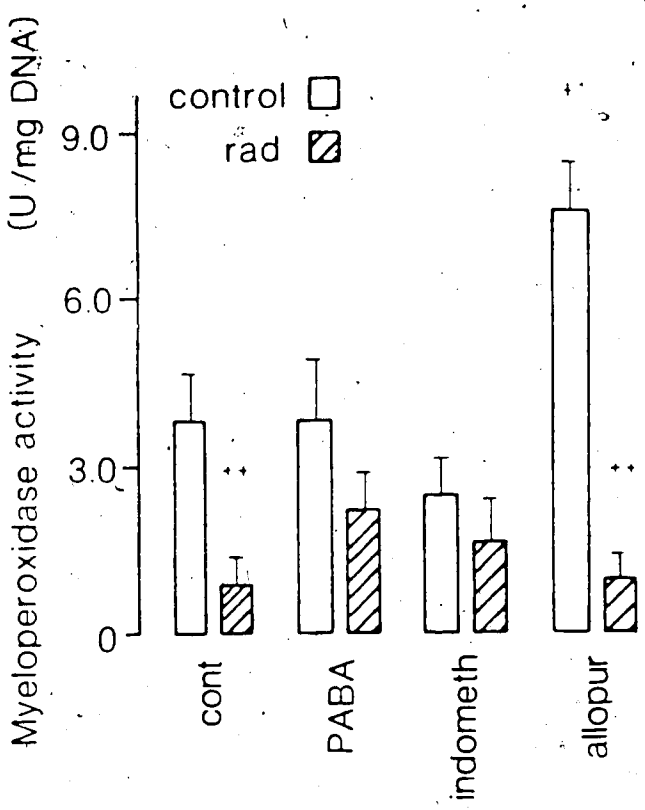

Figure 6.6 Intestinal myeloperoxidase activity in control and irradiated rats with no further treatment (n=7,7), or PABA (n=5,7), indomethacin (n=6,6), or allopurinol (n=5,5) administration. + = $p<.005$, ++ = $p<.001$. Non-irradiated rats compared with Control (far left); irradiated rats compared with non-irradiated control to immediate left.

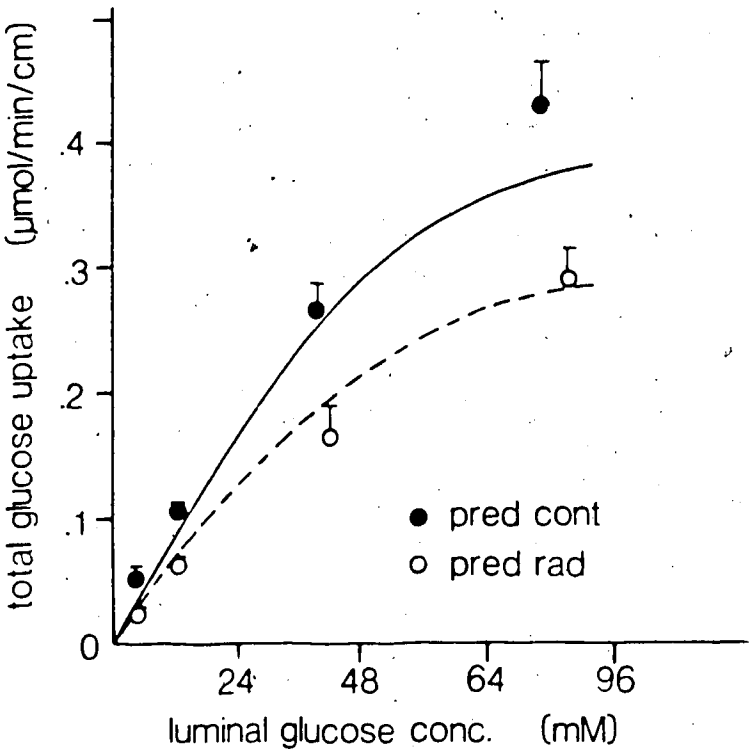

Figure 6.7 Total glucose uptake *in vivo* in control and irradiated rats after administration of prednisolone (PRED) for 3 days. # of observations per point: PRED control, 6; PRED rad, 7. Solid line, uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. Uptake in non-irradiated prednisolone treated rats was significantly higher than in control non-irradiated rats ($p<.05$). Uptake in irradiated prednisolone treated rats did not differ from control irradiated rats.

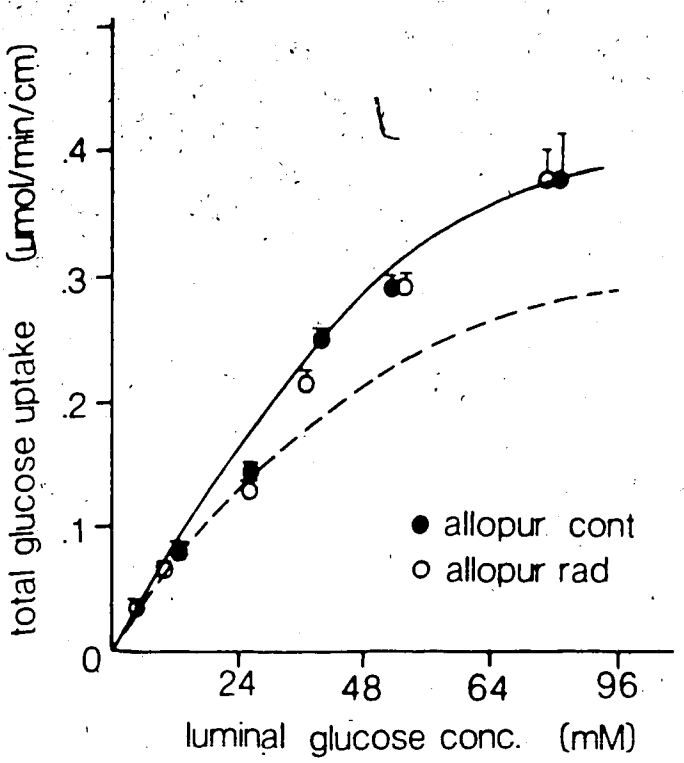

Figure 6.8 Total glucose uptake *in vivo* in control and irradiated rats after administration of allopurinol (ALLOPUR) for 3 days. # of observations per point: ALLOPUR control, 4-6, ALLOPUR rad, 4-5. Solid line, uptake in control non-irradiated rats; broken line, uptake in control irradiated rats. Uptake in both allopurinol treated groups did not differ significantly from that in control non-irradiated rats.

Indomethacin - Glucose and water transport *in vivo* were not significantly changed by indomethacin treatment (Figures 6.6, 6.1). Neither parameter was significantly changed by the addition of irradiation to the treatment. ODC activity was modestly but significantly elevated by indomethacin treatment (Figure 6.3). ODC levels were further elevated by irradiation, and in this group were lower than those in irradiated animals without indomethacin treatment. Myeloperoxidase activity was modestly suppressed by indomethacin, but this did not reach significance (Figure 6.5). The addition of radiation treatment caused no further significant change in myeloperoxidase activity.

Prednisolone - Overall glucose uptake was slightly but significantly increased by prednisolone (Figure 6.7), although water uptake was unchanged. Glucose uptake was significantly reduced by adding the irradiation treatment. Prednisolone increased ODC activity in animals receiving prednisolone alone (Figure 6.3). This was unchanged by the addition of irradiation.

Allopurinol - Glucose uptake was unchanged by allopurinol administration (Figure 6.8) although water uptake was modestly reduced (Figure 6.2). By comparison, glucose uptake was not significantly changed in irradiated animals receiving allopurinol, and water uptake was increased. Water uptake in this group was also higher ($p<.01$) than in irradiated animals not receiving allopurinol. ODC activity was modestly increased by

allopurinol (Figure 6.3), but not further altered in the irradiated group receiving the drug. ODC levels in the latter group were significantly lower ($p<.01$) than in irradiated animals not administered allopurinol. Allopurinol increased myeloperoxidase activity in non-irradiated animals (Figure 6.5), which fell in the group also receiving the radiation treatment.

D. DISCUSSION

The results described here indicate that anti-prostaglandin agents, weak acids, and xanthine oxidase inhibitors have substantially different effects on the function of both the normal and injured intestine. Of special interest here are the effects of the commonly used drug, aspirin, which combines several different properties.

The appearance of diffuse intestinal lesions in the irradiated group receiving aspirin suggests the generation of additional damage not occurring in other groups where intestinal damage has occurred. Adhesion formation commonly complicates surgery of the irradiated bowel, and can be reproduced in experimental animals by the combination of surgical manipulation and irradiation (49).

