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EFFECT OF VITAMIN A DEFICIENCY ON GLUCOSE UPTAKE

IN THE RAT

by

FADIL OENZIL


A thesis submitted for the degree of

Doctor of Philosophy

Section of Human Nutrition

Division of Biological and Health Sciences

DEAKIN UNIVERSITY

May, 1988
DEAKIN UNIVERSITY

CANDIDATE'S CERTIFICATE

I certify that the thesis entitled EFFECT OF VITAMIN A DEFICIENCY ON GLUCOSE UPTAKE IN THE RAT and submitted for the degree of Doctor of Philosophy is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

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Date 16 MAY 1988.
ACKNOWLEDGEMENTS

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SUMMARY

This thesis describes an investigation of the effects of vitamin A deficiency on gut function. The central hypothesis to be tested was that acute vitamin A deficiency affects glucose uptake from the small intestine. The hypothesis was tested using a system involving perfusion of isolated segments of the small intestine in the anaesthetized rat. The system was used to study effects on glucose uptake under steady-state conditions.

In the initial part of the study, experiments were diverted towards setting up the system for measuring steady-state uptake, and determining the relative contributions of active uptake and diffusion. Phenol red was found to be a reliable non-absorbable marker for determining net water movement. Phlorizin, generally at 1 mmol/L, was used as a competitive (reversible) inhibitor of active uptake. It is difficult however to confirm complete inhibition of active uptake by phlorizin because of the limited solubility of the inhibitor.

The kinetics of glucose uptake from intra-luminal maltose were found to be, in general, not significantly different from those applying to the uptake of glucose from an equivalent glucose solution. Maltase activity in the perfused gut segment was found to be sufficient to hydrolyse most of the maltose (80 per cent or more) in the solution being perfused, a much greater proportion than was absorbed. Glucose absorptive capacity, measured on an intestinal dry weight basis, was greatest in the duodenum and progressively less in the jejunum and ileum.

The rate of water uptake from the gut was increased by the presence of glucose in the lumen, and was linked to glucose uptake as shown by the inhibition of water uptake by phlorizin. Uptake of glucose by solvent drag was demonstrated by showing an increased rate of glucose uptake when the rate of water uptake was increased by perfusing a solution of reduced osmotic pressure. In the experiment a low intra-luminal glucose concentration was used to preclude net uptake by diffusion and active uptake was blocked with phlorizin.
This process was further investigated using streptozotocin-diabetic rats in which the diabetes establishes a hyperosmotic blood with hypoglycaemia. Uptake by solvent drag was more obvious in diabetic animals. A back-diffusion (exsorption) of glucose from the tissues to the lumen was also shown; the rate being proportional to plasma glucose concentration.

Vitamin A deficiency was established in weanling rats after 6 - 7 weeks feeding on a diet based on wheat starch, coconut oil, and casein washed with hot ethanol, together with vitamins and minerals. The vitamin A deficiency led to classic eye signs and was reversed by the addition to the diet of retinoic acid (5 μg/g diet). Vitamin A deficiency decreased intestinal mucus production (dry weight) but had no detectable effect on the histology of the villous epithelium as shown under the light microscope. Using perfusion experiments it was shown that vitamin A deficiency had no significant effect on the rate of active uptake of glucose, but that deficiency increased the rate of passive uptake.
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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Vitamin A deficiency is common in many developing countries (Appendix 1). The occurrence and effects of vitamin A deficiency in these countries are related to deficiencies of other nutrients and to the effects of infection and infestation. Diets are frequently low in fat, diarrhoea may be frequent and malabsorption is common. The interaction of protein-energy malnutrition and reduced resistance to infection is now fairly well understood. Deficiencies of zinc, iron, calcium, copper, vitamin B6, iodine and other micronutrients may occur as consequences of poor diet or as a result of malabsorption. Microbial toxins such as aflatoxin in the diet may have further adverse effects on organ and body function.

In developing countries malnutrition is a major cause of death, particularly in the post-weaning to toddler age group. Children not obviously affected by deficiency disease may have a marked degree of nutritional dwarfing. There is considerable difficulty in studying the physiological effects of vitamin deficiency in situations where complex interactions exist. To understand the contributions of the various factors to the total picture it is necessary to isolate as far as possible the separate parts of the various physiological systems involved for closer study.

Deficiency of vitamin A affects a range of organ systems; most notably the processes of vision, growth, reproduction, immunological defence and maintenance of epithelial tissue, especially in the eyes, lungs and gut. It is clear that the mortality rate is high among children affected by vitamin A deficiency, and it is desirable therefore that we should gain as full an understanding as possible of the mechanisms by which vitamin A deficiency adversely affects nutritional and total health status.
As noted above, vitamin A has a role in the maintenance of mucus-secreting epithelial tissues located in the eyes, lungs, gut and reproductive tract. Satisfactory nutritional status is dependent on normal gut function and, whereas malabsorption probably contributes to vitamin A deficiency, it may also be true that vitamin A deficiency can adversely affect the gut and worsen malabsorption. In the studies to be described in this thesis I have set out to test the hypothesis that vitamin A deficiency adversely affects the absorptive capacity of the gut.

The experimental system chosen for testing the hypothesis was measurement of the glucose uptake capacity of perfused segments of the small intestine of the rat. The rat was chosen because of its convenience as a laboratory animal and because a considerable amount is known about glucose transport in this species. The glucose transport system was chosen for study because of its central position in the maintenance of energy balance. In terms of quantity, the principal macronutrient in the diet is usually starch, which after digestion, is absorbed from the small intestine as glucose. Sucrose, also a major energy source, is taken up as glucose and fructose.

Much of the research carried out on glucose transport has used preparations of isolated tissues, and many studies have been concerned with initial rate kinetics. In this study I have examined the effect of vitamin A deficiency on the rate of glucose uptake under steady-state conditions. It was necessary therefore, first to characterise the uptake processes operating under steady-state conditions in normal rats before studying those processes in vitamin A-deficient animals.

1.2 MORPHOLOGY AND FUNCTION IN THE SMALL INTESTINE

An important aspect of the structure of the small intestine is the special means of increasing the epithelial surface area for contact with the intestinal contents. The plicae circulares (circular folds), the villi and the microvilli (brush border) enable an increase of surface area of approximately six hundred-fold (Wilson
1962). The structures and the increase in surface area are illustrated in Figure 1.1. The plicae circulares are folds of submucosa covered by a full thickness of mucosa. The plicae are more prominent in the distal duodenum and proximal jejunum and become less frequent in the ileum.