Aspirin has been reported to reduce water, electrolyte, and glucose uptake *in vivo* (1) and *in vitro* (12), as well as increasing intestinal permeability (34). In the present study, reduced glucose transport occurred with aspirin administration in non-irradiated animals. No further

decrease in transport could be induced by superimposing radiation damage on aspirin-mediated changes, however. Thus an increase in the severity of intestinal damage may occur which is not reflected in quantitative changes in transport *in vivo*. The mechanism of this additional injury is not clear. Aspirin has both antiprostaglandin (44) and weak acid properties, and consequently, both these components were explored individually.

Weak Acid Effects

The intestinal response to PABA was different in several respects from that of aspirin, suggesting that aspirin effects are not due solely to its weak acid properties. PABA administration, unlike aspirin, produced no difference in glucose or water uptake in control rats. In addition, the expected increase in ODC activity after irradiation was abolished.

As a weak acid, PABA readily crosses into the cell in its non-ionized form, where it dissociates and results in a small but significant intracellular acid load (21). The regulation of intracellular pH may be a critical determinant of DNA synthesis. Increases in intracellular pH have been recorded using micropipettes prior to growth (27). This alkaline shift is mediated by Na^+/H^+ exchange, requires sodium in the medium (27), and is mimicked by phorbol esters and other growth promoting agents (45). A specific mutation of hamster fibroblasts lacking the Na^+/H^+ exchanger is only capable of growth in alkaline media (55). It is conceivable

that an intracellular acid load produced by PABA could interfere with the alkalinization required for DNA synthesis. The involvement of pH as a regulator of ODC induction is a novel suggestion, but there is some indirect evidence to support this. Growth promoting agents such as phorbol esters are capable of stimulating ODC activity (8, 66, 70), and also stimulate intracellular alkalinization prior to growth induction (45).

ODC inhibition may also account for the smaller decrease in myeloperoxidase activity following radiation treatment when PABA is administered. Polyamine production by ODC has been linked with the inhibition of lymphocyte migration (10, 46). This inverse relationship has also been observed in Chapter V when the post-irradiation increase in ODC has been inhibited by starvation or by DFMO.

Antiprostaglandin Actions

Unlike aspirin, the administration of indomethacin to irradiated animals did not promote further injury, but actually appeared to be beneficial in its ability to improve glucose absorption as well as glucose-stimulated water uptake (Figures 6.1, 6.3). The suggestion here is that the release of prostaglandins or prostaglandin derivatives by abdominal irradiation can in turn contribute to intestinal fluid secretion (14, 72). This could arise from vasodilation and increased capillary permeability (72, 4), resulting in a pressure-driven secretion (37); a more direct stimulation of active electrolyte secretion could also occur (9, 22, 33,

61).

Prostaglandins can stimulate ODC activity in cell culture (3) and *in vivo* (39). Induction of ODC by agents such as phorbol esters can be inhibited by indomethacin, and this can be overcome by adding exogenous PGE (8, 70). It is possible, then, that the induction of ODC activity observed in these studies three days after radiation treatment could be mediated by prostaglandin release. However, indomethacin was not capable of fully inhibiting the increase in ODC activity that normally occurred after irradiation. This could have been due either to the incomplete inhibition of prostaglandin synthesis, or to the mediation of agents other than prostaglandins in the stimulation of ODC activity.

One such candidate for the induction of ODC activity are the leukotrienes. Mepacrine, a PLA2 inhibitor, blocks phorbol-induced ODC activity, but ODC activity cannot be restored by the addition of exogenous prostaglandins (23, 31). As leukotriene synthesis is only modestly inhibited by indomethacin, it was suggested that these arachidonic acid derivatives of the lipoxygenase pathway were also important in ODC induction (31). Leukotrienes have been shown to have calcium ionophore activity in liposomes (59), and elevated intracellular calcium levels are capable of stimulating ODC activity (36, 50).

Inhibition of prostaglandin synthesis by large doses of either steroidal (35) or non-steroidal (57) anti-inflammatory drugs can induce intestinal ulceration,

which can be overcome by exogenous prostaglandins, especially PGE. Either oral or intramuscular administration can create this injury (43). A dose comparable to that used here was able to delay the onset of castor oil-induced diarrhea in rats (2) without ulcerogenesis, as well as suppressing the inflammatory response characteristic of carrageenan-induced paw edema. Larger doses than those used in the current studies, therefore, could prove to produce further injury.

The use of prednisolone makes it possible to study the inhibition of prostaglandin synthesis by a different mechanism than that used by indomethacin (19). Unlike indomethacin, these studies show that prednisolone increases ODC activity in the non-irradiated intestine. Similar stimulation of ODC by glucocorticoids has been observed in other normal tissues such as mammary gland (48, 73) and liver (6, 13). Aspirin administration also stimulated ODC activity in non-irradiated animals, but unlike aspirin, prednisolone increased fluid and glucose absorption. This improvement in transport is corroborated by evidence from other authors who noted increased transport *in vivo* (5, 60) and in cell culture (29). Whether these effects on transport are connected to the increased ODC activity requires further study. Since both aspirin (44) and glucocorticoids (19) inhibit, rather than stimulate prostaglandin production, it can be concluded that ODC induction by these two drugs in non-irradiated animals is not mediated by prostaglandin

synthesis.

The present experiments indicate that prednisolone and irradiation are not additive in their ability to stimulate ODC activity, and that prednisolone is not able to alter the recovery of glucose or water uptake by the irradiated intestine. This again resembles phorbol-induced ODC induction in the skin, which can be blocked by indomethacin, but not by cortisone (8). The experience with other experimental intestinal injury syndromes offers some corroborating evidence on this point, as cortisone did not influence the development of necrosis (67, 68) or increased permeability (25) following acute intestinal ischemia. On the other hand, Manohar (40) found that the increase in tissue lysosomal acid phosphatase after acute ischemia was blocked by cortisone. The conclusion that can be drawn from the present studies is that doses of prednisolone sufficient to alter intestinal function in the normal bowel are without effect in the irradiated intestine.

The results presented here indicate that the effects of aspirin on the normal and irradiated intestine cannot be accounted for on the basis of either anti-prostaglandin action or weak acid effects of the drug. It is therefore necessary to postulate other mechanisms by which aspirin injury could occur.

Xanthine Oxidase Mediation

By the third day after radiation treatment, at least two generations of cells have been exfoliated from the

villus tips, yet several indications of damage still persist. Superoxide production from ATP breakdown by xanthine oxidase (52) is one mechanism by which free radicals could arise in succeeding generations of cells to produce additional cell injury. Free radicals derived from xanthine oxidase can result in increased vascular permeability (30, 16), histamine release from mast cells (47), and disruption of lysosomal membranes (20).

Xanthine oxidase normally occurs in the cell in the dehydrogenase form, which does not generate superoxide, but can be converted to the oxidase form in the presence of proteolytic activity (41). Recently, Parks has shown that ischemic injury to the intestine could be attenuated by the use of proteolysis inhibitors (53). The importance of proteolysis in radiation damage syndromes may account for the ability of protease inhibitors to improve post-irradiation survival (69, 51). As PGE can stimulate proteolysis (24), increased tissue content of this prostaglandin could contribute to an extended elevation of tissue free radical content.

Aspirin inhibits ATP production in jejunal biopsies from normal human subjects (1) and in bullfrog gastric mucosa (65). Breakdown of ATP by xanthine oxidase with the resulting liberation of superoxide could be one mechanism of injury involved here. Amongst other effects, the generation of free radicals uncouples Ca^{++}/ATPase, thereby increasing intracellular calcium (28), which in turn stimulates ODC

activity (36, 50). This uncoupling is potentiated by low pH, which would also result from aspirin administration.

During recovery from injury, aspirin inhibits ATP supply at exactly the time when the requirements for increased transport are greatest, in order to balance electrolyte loss to initiate new cell production. ATP breakdown and free radical production could therefore contribute to the injury already initiated by irradiation. These studies indicate that the previously reported usefulness of aspirin in treating radiation enteritis (42) is likely restricted to long-term inflammatory complications of abdominal irradiation, and is contra-indicated for the treatment of acute injury.

If superoxide generation by xanthine oxidase is an important mechanism in the prolongation of intestinal injury, the use of free radical scavengers should be able to improve recovery. Unlike direct scavengers such as dimethylsulfoxide (DSMO), the xanthine oxidase inhibitor allopurinol allows the investigation of the extent to which secondary peroxidation contributes to tissue injury. In addition, use of DMSO can also make the interpretation of transport data difficult, as DMSO can alter glucose uptake by increasing its lipid solubility (15).

The improvement in glucose and water uptake produced by allopurinol would seem to indicate that secondary free radical generation indeed plays a role in the injury process begun by radiation exposure. Allopurinol also abolishes the

post-irradiation increase in ODC levels. This could represent either a direct interference with the signals that trigger ODC induction, or an actual reduction in the amount of damage present, thus providing less of a requirement for increased ODC activity and cell proliferation. Direct inhibition of the stimulation of ODC activity after irradiation by the inhibitor DFMO does not result in the improvement in transport parameters, however, nor does ODC blockade by starvation. The changes in ODC levels that result from allopurinol administration is therefore symptomatic of underlying changes in recovery, rather than their cause.

The ability to decrease ischemic damage with superoxide dismutase, DSMO, and allopurinol (52) appears to stress the importance of free radical generation in the mechanism of ischemic damage. Earlier reports demonstrated that allopurinol treatment increased survival of intestinal and renal transplants (67, 68), which likely involved a significant period of ischemia before reimplantation.

The present studies indicate that intestinal recovery from radiation damage can be influenced by several drugs, each of which provides some insight into the injury mechanisms involved. The results suggest that increased intracellular pH may be important in the stimulation of ODC activity by damage, that prostaglandins may be involved in the pathogenesis of intestinal injury, especially where transport changes are involved, and that superoxide

production secondary to the initial radiation insult may act to prolong the damage process. Finally, additional injury can result from the use of aspirin. This is not due to either antiprostaglandin or weak acid effects, but may instead be a result of superoxide generation when ATP production is inhibited by the drug.

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VII. Experiment 5: Substrate Specificity in the Stimulation of Intestinal Ornithine Decarboxylase Activity by Refeeding after Starvation

A. INTRODUCTION

Several adaptive changes occur in the intestine during starvation, including a reduction in the rate of cell proliferation and cell migration (1, 2, 7, 23), as well as a decrease in the rate of actively transported substrates such as glucose (13, 21). Nutritional deprivation is probably not the cause of these atrophic changes, as similar decreases are observed during parenteral nutrition (17, 18, 20, 33). Instead, the absence of luminal nutrients is a more likely explanation for this adaptive response.

Refeeding induces a rapid return to the pre-starvation state (1, 15). Coincidentally, there is a large increase in the enzyme ornithine decarboxylase (ODC) in the intestine, which peaks three to five hours after the reintroduction of food (26). Ornithine decarboxylase, which synthesizes the first of a series of compounds collectively known as polyamines, is known to play a role in the regulation of DNA synthesis (31), and feeding the ODC inhibitor difluoromethylornithine (DFMO) in the drinking water of rats or dogs severely curtails intestinal cell proliferation (24, 37). Polyamines have been noted to stimulate glucose transport in fat cells (22) and in renal brush border vesicles (14), and in addition, DFMO depresses glucose

transport *in vivo*. Stimulation of ODC, therefore, likely plays an important role in the initiation of the recovery of normal intestinal function as well as morphology brought about by refeeding. The exact stimulus for ODC induction after refeeding is uncertain. These experiments were therefore designed to determine if a specific nutrient serves as the trigger for ODC induction, or whether any nutrient is capable of eliciting such a response. A variety of potential stimuli were used to test the ability of substrates with different absorption characteristics to stimulate ODC activity. D-glucose and L-leucine were used as representatives of actively transported compounds whose uptake is dependent on a sodium gradient (19, 27). D-galactose and 3-O-methylglucose were used due to their capability of being actively transported but not metabolized within the enterocyte (5, 11). D-fructose was chosen as representative of transport by a sodium independent system (10), and L-glucose represents a sugar which enters the enterocyte by a predominantly passive route (36).

B. METHODS

Male Sprague-Dawley rats weighing approximately 250-300 g. were starved for three days prior to use. Each animal was anesthetized with pentobarbital (i.p., 60 mg/kg), and maintained as described in Chapter III. A silicon (Silastic, Dow-Corning, o.d. = .047 in) cannula was implanted in the mid-jejunum for the substrate infusions. A

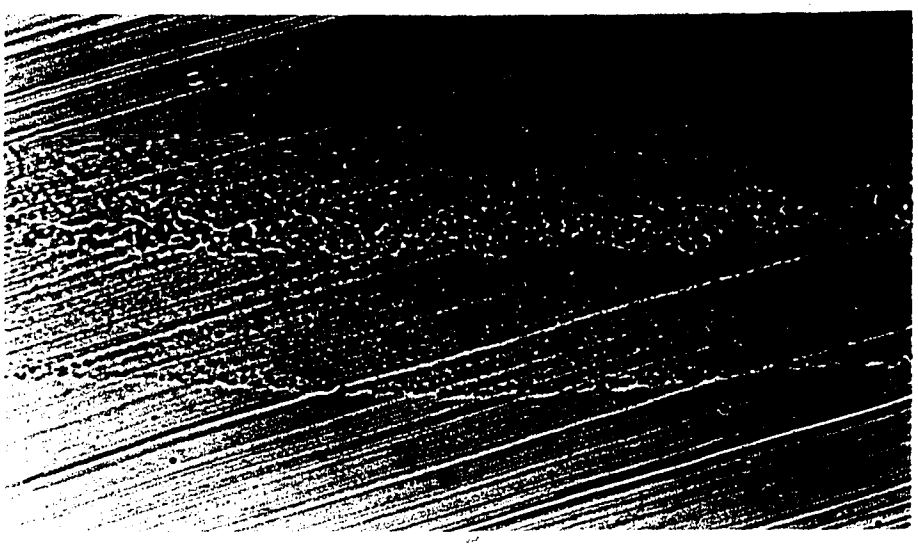
ligature was placed immediately proximal to the cannula insertion to prevent reflux of intestinal contents into the stomach. In one group, the cannula was implanted in the right jugular vein for parenteral substrate infusion. Animals were then infused with one of two concentrations o substrate for four hours at a rate of 5 ml/hour using a Sage infusion pump. Some animals received a 555 mM (10% w/v) solution of either D-glucose, D-galactose, or D-fructose, while others received a 100 mM solution of D-glucose or L-glucose (with or without 2 mM phloridzin), L-leucine, or 3-O-methylglucose. In the latter group, NaCl was added to maintain isosmolarity. Controls were also starved for three days, and were implanted with a jejunal cannula but were not infused.

At the end of the infusion period, the small intestine was removed and placed immediately into ice cold phosphate buffer (NaCl, 136.9 mM; KCl, 2.7 mM; Na_2HPO_4, 4.3 mM; KH_2PO_4, 1.5 mM; pH 7.2). Mucosal scrapings were homogenized on ice in 25 mM Tris buffer (pH 7.2) containing 50 μM EDTA and 2.5 mM dithiothreitol, using a teflon/glass homogenizer (Bauer). The homogenate was centrifuged at 4° C for twenty minutes at 16,000 x g and the supernatant used in the ODC assay according to the method of Bachrach (30). 0.4 ml of supernatant was added to 20 μl (0.25 μCi) of 1-^{14}C-(D,L)ornithine (New England Nuclear) and 80 μl containing cold L-ornithine and pyridoxal phosphate. Blanks used buffer instead of supernatant. The intestinal scrapings

included components from both crypt and villus, as seen by the lack of these structures in a micrograph of the scraping remnant (Figure 7.1).

To arrive at appropriate L-ornithine and pyridoxal concentrations, ODC activity was stimulated by starving an animal and infusing a 100 mM solution of D-glucose as described above. Increasing amounts of L-ornithine and pyridoxal were added to aliquots of homogenate. The L-ornithine concentration used was 0.2 mM (final concentration, 32 nmol/ml) as lower concentrations were rate limited by the L-ornithine concentration and higher ones resulted in too low a specific activity for reliable counting (Figure 7.2). Over the pyridoxal concentration range used, ODC activity appeared to fall at higher concentrations, and therefore a concentration of 1 mM (final concentration, 160 nmol/ml) was chosen as optimal (Figure 7.3). Homogenates from an animal with high ODC activity and one with low ODC activity were serially diluted to test for linearity with respect to DNA concentration (Figure 7.4). The dilution curves were in both cases linear, although they did not pass through the origin. This suggests a lower threshold in sensitivity of the assay to ODC activity, and could reflect a slight, but consistent underestimation of actual enzyme activity.