Numerous microscopic mucosal villi increase the absorptive surface area. Depending on the mammalian species and the level of small intestine examined, villus height and shape may vary. For example, in humans, the villi of the distal duodenum and proximal jejunum may be leaf or finger-shaped and range from 0.5 to 0.8 mm in height, whereas the villi of the ileum rarely exceed 0.5 mm in height and are finger-shaped. In the rat, Dowling and Booth (1967) found that the mean height of the villi in the jejunum was 0.32 mm compared with a mean height of 0.23 mm in the ileum.

Verzar and McDougall (1967) calculated that the increase in absorptive surface area due to the presence of villi was 8-fold in rat. Tasman-Jones et al. (1982) have shown that the composition of the diet, in particular the amount of fibre in the diet, alters villous morphology and therefore affects surface area.

More recently, Keelan et al. (1987) observed that variation in the carbohydrate, essential fatty acid, cholesterol, or protein content of the diet influenced villus height in both control and diabetic animals. Villus height was greater in control rats with low carbohydrate and low protein diets. The greatest villus height was observed in diabetic animals, after feeding a diet high in essential fatty acids and cholesterol, and low in protein.

Peyers patches, patches of lymph tissue, are found along the longitudinal axis of the small intestine on the antimesenteric border. These are located in the submucosa and extend into the mucosa, where they have direct contact with the overlying epithelium (Owen and Jones 1974). At the level of the duodenum, the plicae and the villi are often distorted by small lymphoid nodules, acinar glands, and Brunner's glands. Brunner's glands are located in the
Figure 1.1

Diagram illustrating three mechanisms by which the surface area of the small intestine is increased (Adapted from Wilson 1962).
submucosa from whence they empty their bicarbonate and glycoprotein-rich secretion into the gut lumen through short ducts that terminate at the base of the mucosal crypts. These glands are responsible for the thick coating of mucus which covers the mucosa. Brunner’s glands contain both serous and mucous cells and there appears to be substantial species variation in the structural features of their constituent cells (Fried 1965). However, at the level of the jejunum and ileum, the epithelium is also covered by a layer of mucus which is secreted by goblet cells present in the crypts as well as on the villi. The number of goblet cells in the villi increases progressively from the duodenum down to the end of the ileum and the pH of the mucus also increases towards the ileum (Gilles-Baillien 1983).

The intestinal mucosa can be divided into three layers (Figure 1.2), the muscularis mucosa, the lamina propria and the epithelial cells. The muscularis mucosa is a continuous thin sheet of smooth muscle, three to ten cells thick. Its function is possibly the movement of villi to modulate the unstirred layer adjacent to the absorptive epithelium and to facilitate emptying of the crypt luminal contents by causing luminal compression.

The lamina propria is a continuous connective tissue layer. It forms the core of the villi and surrounds the crypt epithelium. In the lamina propria of mammals there is an abundance of plasma cells, lymphocytes and macrophages, which have important immunologic functions. Also present are eosinophils, mast cells, fibroblasts, small unmyelinated nerve fibres, blood and lymph vessels, and smooth muscle cells.

The villi are covered with a capillary network which carries blood close to the surface. The blood capillaries are composed of endothelial cells that are surrounded by a continuous basal lamina as well as a variable number of pericytes. The endothelial cells have thin walls which contain many diaphragm-covered fenestrations 0.05 to 0.1 µm in diameter.
Figure 1.2

Schematic diagram showing two villi and a crypt in section, illustrating the histological organization of the mucosa of the small intestine (Reproduced from Madara and Trier 1987).
In the capillaries located close to the epithelium the fenestrations tend to face the basal surface of epithelial cells. There is evidence that the diaphragms of the endothelial fenestrae contain the structural equivalents of the small pore system, while the large pore system seems to be restricted to a fraction of the fenestrated population which presumably consists of diaphragm-free or diaphragm-deficient units (Clementi and Palade 1969).

The lymphatics of the lamina propria are centrally located. The lymphatic cells tend to be irregular in shape, consisting almost exclusively of endothelial cells without pericytes. The endothelial cells of the mucosal lymphatics have no fenestrations, their walls are thicker but contain cavoli and vesicles. The basement membrane applied to the external endothelial surface is discontinuous (Dobbins 1966).

Although, when viewed in sections, many connections between adjacent lymphatic endothelial cells appear sealed by tight junctions, gaps of sufficient size to permit the passage of macromolecules and even chylomicra (diameters from 0.1 to 1 μm) are present between some adjacent lymphatic endothelial cells (Casley-Smith 1962, Dobbins 1966, Rubin 1966).

The epithelial cell:
The villi are covered with a continuous sheet of epithelial cells, one cell thick, and the cells surround the crypts. The epithelium is separated from the underlying lamina propria by a thin continuous basement membrane composed of two parts;
(a) a basal lamina that is beyond the resolution of the light microscope and which is in direct contact with the basal surface of the epithelium, and
(b) a deeper layer of reticular fibres and glycoprotein connective tissue, a ground substance that adheres to the lamina propria side of the basal lamina.

The crypt epithelium is formed by;
(a) undifferentiated cells which are actively proliferating and may be seen in mitosis,
(b) mucus-secreting goblet cells,
(c) a few endocrine epithelial cells,
(d) rare caveolated cells,
(e) in many species, particularly in the ileum, occasional cup cells.

Additionally, a specialized epithelial cell, the M (microfold) cell, overlies the apex of the Peyer's patches in the ileum (Owen and Jones 1974).

Intestinal absorptive cells:
The structure of the typical absorbing cell of the mammalian small intestine is shown in Figure 1.3, which illustrates schematically many of the structures that can be seen in a transmission electron micrograph.

The luminal border of the cell is lined with finger-like projections (microvilli), approximately 0.5 to 1.5 μm in length, and 0.1 μm in width, depending on the mammalian species studied. Brown (1962) reported that the microvilli were short, wide and relatively few in number in the crypts of the human jejunum. In the intervillous area and on the villous crest, the microvilli become progressively higher, thinner, and more numerous. Palade and Karlin (1959) calculated that the microvilli of the intestinal villi in the rat, occupy approximately 60 per cent of the free surface of the cell and increase the free surface by a factor of 24.

Other research workers have found that the surface coat or glycocalyx is particularly well developed on the microvillous surface of the mammalian small intestine (Ito 1964, Trier 1969). In transmission electron micrographs, the surface coat has been shown to be attached directly to the outer leaflet of the microvillus membrane (Figure 1.4).

Whether the glycocalyx on the apical surface of absorptive cells, alone or in combination with adherent mucus from goblet cells, serves a protective function remains to be firmly established.
Figure 1.3

Schematic diagram of an intestinal absorptive cell
(Reproduced from Madara and Trier 1987).
Figure 1.4

Schematic diagram of the structural features of the apical plasma membrane, cytoplasm and the junctional complex of the intestinal absorptive cell (Reproduced from Madara and Trier 1987).
1.3 ABSORPTION OF SUGARS

There have been many studies of glucose absorption from the gut, but the majority of these have been of the process of glucose uptake in vitro into small rings of everted intestine (Crane and Crane 1956, Alvarado and Crane 1962, Alvarado 1967, Ferraris and Diamond 1986b), or glucose transport via brushborder and basolateral membranes or vesicles (Hopfer et al. 1973, 1976, Murer et al. 1974, Biber et al. 1983, Dorando and Crane 1984, Bluest et al. 1986) or both methods (Doubek and Ambrecht 1987).