The reaction was carried out in glass scintillation vials with rubber stoppers designed to suspend a plastic well (Kontes Glass Co.) containing filter strips impregnated



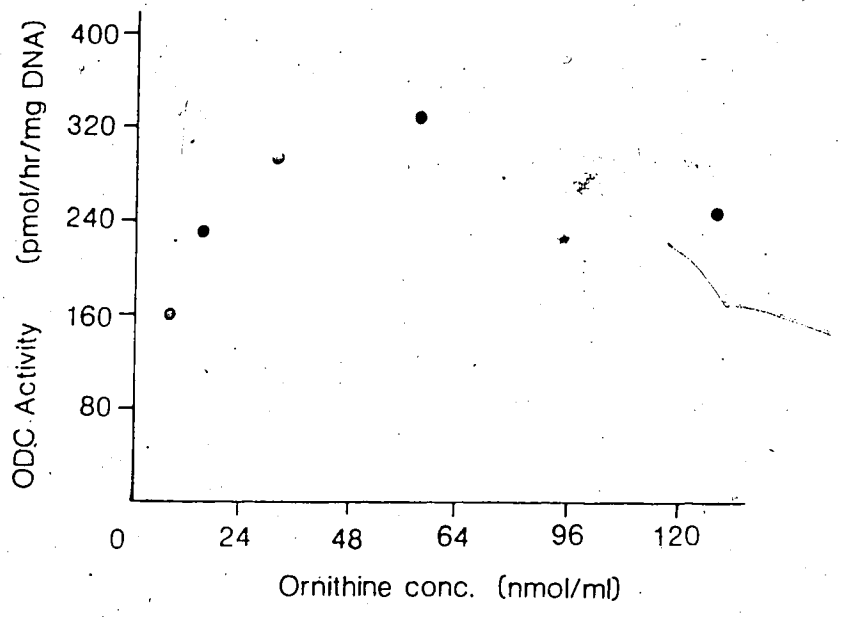
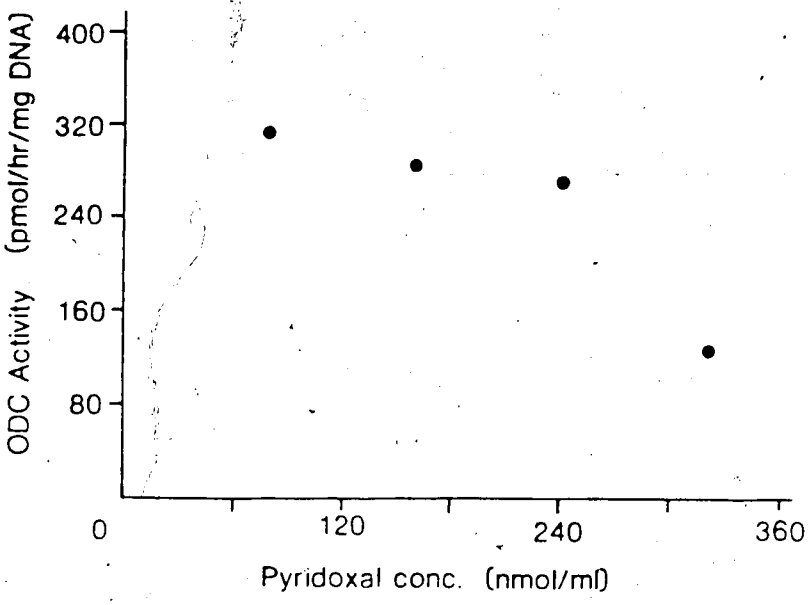


Figure 7.1 (Top) Micrograph of intestinal tissue after scraping for subsequent homogenization. Both crypt and villi are removed. Magnification, 500 X.

Figure 7.2 (Bottom) Effect of medium ornithine specific activity on ornithine decarboxylase (ODC) activity in a typical animal. ODC activity previously stimulated as outlined in Methods.



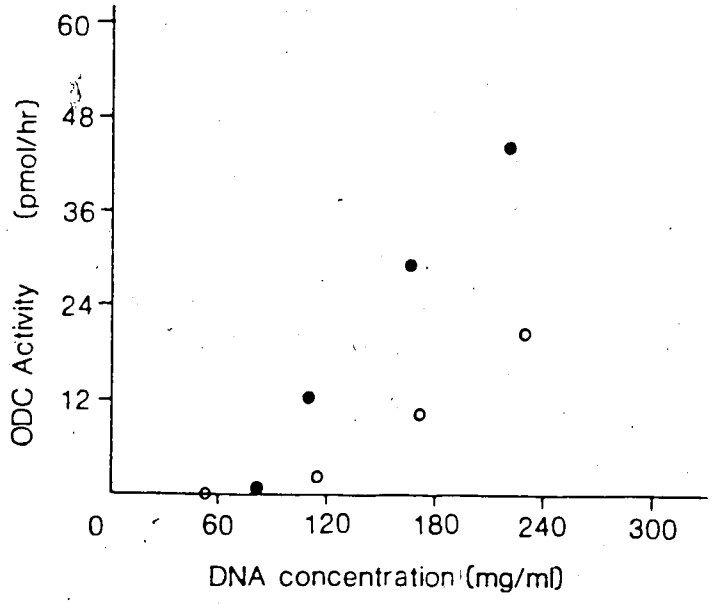
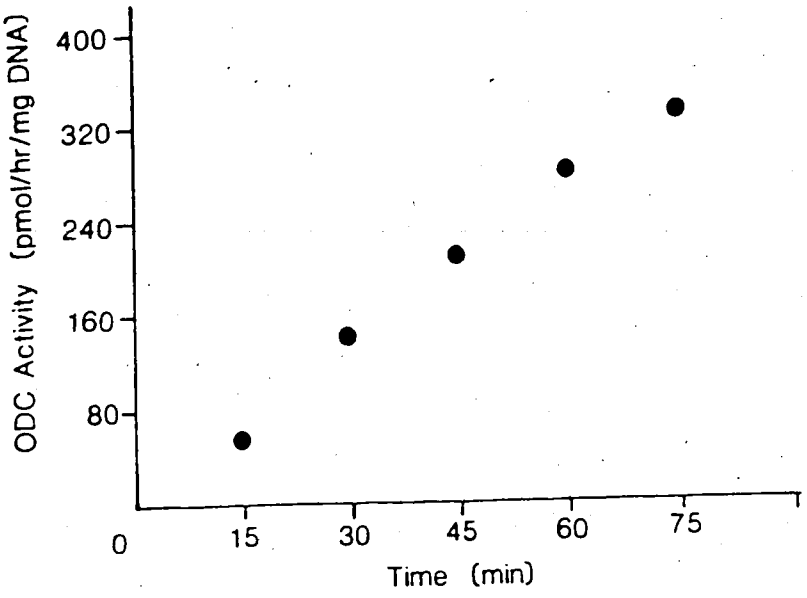


Figure 7.3 (Top) Effect of medium pyridoxal concentration on ornithine decarboxylase (ODC) activity in a typical animal. ODC activity previously stimulated as outlined in Methods.

Figure 7.4 (Bottom) Change in ornithine decarboxylase (ODC) activity with increasing DNA content. Results obtained by serial dilution of homogenate in a typical animal in which ODC activity had been stimulated as described in Methods.

with 200 µl of hyamine chloride. After incubation at 37° C for 60 minutes, the reaction was stopped by injecting 100 µl of 50% trichloroacetic acid into the vial. The reaction was linear with respect to time for the period studied (Figure 7.5). After a further fifteen minutes, the filters were removed and counted for 5 minutes in 5 ml of Aqueous Counting Scintillant (ACS, Amersham) in an LKB Rackbeta. Counting error averaged 1.7%. ODC activity was expressed in terms of CO_2 evolved (corresponding on a molar basis to putrescine produced) per hour per mg DNA.