Historically, intestinal transport processes were first approached by studies performed in loops in vivo and in situ but attention later shifted to in vitro techniques. In order to test the effects of vitamin A deficiency on glucose uptake in the present study, it was decided that the best overall view would be gained by measuring rates of uptake under steady-state conditions in vivo in the whole animal. The advantage of the in vivo method is to examine the whole process of absorption as it typically operates under physiological conditions.

1.3.1 Glucose Uptake into the Blood

Glucose is taken up from the gut into the blood by a complex process. Uptake involves transfer of glucose from the gut into the epithelial cells of the villi, followed by transfer of the glucose across the serosal membrane into the extracellular fluid and subsequent transfer into the blood capillaries. During steady-state uptake at relatively high concentrations of glucose in the lumen, the rate of glucose release from the serosal side of the epithelial cells is approximately equal to the rate of uptake, since the rate of metabolism of glucose is small in proportion to the total flux.

There are at least two and probably three processes by which glucose is absorbed from the gut lumen across the brush border into the epithelial cells (Oenzil and Read 1986). These include active (stereospecific, energy-dependent) uptake, diffusion (Figure 1.5) and uptake by solvent drag. The relative importance of these
Figure 1.5

Hypothetical figure showing kinetics of active and net diffusional uptake of glucose in the jejunum (Based on data from Vinardell and Bolufer 1984).
processes varies according to glucose concentration and location in the gut. Glucose exit from the epithelial cells to the portal blood across the basolateral membrane occurs by simple diffusion plus facilitated diffusion (Hopfer et al. 1976, Murer et al. 1974, Wright et al. 1980). The degree of vasodilation of the splanchnic vascular bed probably also influences the rate of uptake, but this aspect has received little study as yet.

1.3.2 Absorption Against a Concentration Gradient

In the overall process of uptake from the gut, glucose can be actively concentrated against a concentration gradient using metabolic energy in the form of ATP. Gradients up to eight-fold are measured in fresh tissue (Kümich 1970). Net uptake of glucose by diffusion also occurs if the concentration in the lumen is higher than that in the blood. Investigations of the mechanism of uptake have been designed to study separately the transport processes at the mucosal and serosal membranes of the epithelial cells. Details of the mechanisms of uptake have been obtained from studies on cells and tissues from the mouse, rat and chicken as well as tumour cell lines. The most detailed information has been obtained from studies on chicken gut epithelial cells and current hypotheses are based substantially on those studies (Kümich and Randles 1975a, 1975b). There is some, though limited, evidence that there may be differences between the kinetics of transport in the chicken and the rat (Kümich 1981).

Investigations of the mechanisms of uptake of glucose have been designed to study separately the transport processes at the mucosal and serosal membranes of the epithelial cells. At the mucosal or brush border membrane both active transport and passive diffusion occur (Alvarado and Crane 1964). The active transport process, located in the mucosal membrane, drives active uptake as a whole, while transport of glucose across the serosal membrane is driven by the downhill gradient of glucose concentration from the cells to the sub-epithelial tissues. Active transport appears to involve a protein carrier with specificity for glucose, galactose and a limited range of other sugars (Crane 1960).
The driving force for active uptake appears to be the electrochemical gradient of sodium ions (Na\(^+\)) established across the brush border membrane (Crane and Krane 1956, Gibb and Eddy 1972, Kimmich 1981). The gradient is established by the activity of the ATP driven Na\(^+\)-K\(^+\) pump located on the lateral serosal membrane of the columnar epithelial cells. Na\(^+\) is extruded from the cell and leaks through the tight junctions between cells to form the gradient between the luminal surface and interior of the cell. Transport across the membrane involves a carrier with binding sites for sodium ions and for glucose. The glucose binding site can also bind and transport galactose and a limited range of other sugars. The carrier, which possibly carries a negative charge, binds glucose together with two sodium ions to form a complex which then has a positive charge (Kimmich and Randles 1980). The complex with its positive charge is driven to the inner face of the membrane by the potential difference existing across the membrane. At the inner surface the complex dissociates, transferring glucose and sodium to the interior of the cell. The carrier, having a negative charge is driven back to the luminal surface by the potential difference and the process repeated. The hypothetical mechanism is described in Figure 1.6 (Kimmich and Carter-Su 1978).

In recent papers Restrepo and Kimmich (1985a, 1985b) have described the simplest mechanism as an ordered ter-ter model with a binding order (Na\(^+\)+glucose+Na\(^+\)) in which carrier translocation across the membrane is the rate-limiting step (Figure 1.7).

Glucose is concentrated within the epithelial cells and subsequently crosses the serosal membrane to the extracellular fluid. At least in the chicken there appear to be three processes involved in transfer across the serosal membrane, none of these processes is energy dependent. These include a sodium-dependent process, a sodium-independent process and diffusion (Kimmich 1977, Kimmich and Randles 1976). Kimmich and Randles (1979) calculated that for intestinal epithelial cells of the chicken which had accumulated 3-0-methyl glucose to a steady state in a 1 mmol/L solution, the contribution of the three processes to transfer across the serosal membrane would be 50 per cent via the Na\(^+\)-independent process, 30
Figure 1.6

Hypothetical scheme illustrating the mechanism of transport of glucose and sodium across the villous epithelial membrane. Driving force is provided by the transmembrane potential difference acting on a charged carrier molecule.
Hypothetical scheme showing an ordered ter-ter model with an order N:S:N for the binding of substrates to a carrier, and subsequent transport to the inner surface of the villous epithelial membrane. Translocation of carrier (E) across the membrane is rate-limiting. This is stated to be the simplest mechanism that is compatible with observed kinetics of sugar fluxes; $N_0$ and $N_1$ represent extra-cellular and intra-cellular sodium respectively, and $S_0$ and $S_1$, extra-cellular and intra-cellular sugar respectively (Reproduced from Rostrepo and Kimich 1985a, 1985b).
per cent by reverse transfer on the Na⁺-dependent process and 20 per cent by diffusion. The relative contributions of these pathways in the rat is unknown and probably depends on intra-cellular concentration and hence on luminal concentration.