DNA was assayed as previously described by Holt (16). 100 µl of the original homogenate was extracted in 0.5 ml of cold 0.8 M perchloric acid for 30 minutes, and centrifuged in a Beckman microfuge for two minutes. The pellet was washed with 0.5 ml of 0.4 M perchloric acid and recentrifuged, and washed again 0.5 ml of 0.4 M perchloric acid and hydrolyzed at 70° C for thirty minutes. After cooling, 0.5 ml of Burton's reagent was added and colour was allowed to develop overnight. Burton's reagent consisted of 100 ml glacial acetic acid and 1.5 ml H_5SO_4 (Fisher) with 1.5 g diphenylamine and 0.5 ml glutaraldehyde. Samples were read at 600 nm in a Phillips spectrophotometer and compared against a standard curve using calf thymus DNA as a standard. Unless otherwise noted, reagents were from Sigma. A typical standard curve is shown in Figure 7.6. The standard curve was linear over the range of sample DNA assayed, and had a Coefficient of Variation of 1.2%. The



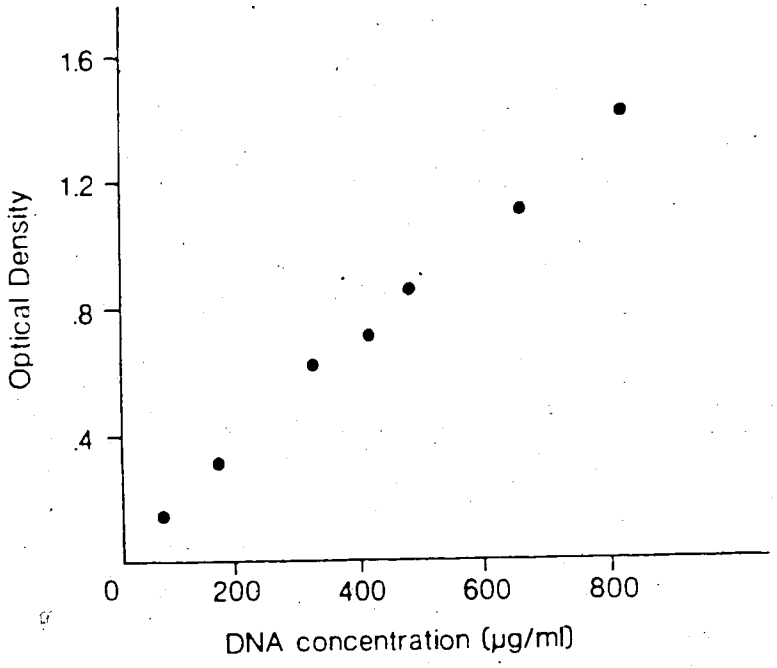


Figure 7.5 (Top) Change in ornithine decarboxylase (ODC) activity over time in a typical animal in which ODC activity had been stimulated.

Figure 7.6 (Bottom) Typical DNA standard curve, using calf thymus DNA.

standard curve was highly reproducible and curves constructed two years apart were superimposable.

C. RESULTS

555 mM D-glucose administered luminally elicited a substantial increase in ODC activity compared to fasted controls, whereas the same solution delivered intravenously failed to stimulate enzyme activity (Figure 7.7). D-galactose stimulated ODC activity to the same degree as D-glucose, whereas no difference from controls was observed when fructose was infused. At the lower substrate concentration (Figure 7.8), D-glucose was still able to stimulate ODC activity, although to a significantly lower degree than with the 555 mM solution. Enzyme activity was also elevated by 3-O-methylglucose and by L-glucose, but not by L-leucine. Preliminary experiments have indicated that the amino acids L-valine and L-lysine are also without effect in eliciting increased ODC activity. Enzyme induction by either L- or D-glucose was abolished by simultaneously infusing 2 mM phloridzin.

D. DISCUSSION

The results clearly indicate that ODC stimulation by refeeding after starvation is dependent on the administration of specific substrates. Further, these substrates must be present in the lumen, as intravenous D-glucose does not elicit the response that can be observed

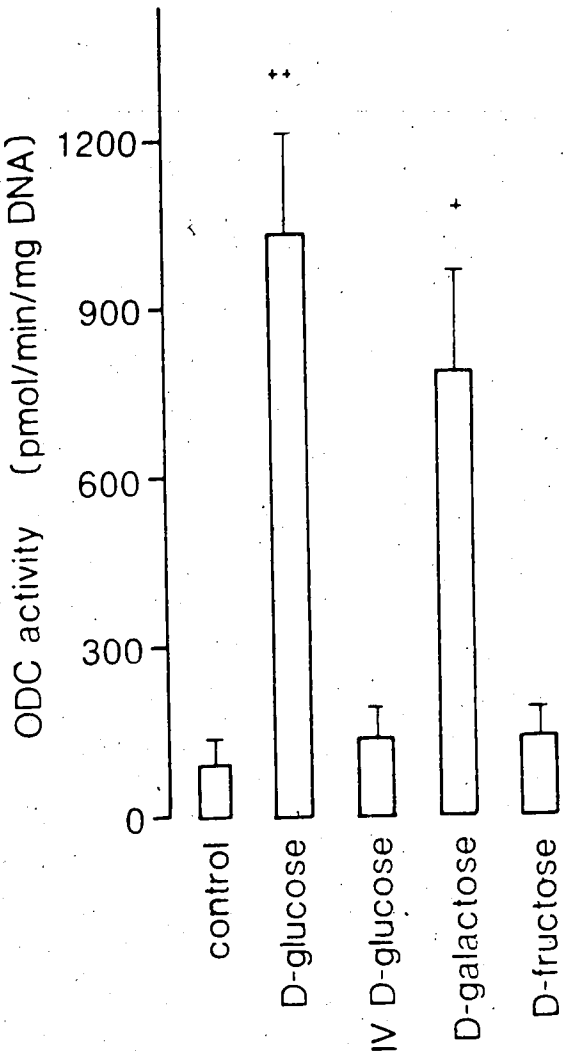


Figure 7.7 Intestinal ornithine decarboxylase (ODC) activity in control rats and in rats receiving an intestinal perfusion of 555 mM (10% w/v) D-glucose, D-galactose, D-fructose, or an intravenous infusion of 10% D-glucose. All rats had been previously fasted for 3 days. + = $p<.005$, ++ = $p<.001$ cf. control.

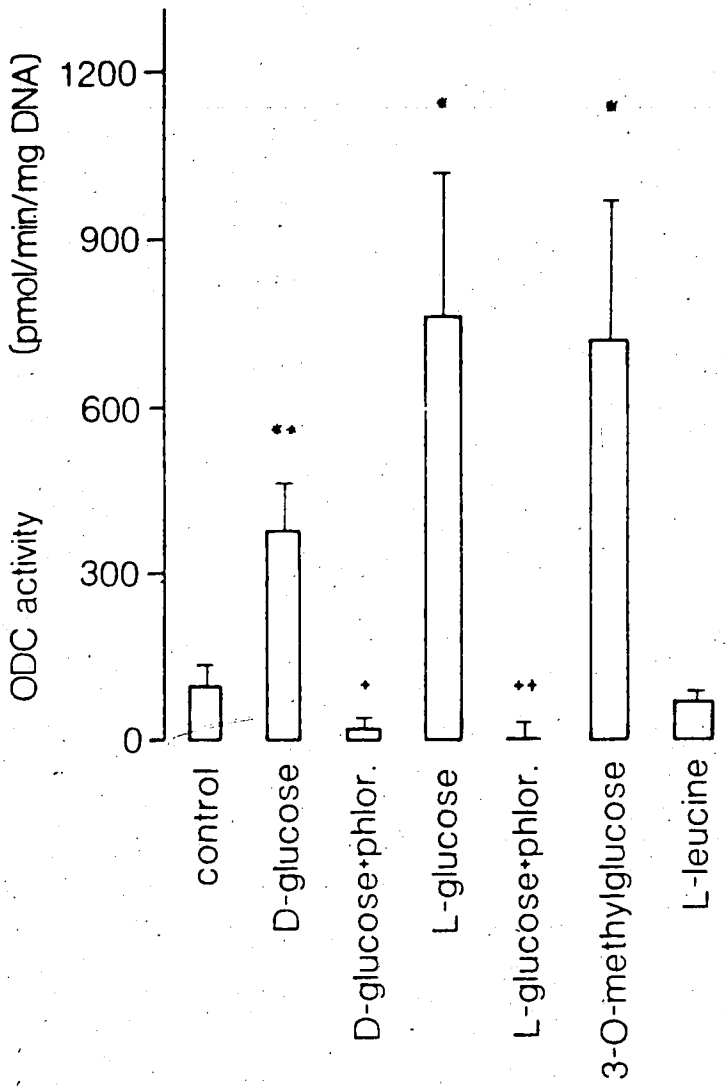


Figure 7.8 Intestinal ornithine decarboxylase (ODC) activity in control rats and in rats receiving an intestinal perfusion of 100 mM D-glucose, 3-O-methylglucose, D-glucose + 2mM phloridzin, L-glucose, L-glucose + 2mM phloridzin, or L-leucine. All rats had been previously fasted for 3 days. * = $p<.05$, ** = $p<.01$, + = $p<.005$, ++ = $p<.001$ cf. control.

even by luminally infusing a quantitatively smaller amount of D-glucose. The amount of glucose infused intravenously is sufficient to substantially increase the plasma insulin levels to over 400 μU/ml in rats (4), and it can therefore be stated that endogenous insulin release, at least over the time scale studied here, is not a factor in ODC induction. It has been noted that subcutaneous injection of insulin was able to elevate ODC levels (26). However, considering that the amount of insulin delivered (5 U/kg) probably resulted in substantial hypoglycemia in the rats tested in that report, a reflex catecholamine release was likely involved in those studies. As epinephrine is able to stimulate ODC activity (3, 25), this action of insulin is likely dependent on catechol mediation.

Most amino acids are actively transported by a sodium dependent mechanism (27), and their inability to induce ODC activity would therefore appear to rule out the possibility that sodium co-transport *per se*, or changes in potential difference serve as the mechanism of ODC stimulation. A previous report has indicated that no increase in ODC activity occurs when rats were fed a high protein diet despite a increase in villus height (32). Such a result would be expected on the basis of the present findings. Since such dietary manipulation usually occurs at the expense of carbohydrate content in the diet, the replacement of one nutrient capable of stimulating ODC activity with one that shows no such capability is unlikely to result in ODC

stimulation. The increase in villus height on such a diet must therefore be due to other factors. Such factors may not necessarily depend on increases in cell proliferation, as decreased cell migration and decreased enterocyte exfoliation could result in similar obervations.

D-galactose and 3-O-methylglucose both use the glucose carrier and are both actively transported against a concentration gradient (36), although they are not metabolized within the cell (5, 11). The ability of both sugars to stimulate enzyme activity cannot therefore be attributed to either the action of a glucose metabolite or to any enzyme in the metabolic pathway of glucose. Fructose, however, does not use the glucose carrier, and is not dependent on sodium as part of its uptake mechanism (10).

Since fructose, as well as the amino acid tested, was incapable of stimulating ODC activity, it would seem that the enzyme induction depends on substrates using the hexose transport system. However, the L-glucose stimulation of ODC levels provides a potential problem in this analysis. Central to this paradox is whether L-glucose enters the cell by a passive diffusion process, or if it is actively transported. Some investigators have reported that L-glucose uptake *in vitro* shows some sodium dependence, and that its transport can be partially inhibited by phloridzin (6, 8). Feeding patterns may themselves alter the properties of L-glucose transport, as accumulation of L-glucose against a concentration gradient has been reported using everted sacs

from rats which had been previously starved (28, 29). This uphill transport was not observed in fed animals, and it could be inhibited by D-glucose.

While the transport rate of L-glucose is only a fraction of that for D-glucose (8), the absolute amount of substrate transported may not be the rate limiting step for hexose stimulated ODC induction, since both D and L-glucose elicit a qualitatively similar rise in enzyme activity despite being transported at considerably different rates. Similarly, 3-O-methylglucose has a lower affinity for the glucose carrier (12), and is transported to a lesser degree than D-glucose, yet is equally competent in stimulating ODC activity when presented in equimolar concentrations at the luminal membrane surface. This is not due simply to the saturation of ODC stimulation by hexoses, as higher ODC levels could be obtained by infusing higher concentrations of D-glucose. The observation that D-glucose elicited the smallest increase in ODC activity of the sugars tested at the 100 mM concentration may even imply that the metabolism of the sugar may terminate its own ability to trigger ODC activity.

ODC stimulation by both L- or D-glucose could be inhibited by phloridzin, a specific inhibitor of the glucose transporter (10). Since phloridzin does not enter the cell (34), and does not therefore interfere with the stimulation process intracellularly, it appears that the binding of hexose to a membrane component is a critical part of this

process. It remains to be determined whether this component is the glucose carrier itself, which displays greater specificity for glucose transport than for triggering ODC action, or if a separate and less stereospecific hexose receptor is involved here.

The results obtained here are consistent with previous work with parenterally fed rats in which luminal nutrition was required to maintain optimal intestinal function (20), as well as parameters such as villus height. Previous investigators were puzzled by the fact that intestinal function could also be maintained by compounds such as 3-O-methylglucose which have no nutritional value (9, 35). The present study would appear to indicate that if ODC activity is connected with intestinal function and morphology, maintenance of these parameters may depend not on the nutritive value of the luminal contents, but rather on whether they have the proper structural configuration to induce ODC activity.

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VIII. General Discussion

A. Summary

In general, the overall purpose of these studies was to determine how the intestine responds to and recovers from radiation injury and what factors are required to trigger the repair process. The observations and conclusions listed below may be drawn from the data obtained.

Experiment 1 was designed to determine if some features of intestinal function were more affected by radiation damage than others. It was found that

1. Active transport of hexoses *in vivo* was decreased 3 days post-irradiation, but normal within a week, whereas active leucine uptake required more than 7 days to return to control levels. No change in the passive uptake of either L-glucose or D-leucine was observed.

2. Increased sensitivity to phloridzin, the specific inhibitor of active glucose transport, was observed 3 days after abdominal irradiation. Lower concentrations of this compound were required after damage in order to achieve similar inhibition of glucose transport to controls. This demonstrates that care must be taken in the choice of the appropriate dose of phloridzin to inhibit active glucose uptake. This is especially true where intestinal injury is likely to be present.

3. Intestinal ornithine decarboxylase activity is elevated for more than seven days after radiation exposure, while peroxidase activity is decreased for a similar length of time. Thus active transport, glucose stimulated water uptake, and villus height, all of which do not differ from controls 7 days post-irradiation, serve as poor indicators of recovery from intestinal injury, as other parameters of intestinal function remain altered for a longer period.

The purpose of Experiment 2 was to determine whether irradiation results in a change in cell migration rate on the villus. The data indicate that

1. Earlier appearance on the villus, no change in migration rate, and shorter villi 3 days after irradiation resulted in cells reaching the villus tip at a younger age. Thus, any enzyme or transport system which requires a fixed time or age to be expressed or activated, would likely be adversely affected by these changes.

2. 7 days after radiation treatment, the migration rate was increased despite the lack of significant difference in villus height from controls. It was suggested that this may represent increased cell loss balanced by an increase in cell regeneration.

The intent of the third experiment was to determine whether intestinal changes occurring after radiation injury could be

attributed to the restriction of food intake and to study the extent to which recovery was dependent on the activity of ODC. This experiment revealed that

1. In the absence of prior irradiation, tissue weight was depressed more by starvation or DMFO administration than by irradiation.

2. The peak in ODC activity induced by radiation treatment is prevented by starvation. Thus, irradiated animals must normally continue feeding, as the ODC peak would not otherwise occur. The induction of ODC activity therefore requires continued food intake.

3. Despite continued food intake, glucose uptake and villus height were reduced in the upper gut of animals administered DFMO and this coincided with negligible ODC activity. Normal intestinal function and morphology, at least in the upper intestine, therefore requires ODC activity. Further, glucose uptake and villus height remained reduced 7 days after irradiation when DFMO was administered. Thus, ODC is also required after injury for these parameters to return to normal.

4. ODC and peroxidase activity were inversely related in these experiments. Suppression of ODC activity by DFMO or starvation with or without prior radiation treatment increased peroxidase activity. Polyamines may therefore play a role *in vivo* in the control of

leukocyte infiltration of the small intestine.

To determine if the course of recovery from intestinal damage could be altered by pharmacological agents, Experiment 4 combined radiation treatment with the administration of several drugs.

1. PABA and allopurinol abolished the ODC peak in irradiated animals while indomethacin reduced the increase.

2. Aspirin and prednisolone caused an increase in ODC activity in unirradiated animals, but this increase was not additive with that seen after radiation treatment.

3. Aspirin administration decreased glucose and glucose-stimulated water uptake in intact animals and caused the formation of intestinal adhesions in irradiated animals. As this differed from the outcome when rats received either indomethacin or PABA, these effects cannot be attributed to either antiprostaglandin activity or weak acid effects. It was suggested that the detrimental aspects of aspirin treatment arose from its inhibition of ATP production and that this made the outcome of the radiation-induced injury more severe.

4. Allopurinol and indomethacin prevented the decreases in glucose and glucose-stimulated water uptake after abdominal irradiation. Thus, prostaglandin synthesis

and the production of free radicals by xanthine oxidase play a role in the transport changes observed with radiation injury.