Uptake processes in which a carrier is involved have kinetic characteristics similar to those of enzymes. A maximum velocity of transport \( V_{max} \) can be determined as well as a \( K_m \); \( K_m \) being the substrate concentration giving half maximum transport. For the overall uptake process however, kinetic analysis is complicated because several transport processes are involved. During steady state uptake, the relative contribution of each process to total flux will vary with substrate concentration. Flux across the mucosal membrane is also affected by both the sodium gradient and the transmembrane voltage. Other potential substrates in the medium, for example amino acids, which can affect the sodium gradient or transmembrane voltage, can also affect the rate of sugar uptake.

1.3.3 Glucose Uptake by Diffusion

Little is known of the mechanism of glucose uptake by diffusion. Substances are absorbed passively depending on concentration gradients and the permeability of the intestine for that substance. The process is apparently two-way, with movement of molecules from plasma to gut lumen, as well as from lumen to plasma.

Alvarado and Crane (1964) observed that about 80 per cent of glucose uptake by evicted sacs is inhibited by phlorizin and the residual uptake is non-concentrative and shows no evidence of saturability. The characteristics of residual uptake observed are those of a diffusion process, and in the following discussion this process is referred to as diffusion although the nature of the process is not yet clear. Since active uptake is saturable, it would be expected that the proportion of uptake accounted for by diffusion would increase with substrate concentration, as substrate concentration approaches \( V_{max} \) (Warden et al. 1980). Net uptake by diffusion will become zero as substrate concentration in the gut lumen is reduced.
to the level normally present in tissue fluids. It has been suggested that following a meal the predominant mechanism of glucose and amino acid absorption in the more permeable jejunum is passive. Murakami et al. (1977) suggested that the diffusive pathway has greatest quantitative importance in the intestinal absorption of glucose in rat jejunum after a meal which establishes an intraluminal glucose concentration of 50 mmol/L.

1.3.4 Glucose Uptake by Solvent Drag

When net water absorption occurs, passively absorbed sugars may be carried inwards by solvent drag. It is possible that glucose also enters the villous tissue by 'solvent drag' as water is drawn through the tight junctions to the hyperosmotic solution in the lateral intercellular spaces (Csaky and Autenrieth 1975, Vinardell and Boulfer 1983).

1.3.5 Inhibition of Transport by Phlorizin

Inhibition of glucose uptake by the glycoside, phlorizin was observed very early (Nakazawa 1922) and this inhibitor has frequently been used for studies of the intestinal absorption of sugars (Alvarado and Crane 1962, Diedrich 1966, 1968, Ponz and Lluch 1955). Phlorizin is effective at low concentration at the luminal surface of the gut epithelial cell but does not penetrate the brush border membrane (Crane 1968, Stirling et al. 1972). It is a competitive inhibitor of the active transport of glucose, thus inhibition is reversible and varies with substrate concentration (Alvarado and Crane 1962, Rodriguez et al. 1982).

Alvarado and Crane (1962) in their experiments, showed competition between phlorizin and glucose for the glucose-specific site on a carrier in the brush border. Phlorizin is believed to interact with the D-glucose transport protein at two distinct sites; a sugar binding-site, which is involved in the translocation of monosaccharides and an aglycone-binding site, which is hydrophobic (Diedrich et al. 1975). Phlorizin is slowly hydrolysed at the
mucosal membrane and the glucose residue transported into the cell (Wardon et al. 1980).

Phlorizin, formed by the hydrolysis of phlorizin, is also an inhibitor of active transport, but is slow acting, non-competitive and less than four per cent as effective as phlorizin (Alvarado 1970, Diodrich 1966). Blum et al. (1975) showed that phlorizin inhibits glucose uptake even in lactase-deficient human subjects and, since the lactase protein carries phlorizin hydrolyase activity, phloretin cannot be the principal inhibitor of glucose transport because it is not formed in lactase deficiency.

1.4 FACTORS AFFECTING GLUCOSE ABSORPTION

1.4.1 The Unstirred Water Layer and the Role of Mucus

The exact relevance of the mucous layer to intestinal transport processes is far from established. Its role is apparently more important than just to maintain an unstirred water layer on the mucosal surface of the epithelium. The solute concentration adjacent to a membrane differs from its bulk solution value as a result of intervening unstirred layers.

Dainty (1963) concluded that in any situation in which a biological membrane or cell surface is immersed in a solution, there exist concentric layers of water, extending out from the aqueous-lipid interface, that are not in equilibrium with the remainder of the bulk water phase, and that these constitute a relatively 'unstirred' water layer, through which solute molecules must move by simple diffusion.

As molecules move from the bulk water phase into the cell, they meet two major resistances; the unstirred water layer and the lipid layer of the cell membrane (Figure 1.8). The rate of diffusion (J) of a molecule across the unstirred water layer will be determined by its free diffusion coefficient constant (D), its concentration in the bulk water phase (C₁), and the thickness of the unstirred layer (d) according to formula;
Diagrammatic representation of the unstimred water layer adjacent to the cell membrane of the intestinal absorptive cell, where:

- $C_1$ = concentration in the bulk water phase;
- $C_2$ = concentration at the aqueous-lipid interface;
- $C_3$ = concentration just inside the cell membrane;
- $d$ = thickness of the unstimred layer.

(Reproduced from Dietschy et al. 1971).
\[ J = \frac{D}{d} (C_1 - C_2) \]  

(Dietschy 1973).

For a passively absorbed molecule, the rate of movement across the lipid cell membrane, in turn, will be determined by the permeability coefficient for the molecule \( P \) and its concentration just inside the cell membrane \( C_3 \) as given by the formula:

\[ P (C_2 - C_3) \]  

During passive uptake, flux of the molecules across the unstirred layer must equal that across the cell membrane so that the rate of tissue uptake, \( J \) is given by the relationship:

\[ J = \frac{D}{d} (C_1 - C_2) = P (C_2 - C_3) \]  

It should be noted that the unstirred water layer and the cell wall may be considered as two membranes in series where the permeability of the latter is denoted by term \( P \).

There are two limiting situations that may be encountered during passive absorption across biological membranes. If \( D/d \) is very small relative to \( P \), then diffusion across the unstirred water layer becomes primarily rate-limiting. In this case \( C_2 \) approximately equals \( C_3 \) so that the rate of absorption into the cell will be given by the formula:

\[ J = \frac{(D/d)}{(C_1 - C_3)} \]  

If the value of \( D/d \) is very large relative to \( P \), the cell membrane penetration will be primarily rate-limiting. Since, in this case, the value of \( C_2 \) will be nearly equal to that of \( C_1 \), the rate of absorption into the cell will be given by the expression:

\[ J = P (C_1 - C_3) \]
For a molecule that is absorbed into the cell predominantly by a carrier-mediated process, as the concentration of $C_2$ at the cell membrane rises, the quantity transported per unit time rises along a hyperbolic curve until a maximum rate of transport is reached and will equal:

$$J = \frac{(V'_{\text{max}})(C_2)}{K'_m + C_2}$$  \hspace{1cm} (6)

$V'_{\text{max}}$ is the apparent maximal transport velocity, and $K'_m$ is the apparent Michaelis-Menten constant for the carrier-mediated process. In this circumstance, the rate of absorption ($J$) of a molecule from the bulk water phase into the cell is given by the expression

$$J = \frac{(D/d)(C_1 - C_2)}{K'_m + C_2}$$  \hspace{1cm} (7)

with diffusion and carrier-mediated transport in series. Again, whether the unstirred layer or the carrier-mediated cell membrane penetration step primarily is rate-limiting to overall absorption will depend upon the value of $D/d$ relative to $V'_{\text{max}}$ and $K'_m$ for a particular solute molecule.

Dietschy and Westergaard (1975) have noted that in several flat membranes examined, the unstirred layer varied from approximately 90 to 400 $\mu$m. However, in vivo, the unstirred layer may be much thicker due to, among other things, the presence of mucus.

The thickness of the unstirred water layer ($d$) can be determined directly by measuring the time necessary to reach half the steady-state potential difference generated by an osmotic or electrolyte gradient across the small intestine (Diamond 1966). In everted rat jejunum, the effective thickness of the mucosal unstirred layer can be reduced by vigorous stirring, from $198 \pm 5$ $\mu$m to $141 \pm 4$ $\mu$m, and in the ileum, from $217 \pm 5$ $\mu$m to $159 \pm 5$ $\mu$m (Wilson and Dietschy 1974). In rabbit jejunum it varies in thickness from 334 to 115 $\mu$m. As the stirring rate was changed from 0 to 1200 rpm, the total thickness reached a minimum of 250 $\mu$m at 1200 rpm (Westergaard and Dietschy 1972, 1974). Lukie et al. (1974) found that, at a stirring
rate of 500 r.p.m., the mean thickness of the diffusion barrier in rabbit jejunum was approximately 150 \( \mu \text{m} \).

Winne (1976) found the effective unstirred layer thickness in rat jejunum perfused in vitro to be at least 530 \( \mu \text{m} \). The apparent unstirred layer thickness in the healthy human jejunum has been found to be \( 632 \pm 24 \mu \text{m} \) (mean \( \pm \) SEM, Read et al. 1976, 1977). For the human intestine, the unstirred layer was calculated to be \( 632 \mu \text{m} \) thick in the review by Davenport (1982).

Recently, Sparso et al. (1984) measured the thickness of the unstirred water layer in the normal upper duodenum in 11 healthy volunteers using a flow rate of 176 mL/minute and found it to be \( 351 \pm 13 \mu \text{m} \) (mean \( \pm \) SEM).

Obviously there is no sharp boundary between the bulk water phase and the unstirred water layer, as shown diagrammatically in Figure 1.8. In the case of the small intestine the unstirred water layer presumably consists of a series of water lamellae extending outward from the mucosal cell membrane, each progressively more stirred, until they blend imperceptibly with the bulk water phase of the intestinal contents.

Passive absorption rates are represented as permeability coefficients \( P \), i.e. the mass of solute absorbed per unit time per unit surface area per unit concentration of the solute in the bulk water phase.

\[
P = \frac{J}{C_1}
\]  

This relationship is derived from equation 5 (after assuming that \( C_3 \) is near zero and can be ignored) and, therefore, is valid only in the special circumstances where \( D/d \) is large relative to \( P \) so that the value of \( C_2 \) approximately equals that of \( C_1 \).

Under conditions where diffusion across the unstirred layer is relatively rate-limiting, the value of \( C_2 \) is significantly less than \( C_1 \), and \( P \) values calculated from this equation will be
underestimates of the true permeability coefficients. Thomson (1979a, 1979b) formulated an equation which described $J_d$ (undirectional flux) under conditions of varying effective thickness or surface area of the unstirred water layer. The free diffusion coefficient of the probe molecule and the distribution of transport sites along the villus are shown in Figure 1.9.

In summary, the unstirred water layer may significantly alter the parameters of both diffusional and carrier-mediated transport of glucose. The exact relevance of the mucous layer in the intestinal transport process is far from established.

With a mucous layer depth of about 500 μm for rat jejunum (Winne 1976), compared to the height of an enterocyte (25 μm), the mucous layer would probably have an important part to play in the overall transport process. Indeed the effect of the unstirred water layer on kinetic parameters of active transport processes has been studied by a number of workers (Wilson and Dietschy 1974, Winne 1977, Thomson 1979b, Thomson and Dietschy 1980, Yusa et al. 1986).

Other functions of gut mucus:
Gut mucus has usually been assigned rather general functions such as protection and lubrication. The mucous layer is generally immiscible with solutions of most other macromolecules. This would fit in with the idea of mucus as a protective coating, keeping enzymes, toxins and other potentially harmful macromolecules away from the mucosa, but allowing free diffusion of small molecules (Clamp 1980).

Mucous membranes are also protected by secretory IgA antibodies. Another point of interest is the fact that the mucous layer would maintain a slightly alkaline pH, different from the pH in the lumen, which could also promote the movement of certain molecules (Gilles-Baillien 1983). Table 1.1 shows a number of protective functions both independent of and in association with the immune system. No one defence mechanism is absolute and there is considerable overlap between the various roles.
Figure 1.9

Diagrammatic representation of the major barriers to active transport at different sites on the villus. The unstirred water layer can be assigned values for an effective thickness (d) and an effective surface area ($S_w$) so that the resistance of this layer to molecular diffusion is related to the ratio of $d/S_w$. It is likely that the resistance of the unstirred water layer varies over transport sites present at different locations along the villus. For the mathematical presentation of this problem the villus was arbitrarily divided into 10 segments of equal height, and the rate of uptake and the dimensions of the unstirred water layer appropriate for the transport sites at each of these levels have been designated as $J^m_j$, $S^m_w$, and $d^m$, respectively (Reproduced from Thomson 1979a).
Table 1.1

A brief summary of possible defensive functions of mucus secretions.