Specific nutritional stimuli for ODC activity were investigated in Experiment 5 by perfusing rats starved for 3 days with specific substrates.

1. ODC activity was stimulated by luminal, but not intravenous glucose. Thus the enzyme response has a requirement for a specific sidedness of substrate entry.

2. Nutrient specificity was required for the stimulation of the enzyme as fructose or any amino acid tested were without effect.

3. ODC activity could be stimulated by the non-metabolized substrates 3-O-methylglucose and galactose, as well as substrates such as L-glucose which are not actively transported.

4. Stimulation of ODC could be abolished by phloridzin, suggesting the mediation of a cell surface event. Thus ODC is triggered either by the glucose carrier without requiring sodium co-transport or by a separate glucose receptor, which shows less stereospecificity than the carrier.

B. Intestinal Recovery after Radiation Injury

If ornithine decarboxylase induction is indicative of an underlying recovery or development process, repair is already underway in the intestine 3 days post-irradiation despite other overt signs of damage, namely decreased transport activity, reduced villus height, and lower peroxidase content. Further, this process continues for up to 14 days or more despite the return to normal of many of the parameters of function and morphology. Inhibition of ODC activity by DFMO administration for 7 days prevents the recovery of normal transport function and villus height remains depressed.

The importance of polyamines in the recovery process is suggested by the fact that many of the properties central to the repair process are displayed by polyamines. Polyamines can stimulate DNA and protein synthesis (54), stabilize the membrane against lysis (53) and may inhibit the formation of lipid peroxides in the membrane (33).

The data from these experiments allow some inferences to be made about the stimuli for ODC stimulation and the induction of other repair processes. Luminal nutrition is definitely one of possibly several factors which can trigger ODC induction. This can be seen in the elimination of the radiation-induced ODC peak by starvation. The inability of intravenous glucose to evoke ODC activity after starvation further suggests that this nutritional stimulus is specific to one side of the enterocyte. It can be argued that

starvation could result in a depletion of molecules essential for new cell production. While parenteral feeding has not prevented mucosal atrophy in normal animals (20, 30, 35, 62), future studies obviously could take a more definitive approach to this issue by determining if intravenous nutrient also delays the return of transport function and histological appearance after abdominal irradiation. It must be emphasized that delayed recovery does not necessarily mean delayed repair, and it will be suggested below that an excessive luminal workload may actually contribute to further damage.

It would appear that cell alkalinization is also a necessary pre-condition for ODC induction, as administration of the weak acid PABA prevents the increase in ODC activity expected after injury. The total impact of this inhibition is unknown. On the one hand, only the peak activity of the enzyme might be affected, allowing sufficient polyamine production to permit the gradual, although slower, return of function. On the other hand, changes in intracellular pH may be an obligatory first step in the triggering of cell proliferation. More extended treatment with PABA should be able to answer this. What intracellular events connect cell damage with intracellular pH changes, remain uncertain.

Another candidate for the mediation of ODC induction is the free radical content of the cell. The ability of allopurinol to eliminate the increase in ODC activity after injury implies that radicals produced by xanthine oxidase

could activate ODC activity after damage. As the radical "wave" produced by radiation is extremely brief (14), the continued generation of these molecules as a byproduct of ATP breakdown (23, 57) would serve to prolong the injury process. Only one pathway of ODC induction is likely inhibited by allopurinol, as preliminary studies not shown here indicate that the drug does not block ODC induction by luminal glucose after starvation.

No information is available from these experiments concerning the nature of the free radical(s) (i.e., superoxide, hydroxyl radical, or lipid peroxide), involved in this process. Xanthine oxidase could be generating radicals at a relatively slow rate and in this respect could resemble damage created by low radiation dose rates, which results in cell lethality from lipid peroxide-related membrane damage rather than by DNA damage (34). Since the quantity of radicals produced at low dose rates is small, they are more likely to interact with lipid molecules in the membrane than with other radicals in termination reactions. Once lipid peroxides are formed, conventional free radical scavengers may prove ineffective in their removal. Using a synthetic lipid system, Raleigh (55) noted that hydroxyl radical scavengers such as DMSO or mannitol were relatively ineffective in removing lipid peroxides, although alpha tocopherol was effective and could act synergistically with hydroxyl radical scavengers.

Some evidence for the direct activation of ODC by tissue damage comes from studies on tumour growth. Heby and Andersson (26) found that a constant increase in serum and tumour fluid polyamine content occurred in mice with Ehrlich ascites tumours, despite a decreasing rate of growth as the tumour occupied a larger mass. This rate decrease is a common phenomenon in tumours and results from cell death in the centre of the tumour as a result of poor vascularization. Those authors concluded that cell death was responsible for the unexpected ODC induction. The intracellular mechanism by which this occurs is largely speculative. Peroxide production could result in membrane disruption (34) and interference with enzyme activity in the cell (24). This in turn could increase the membrane permeability to calcium, which in turn can stimulate ODC activity (37, 49).

In light of claims of the cytoprotective properties of prostaglandins (36, 45, 56), the finding that indomethacin appeared to improve transport properties *in vivo* appears puzzling. It is possible that such action only reflects a temporary amelioration of the fluid secretion (6) and proteolysis (22) that may result from the radiation-induced release of prostaglandins (9). Indeed, within 24 hours of radiation exposure, there is an increase in NADPH ketoreductase, a glutathione dependent enzyme, and PGF levels rise as PGE concentrations decline (64). Unlike PGE, PGF is vasoconstrictive (16), and can block histamine

induced permeability (63), thereby contributing to fluid absorption (6).

Prostaglandins have been purported to exert a cytoprotective effect on gastrointestinal tissue by increasing the phospholipid composition of the membrane (38). Phospholipids such as lecithin have strohgly negative head groups, which could bind positively charged polyamines, and thereby adding to membrane stability. However, Keelan (21) found that membrane phospholipid concentration increased only in the ileum after irradiation of the rat intestine. The significance of this regional effect on overall intestinal repair is uncertain.

Gut hormones such as enteroglucagon are capable of producing intestinal hyperplasia (7) and intestinal growth in conditions such as lactation could be due in part to humoral agents. Enteroglucagon levels are elevated under these conditions (28), and Thiry-Vella loops in lactating animals show similar changes to segments of intestine exposed to luminal nutrition (17, 18). In contrast, humoral agents may not play as significant a role in repair as in adaptation. The release of a wide variety of gut hormones is depressed in celiac disease, and does not fully return until other signs of damage have disappeared after maintenance on a gluten-free diet (39). Enteroglucagon levels are elevated in celiac disease, but this appears to be a result of most of the disease occurring in the proximal bowel, whereas most enteroglucagon-secreting cells are located distally (8). In

the case of radiation injury, histological appearances would suggest that the distal gut is also affected by this kind of injury, and would therefore probably also involve damage to enteroglucagon cells.

Evidence from the cell migration rate studies suggest that cell death occurs at a greater rate than normal for at least 7 days after irradiation, coinciding with the duration of the elevation in ODC levels. At 3 days post-irradiation, the migration rate is unchanged from controls, yet villi are shorter. This combination implies cell loss, as villi would otherwise be of normal height. A smaller cohort of cells could be produced by the crypts due to a smaller stem cell pool, but this would only account for fewer or narrower villi, and not the premature loss of cells at the villus tips. By comparison, the migration rate at 7 days is increased, yet villus height is normal. Likewise, increased cell loss must be present here, for the villi would otherwise be expected to be taller.

What then causes this protracted loss of cells? Some of the damage may simply be the expression of delayed cell lethality arising from the original radiation dose. Puck (52) noted that lethally damaged cells could still proliferate for several generations before dying. Assuming a 12 hour hiatus in mitosis and earlier cell loss the first 3 days after irradiation, at least 4 to 5 generations of cells have been exfoliated by the seventh day. If cell division (which occurs in the crypts) is the point when defective

cells are most susceptible to lethal effects (25), the question still remains as to why a significant amount of cell death occurs on the villus tips.

The depression of DNA synthesis that results from irradiation can be mimicked by administering colchicine. This results in decreased brush border lactase activity less than eight hours after drug administration (27), which is too soon to be caused by the upward migration of defective cells. Curran (15) has suggested that this reflects a decrease in the synthesis of new transporter in the damaged villus cells. Indeed, new protein synthesis does appear to be required for villus integrity, as its inhibition by cycloheximide results in cell extrusion from the villus tips beginning as early as three hours after administration (3). Disruption of the sodium gradient may occur after irradiation, but the short circuit current (a measure of sodium co-transport) is unchanged in rabbits 50-200 hours following 20 Gy. of X-rays (44), implying that the sodium transporter is relatively rapidly repaired or replaced if it is damaged by this tr tment.