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucus glycoprotein</td>
<td>Exclusion Non-mucin-like molecules</td>
</tr>
<tr>
<td></td>
<td>Trapping Toxins, viruses, bacteria, worms</td>
</tr>
<tr>
<td></td>
<td>Non-specific 'Stickiness'</td>
</tr>
<tr>
<td></td>
<td>Specific Spurious attachment sites</td>
</tr>
<tr>
<td>Mucus release</td>
<td>Direct For example by toxins</td>
</tr>
<tr>
<td></td>
<td>Indirect Immune complexes</td>
</tr>
<tr>
<td></td>
<td>IgE-mediated Macrophage secretagogue</td>
</tr>
<tr>
<td></td>
<td>Clearance (Ciliary action)</td>
</tr>
<tr>
<td></td>
<td>Peristalsis</td>
</tr>
<tr>
<td>Non-mucin components</td>
<td></td>
</tr>
<tr>
<td>Bicarbonate ion</td>
<td>pH gradient (stomach and duodenum)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Bacteriocidal</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Reduces free ion concentration</td>
</tr>
<tr>
<td></td>
<td>Bacteriostatic for iron-requiring micro-</td>
</tr>
<tr>
<td></td>
<td>organisms</td>
</tr>
<tr>
<td></td>
<td>Effect on hydroxyl radical production</td>
</tr>
<tr>
<td>Secretory IgA</td>
<td>Micro-organisms more 'mucophilic'</td>
</tr>
<tr>
<td></td>
<td>Attachment to mucosal surface hindered</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td>Polymorphs and</td>
<td>Phagocytic action</td>
</tr>
<tr>
<td>macrophages</td>
<td>Production of oxygen radicals</td>
</tr>
<tr>
<td></td>
<td>Secretagogue</td>
</tr>
</tbody>
</table>

(Reproduced from Clamp 1986)
1.4.2 Intestinal Permeability

The permeability barrier of the small intestine:
Permeability is the property of a membrane which allows movement of molecules across it, and is defined as the quantity of solute to cross a unit area of membrane in a given time. Absorption is not movement of solute across a membrane in a given time. There are several definite anatomical and physiological barriers to permeation including:

(a) an unstirred water layer,
(b) the epithelial cell (enterocyte) layer, which provides a series of barriers including the apical cell membrane, the intracellular fluid, the basolateral cell membrane, and the intercellular space,
(c) an epithelial basement membrane,
(d) the capillary endothelium.

As already discussed, the unstirred water layer decreases permeation of passively transported substances and alters the absorption kinetics of actively transported molecules.

Routes and mechanisms of permeation through the enterocyte:
1. Passive transport;

(a) simple diffusion across the lipoprotein cell membrane, which depends on the solute's lipid solubility, polarity, molecular size and shape. The rate of diffusion is proportional to the concentration gradient existing across the membrane,

(b) movement of solute through water-filled pores in the membrane, which depends on the solute's molecular size and shape and on its diffusion coefficient within the pore. This process is under the influence of osmotic pressure or solvent drag and is sometimes termed filtration.

2. Carrier-mediated transport;

(a) active transport,

(b) facilitated diffusion, in which energy is not used and solute movement follows a concentration gradient.
Any substrate crossing the small intestinal epithelium passes either by a transcellular route, passing through the enterocytes, or by a paracellular route, passing between the cells. The main paracellular route is probably through the tight junctions (Schultz 1977a).

Passive permeability of the small intestinal epithelium: If no specific carrier-mechanism exists, the principal factors determining whether a molecule will permeate the small intestine are lipid solubility and molecular size. If the molecule has a low solubility in lipid, the critical factor is molecular size, expressed by weight, radius or molecular shape, and most importantly, volume (Wright and Pietras 1974). Hober and Hober (1937) tested the hypothesis that although the mucosal cell membrane of the enterocyte is lipoidal in nature, cell membranes are interspersed with water-filled pores giving a sieve-like structure through which small molecules of non-lipid-soluble substances can diffuse.

Small molecules are absorbed more rapidly than larger ones, and beyond a certain size, corresponding to a molecular weight of about 180, which gives a molecular radius of approximately 0.4 nm, no penetration occurs. The calculated equivalent pore radius of rat small intestine was 0.4 nm (Lindemann and Solomon 1962). The effective pore radius of the jejunum was calculated to be about 0.7 nm, which is about twice that found in the ileum (Fordtran et al. 1965). Davis et al. (1982) concluded that, in the jejunum, the pathways for passive ion movement appear to be highly permeable to both sodium and chloride with some cation selectivity. In the ileum this pathway is much more cation selective, predominantly because of a relative impermeability to chloride.

Water-soluble lipophobic molecules of radius greater than 0.7 nm and with molecular weights in the range 60 - 33,000 have been shown to permeate the small intestinal epithelium of rabbits (Loehry et al. 1970, 1973). Loehry et al. (1970, 1973) suggest that there is a progressive fall in permeability from the duodenum to the terminal ileum. The results of studies of differential absorption of various sized inert molecules are also consistent with the existence of
multiple pores of different sizes (Wheeler et al. 1978). The theoretically determined aqueous pores are thought to correspond with the anatomically demonstrable tight junctions (Schultz 1977b) and there is evidence that the tight junctions and intercellular spaces are the main route of permeation of water-soluble lipophobic molecules, including macromolecules (Wright and Picrostas 1974, Schultz 1977a). Water and passive ion movements are mainly through the same paracellular pathways (Schultz 1977b). Another possible paracellular pathway is through the zone of exfoliation at the villous tip (Cline et al. 1976).

Studies of the small intestines of rats suggest two routes of passive permeation, diffusion across the membrane pores and passage through extracellular pathways, and absorption may be related to cell shedding (Cobden et al. 1981). The evidence for two routes of passive permeation of lipid insoluble molecules in man, comes from the study of untreated coeliacs, in whom the absorption of small molecules is reduced and the absorption of larger molecules is increased (Cobden et al. 1978, Ukabam and Cooper 1984). The basal membrane of the enterocytes lies on an epithelial basal lamina which appears as a continuous line, about 6 µm thick. This basement membrane is composed of fine fibrils (Palade and Karlin 1959).

Permeation through the capillary endothelium: The capillary endothelium consists of a layer of endothelial cells surmounted by a continuous basement membrane (Figure 1.10). Capillary permeability can be altered by changes in blood flow and pressure, and by a variety of stimuli including hormones, drugs, toxins and hypertonicity.

There appear to be two barriers to the passage of macromolecules through membranes which are freely permeable to small solutes; (1) between the vascular lumen and the lamina propria, i.e. the capillary wall, and, (2) between the lamina propria and the epithelium, i.e. either the epithelial basement membrane or the epithelial mucosal cell membrane.
Figure 1.10

Diagram of transport pathways in capillaries of the alimentary tract. Transport pathways: 1, cell membrane; 2, open fenestrae; 3, diaphragmed fenestrae; 4, intercellular junction; 5, pinocytotic vesicles; 6, transendothelial channels; 7, basement membrane.

(Reproduced from Granger and Barrowman 1983).
While the barrier on the epithelial side is more selective than that on the side of the endothelial cells (Kingham et al. 1978), Ukabam and Cooper (1984) showed that the normal human small intestine is permeable to significant quantities of small molecules of molecular radius 0.4 nm or less, for example 15.9 ± 0.88% (mean ± SEM) of orally administered mannitol was excreted via urine in normal subjects, but it is much less permeable to larger molecules; only 0.15 ± 0.01% (mean ± SEM) of orally administered lactulose was excreted.

However, the human small intestine is permeable to molecules up to the size of polyvinyl-pyrrolidone (PVP) having a molecular weight of 33,000 or greater (Lochry et al. 1973). Laker and Menzies (1977) found that the administration of lactulose in hypertonic solution, increases its absorption in the human intestine. Ideally, there is a need for intestinal perfusion studies to provide much more detailed information about permeability, but the number of available inert permeability markers that permeate the small intestine is small.

The ideal, passive, permeability marker should be:

(a) non-toxic,
(b) hydrophilic,
(c) lipophobic,
(d) absorbed entirely by passive diffusion,
(e) not modifiable by enzymes,
(f) absent from the diet,
(g) not produced endogenously,
(h) not metabolised,
(i) rapidly excreted,
(j) limited to the extracellular space,
(k) easily measured with sensitivity, accuracy and precision in body fluid (Cooper 1984).

The most useful clinical data has been obtained from studies with disaccharides (e.g. lactulose, cellobiose), hexoses (e.g. L-rhamnose), sugar alcohols (e.g. D-mannitol), and $^{51}$Cr-EDTA. However none of the sugars or mannitol, completely fulfils all the features
of an ideal permeability probe (Cooper 1984). Polyethylene glycol (PEG) 400 has been claimed to be the ideal permeability probe (Chadwick et al. 1977a, 1977b), but is is lipid-soluble and its absorption is not related to molecular size (Ukabam and Cooper 1984).

Ukabam and Cooper (1984) found that in normal subjects, about 0.1 per cent of ingested lactulose (molecular weight 342) is excreted in the urine in 6 hours, compared to about 35 per cent of ingested PEG (molecular weight 374). Cooper (1984) has suggested that an abnormality of small intestinal passive permeability might play a part in the etiology of the poorly understood diarrhoea of diabetes mellitus.

1.5 ROLES OF VITAMIN A

The term vitamin A is used as a generic descriptor for retinoids having the biological activity of retinol. There are a number of these compounds but those of central interest with regard to the physiological activity of vitamin A are the related structures, retinol, retinal and retinoic acid (Olsen 1988, footnote ref.1). The structure is made up of a polyisoprenoid attached to a cyclohexenyl ring. Retinol and retinaldehyde are interconvertible by oxidation and reduction; retinol can be oxidized to retinaldehyde and on to retinoic acid. Retinal can be re-reduced to retinol however the oxidation step to retinoic acid is not reversible and retinoic acid is rapidly degraded (Figure 1.11).

There are also many compounds which exhibit pro-vitamin A activity; most of these being plant carotenoid pigments (Olsen 1988, footnote ref.1). Beta-carotene has twice the pro-vitamin A activity of other

Footnotes
Figure 1.11  Structures of vitamin A

Figure 1.12  Conversion of beta-carotene to vitamin A

Vitamin A appears to have two distinct roles in the body; one in the visual process in which retinol combines with the protein opsin to form the photosensitive pigment rhodopsin, and a second as a hormone, probably mainly as retinoic acid, which has key roles in tissue differentiation and development.

Deficiency of vitamin A is associated with night blindness, xerophthalmia, growth retardation, follicular hyperkeratosis, abnormalities in taste and smell, anaemia, and anorexia. There is also increased susceptibility to infection and to carcinogens.

In developing countries xerophthalmia in children under five years of age is the most obvious manifestation of vitamin A deficiency. Xerophthalmia is the general term used to cover the ocular manifestations of vitamin A deficiency, including conjunctival and corneal xerosis, keratomalacia, and night blindness (Sommer 1982). The relationship of vitamin A deficiency to xerophthalmia relates to the requirement for vitamin A for the maintenance of epithelial tissues as well as for resistance to infection.

Vitamin A is required for tissue growth, including development and maintenance of epithelial tissues (eye, lung, GI and uro-genital tracts) as well as for bone growth and development (Wolbach and Howe 1925, Sherman 1961, DeLuca et al. 1972, Zile et al. 1980, Tseng et al. 1984). Tseng et al. (1984) reported that corneal and conjunctival epithelia became heavily keratinized, forming multiple layers of superficial, anucleated cornified cells, but oesophageal epithelium underwent only minor morphological changes. Zile et al. (1981) found that although the cells of intestinal epithelium appear to remain normal in mild vitamin A deficiency, other workers (DeLuca et al. 1969, Rojanapo et al. 1980, Olson et al. 1981) have reported that the number of goblet cells is reduced.
Deficiency of vitamin A and carotene in the diet of rats tends to increase their susceptibility to hyperplastic and other abnormal conditions of the periodontal epithelia and connective tissue (King 1940), as well as salivary gland pathology (Sherman 1961). Sherman (1961) found a reduction in the mitotic index of epidermal, corneal and tracheal tissues. He concluded that vitamin A is a factor in the regulation of the mitotic activity of epithelial cells. Vitamin A was suggested to have a role in the regulation of cell division in the small intestine of the rat (Zile 1977), as well as in the colon, trachea and skin (Zile 1980). Sasaki et al. (1980) suggested that the effect of retinoic acid on cellular adhesion may well be related to its effect on the biosynthesis of cell surface glycoconjugates.

Vitamin A deficiency has been shown to cause squamous metaplasia in the hamster tracheal epithelium, a process similar to that induced by chemical carcinogens (Harris et al. 1972). Cohen et al. (1976) found squamous metaplasia of the urinary bladder and a high incidence of cystitis, ureteritis and pyelonephritis in vitamin A-deficient rats. Deficiency appeared to accelerate the carcinogenic process initiated by a urothelial carcinogen.

Chronic dietary deficiency of vitamin A slightly increased the incidence of tumors, and appeared to reduce the induction time of intestinal tumors following intragastric administration of chemical carcinogens in experiments with rats (Rogers et al. 1973). The dietary administration of retinyl acetate significantly prolonged cancer latency and decreased the average number of mammary cancers per animal (Thompson et al. 1979). Recently, Hicks et al. (1982) demonstrated that retinoic acid inhibited the development of bladder cancer in rats pretreated with carcinogens.

Mammalian cells are known to contain specific retinol-binding and retinoic acid-binding proteins, though their role remains obscure (Chytel and Ong 1984). Recent work has shown that retinoic acid can combine with a protein receptor to form a complex which can bind to a regulatory sequence of DNA, activating gene expression (Giguere et
al. 1987). It also appears that a gradient of retinoic acid is able to act as a factor controlling differentiation in the chick wing (Thaller and Eichele 1987).

Vitamin A has been termed the anti-infective vitamin. Although the epithelial changes and reduced mucus production are primary factors in decreased resistance to infection, alteration of both the humoral and the cell-mediated immune responses probably also contributes to lowered resistance to infection.