Other factors, however, could also account for the prolonged loss of cells which could be related to the elevation of xanthine oxidase activity. Evidence from the vascularly perfused kidney suggests that tissue damage could be dependent on the functional workload of the tissue. (Functional workload is defined here as the increased activity of cell processes requiring ATP, active transport

in the enterocyte being a particularly important example.) Treating the perfused kidney with the antibiotics nystatin or amphotericin resulted in an increase in membrane permeability and an increase in the number of necrotic cells (12). This was associated with more ATP consumption. When ouabain was used to inhibit metabolic activity, and hence ATP consumption, less necrosis occurred.

In the intestine, any change in permeability in the tissue can involve increased electrolyte transport to maintain fluid balance. Indeed, there is some suggestion from the osmotic p.d. studies, that the intestine is more permeable to fluid flux, at least 7 days post-irradiation. As blood supply may be decreased after radiation damage (19), energy supply may be insufficient to meet the demand and ATP breakdown by xanthine oxidase (42) could occur, generating free radicals, and causing further damage. Further decreasing ATP production (hence increasing adenosine breakdown) by agents such as aspirin, may therefore increase the damage produced by the original insult.

The generation of new cells to replace dying or defective cells is an important repair process, and its interruption is likely to have deleterious effects. Inhibition of cell proliferation by restraint stress in mice is reflected in decreased radiolabelled thymidine labelling of mitotic figures, and results in ulceration of the gastric mucosa (32). Protein synthesis, assessed by radioactive

leucine incorporation, remains normal, however. Starvation also results in a decrease in cell proliferation (1, 4), but no overt tissue damage other than hypoplasia was observed either with or without concurrent radiation treatment. Unlike restraint stress, however, starvation involves less demand on the intestine, and less transport activity is required. This may help explain some of the early studies on radiation mortality, where fewer animals survived treatment if they were force fed to make up for the reduction in food intake following irradiation (57).

If more damage occurs as the workload increases, the success of parenteral nutrition in damage syndromes such as Crohn's Disease (29) may be due to the decrease of the workload of the intestine, resulting in less damage and a better capacity for the intestine to repair itself. Some improvement has been reported with the use of elemental diets in experimental animals following irradiation (46), ischemia (10), or administration of the cytotoxic drug 5-fluorouracil (5-FU)(11). Such a diet is absorbed over a minimal length of intestine, and thereby serves to reduce the workload in much of the remaining gut (47). Gardner (21) has reported that such a diet did not prevent the decrease in water uptake or peptide hydrolase activity following 5-FU, yet this diet substantially reduced the amount of tissue necrosis observed (11). In the current studies, the combination of aspirin and irradiation produced no further decrease in transport activity, yet intestinal damage could

be observed on a macroscopic scale. This merely underscores the need of a more consistant definition for damage. Functional parameters alone may be an inadequate measure of injury.

Damaged tissue may be susceptible to bacterial invasion (60), and the release of oxidizing agents by neutrophils (5) may result in cell destruction which does not differentiate between invading bacteria and rapidly proliferating enterocytes. The ability of the products of polyamine metabolism to inhibit tissue infiltration by immune elements *in vivo* (2, 13, 48) may be important in limiting additional tissue destruction by the immune response. Polyamines can inhibit serotonin release (59) and prevent serotonin-mediated edema (50) demonstrating that mediators of the inflammatory response are controlled as well as the cellular elements.

C. Implications for Other Intestinal Injury States: Uremia

Uremia could offer an interesting perspective on the relevance of polyamines to the repair process in the intestine. When renal insufficiency results in a high concentration of urea in the circulation, the intestinal consequences include inflammatory infiltration and decreased activity of brush border enzymes such as alkaline phosphatase. Cell proliferation is reduced as a result of an increase in the length of the cell cycle time, especially of the S phase (40, 43). These changes are also associated with

epithelial erosions and bleeding.

While these effects are often attributed to the cytotoxic effects of ammonia produced from urea, the possibility that urea itself could be the mediator has never been conclusively excluded. Urea is one of the products of arginase, which also produces ornithine, the essential substrate for further polyamine synthesis. The possibility of substrate inhibition of arginase could provide insight into the intestinal symptoms observed with uremia. Polyamine depletion resulting from ornithine deficiency could result in decreased enterocyte proliferation and increased inflammatory infiltration in the intestine. Polyamine levels are actually elevated in the plasma with uremia (61), but this is likely due to inadequate excretion of the predominantly conjugated form of circulating polyamines. These circulating polyamine conjugates could be capable of growth suppression at the local level (2). DAO is also elevated in the serum, possibly suppressing growth in fast growing tissues such as the intestine. In addition, DAO activity is reduced in the kidney (61), which could reflect the lack of substrate at the local level. Thus the unavailability of polyamines to promote growth combine with the abundance of polyamine metabolites suppressing growth to prevent cell proliferation.

In vivo, Sterner and coworkers (58) found a decrease in amino acid uptake, but no change in dipeptide absorption in the uremic intestine, although only a single

concentration was tested. In contrast, no changes were noted *in vitro*. It is quite possible that the effects of uremia are rapidly reversible in a way that would affect *in vitro* results. Glucose uptake is reduced in isolated cells taken from uremic tissue immediately after harvesting, but subsequent washing of the tissue and further culturing results in a return of normal transport function (41). This could simply be a result of the removal of urea from the medium and a resumption in polyamine synthesis, which is capable of rapid induction after the removal of inhibitors. Differences between amino acid uptake and dipeptide uptake could arise from the sodium-dependent nature of amino acid transport, whereas dipeptide transport occurs by a sodium independent process. This would be an important factor if inhibition of polyamine synthesis affects only sodium dependent processes. Such explanations remain speculative, however, and await further information on the effects of polyamine depletion on normal intestinal tissue *in vivo*.

D. Conclusions

In summary, the experiments described here make several important statements about the nature of damage and recovery of the intestine after radiation exposure. Of greatest importance is that different components of intestinal function and morphology recover at dissimilar rates. The passive uptake of glucose and leucine *in vivo*, for instance, appears to be largely unaffected by the damage

process. By comparison, active glucose transport *in vivo* is depressed by radiation damage, and requires up to 7 days to return to normal, whereas normalization of active leucine transport may take up to two weeks. Permeability changes appear to have a lag in expression, as they do not appear until 7 days post irradiation, and disappear by the 14th day. Of these parameters, only active glucose transport can be correlated with the time course of the reduction and subsequent recovery of morphological parameters such as villus height. Further, the appearance of overt damage can occur in the absence of further reductions in transport *in vivo*, as seen after aspirin administration in irradiated animals.

Cell migration studies suggest that increased cell loss could still be present after injury even when histological features such as villus height have returned to normal. Ornithine decarboxylase activity in intestine could be a good indicator of cell proliferative activity, and requires two weeks or more to return to pre-irradiation levels. ODC activity is required for the maintenance of normal intestinal morphology and function, and inhibition of this enzyme delays recovery from injury. Luminal nutrition is required for induction of the intestinal ODC activity after injury, and this stimulus appears to be restricted to the action of hexoses. Passively transported and non-metabolized hexoses are also able to stimulate the enzyme, so the induction mechanism may not involve the

active transport process *per se*. In addition, ODC induction likely requires intracellular alkalinization to occur, as it can be inhibited by weak acids. Inhibition of the generation of free radicals by xanthine oxidase is also capable of inhibiting the induction of ODC by intestinal radiation damage. Hormones such as prednisolone and damaging agents such as aspirin are capable of stimulating ODC activity in normal animals, but do not appear to produce much change in the response of the enzyme to injury.

Polyamine production may play a role in the infiltration of the intestine by leukocytes, as changes in ODC and peroxidase activity often occur in opposite directions. This is particularly prominant when ODC induction has been inhibited by DFMO or by starvation.

Finally, prostaglandin release seems to be involved in the pathophysiology of intestinal radiation injury, as indomethacin administration improves fluid and glucose transport, at least in the short term. Continued production of free radicals by xanthine oxidase, well after the initial injury, could also contribute to the damage process, as its inhibition by allopurinol results in improvements in transport *in vivo*. Further investigation of the damage and repair processes suggested by these studies could result in the development of a more scientifically based approach to the treatment of intestinal damage.

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