Recently also Smith et al. (1987) have suggested that vitamin A deficiency is associated with a functional defect of the immune system. A study carried out on Indonesian children showed that xerophthalmia was associated with increased risk of respiratory disease and diarrhoea (Sommer et al. 1984). The present analysis of data from the same longitudinal study indicates that the relationship applies in both directions: children with respiratory disease and/or diarrhoea are at increased risk of developing xerophthalmia (Sommer et al. 1987).

There are two major sources of vitamin A in the diet. Animal products provide vitamin A as retinol, mostly in the form of retinyl palmitate. Eggs, milk and milk products, organ meats and fatty fish are good sources, while meat and low-fat fish contribute trace amounts. Plant foods provide pro-vitamin A in the form of carotenoids, supplied from orange-yellow vegetables and fruits, and green leafy vegetables.

In the developing countries of South-East Asia, including Indonesia, animal products such as eggs, milk and milk products, meat and fish, make up a much smaller fraction of the total diet than is the case in Western countries. As a consequence the intake of vitamin A as retinol is relatively low. Diets in these countries are based predominantly on polished rice as the staple which contributes the major part of the food energy but no vitamin A. In addition to rice the diet contains relatively small quantities of animal products.
Vitamin A and Protein-Energy Malnutrition

Protein-energy malnutrition (PEM) is a major problem in areas of many developing countries including those of South-East Asia. It characteristically affects infants and children of less than five years of age, and is associated with poverty, famine, a restricted food supply, infection and inappropriate feeding practices for young children.

When food energy intake is less than required, dietary protein is used for energy and there is a loss of lean tissue from the body. Growth is retarded, immunological defense reduced and digestive function impaired. Frequent infections, often gastrointestinal, and chronic worm infestation compound the problem by producing malabsorption and diarrhoea, anorexia and increased basal metabolism (Hansen 1982, footnote ref.1).

Vitamin A deficiency and PEM are associated in several ways. The environment which leads to a restriction of food intake and the occurrence of PEM is likely to restrict the intake of retinol since animal product foods are less available and more expensive. PEM results in reduced digestive function with pancreatic exocrine deficiency, villous atrophy and impaired nutrient absorption. The absorption of the fat-soluble carotenoids is reduced, as well as the efficiency of conversion of carotenoids into retinal (Hansen et al. 1982, footnote ref.1).

Footnotes:
In addition, the level of retinol-binding protein in the plasma is reduced, so retinol is less efficiently mobilised from the liver (McLaren 1984, footnote ref.1).

Vitamin A deficiency is of major importance as a leading cause of childhood blindness and mortality, and although it is known to be associated with PEM, malabsorption and reduced immunological defence, little is known about the direct effects of vitamin A deficiency on gut structure and function. Since vitamin A deficiency usually occurs as a component in a complex of nutritional deficiencies, it is important to determine whether vitamin A deficiency itself can precipitate digestive dysfunction and malabsorption, and therefore malnutrition.

1.6 AIMS OF THE PRESENT STUDY

The central hypothesis to be tested in this study is that acute vitamin A deficiency interferes with glucose uptake from the small intestine. Such interference should show up as a decreased efficiency of uptake of glucose from the lumen to the tissues.

While in vitro studies are necessary to study the basic processes involved in glucose uptake from the small intestine they do not allow a description of the near-steady state which exists in the living intact animal. The intention in the present study was to use a technique which allowed a description of the effect of vitamin A deficiency on the rate of glucose uptake under steady state conditions in vivo.

A number of subsidiary studies had to be carried out to clarify the contribution of active and passive transport processes to total glucose uptake.

Footnote
These subsidiary studies were:

1. to establish a perfusion technique allowing measurement of the rate of glucose uptake under steady state conditions;
2. to establish that phlorizin could be used as competitive (reversible) inhibitor to block active uptake under steady state conditions;
3. to establish that glucose uptake from maltose, the end product of starch digestion by pancreatic enzymes, was not kinetically different from glucose uptake from a glucose solution;
4. to establish whether glucose uptake by solvent drag represented a significant proportion of the total diffusional uptake;
5. to establish the technique for producing vitamin A deficiency in rats.

The findings of the study as reported in this thesis, do not support the hypothesis; while significant differences were found between the characteristics of glucose uptake in control rats and those with acute vitamin A deficiency, the rate of glucose uptake showed a slight increase rather than a decrease. There was no evidence of decreased efficiency of glucose uptake.
CHAPTER 2

GENERAL METHODS

2.1 MATERIALS

Maltose, glucose, glucose standard solution, phlorizin, streptozotocin and amyloglucosidase were obtained from Sigma Chemical Company (St Louis, U.S.A.). Pentobarbitone was from May and Baker Ltd. (Melbourne). Phenol red (phenolsulphonphthalein) was from Ajax Chemicals (Sydney). Maltose hydrate contained less than 2 per cent maltotriose, less than 0.5 per cent glucose and one H₂O per mole when assayed. Glucose (α-D, +), anhydrous, grade 3, contained approximately 5 per cent beta-anomer. Phlorizin (phloretin-2-beta-D-glucoside) contained 3H₂O per mole as assayed. Amyloglucosidase (E.C.3.2.1.3) from Aspergillus, had a stated activity of approximately 70 units/mg protein. ¹⁴C-Polyethylene glycol 4000 in aqueous solution containing 3 per cent ethanol, activity 10 mCi/g; D-¹⁴C glucose in aqueous solution containing 3 per cent ethanol, activity 3 mCi/mmol, and D-(6-³H) glucose in aqueous solution, activity 30.2 Ci/mmol, were purchased from Amersham (U.K.). Glucose Rapid Test Kits were purchased from Roche Products Pty. Ltd. (Sydney).

2.2 PERFUSION SYSTEM

Rates of uptake of glucose from glucose solutions in the small intestine of the anaesthetised rat were measured using a single pass perfusion technique similar to that used by Schwartz and Levine (1980). The rate of glucose uptake was determined by measuring the difference in the concentrations of sugars in the solutions entering and leaving a cannulated gut segment together with the measurement of phenol red (phenolsulphonphthalein) concentration to correct for water uptake or loss from the animal. The method is illustrated in Figure 2.1.
Figure 2.1

Diagram illustrating method for single pass perfusion of a small intestine segment in vivo.
Male Sprague Dawley rats were used in the weight range 230 to 360 g. The rats were fasted for 18 - 22 hours prior to use and allowed water ad libitum. Rats were anaesthetised with pentobarbitone solution (6 mg per 100 g body weight) injected into the intra-peritoneal cavity and then subcutaneously with an additional half measure of the dose. The animals were maintained at a body temperature of $37^\circ$C $\pm$ 0.5$^\circ$C on a heated metal table together with an overhead lamp and monitored with a digital rectal thermometer.

2.3 PREPARATION OF GUT SEGMENTS

A laparotomy was performed by cutting the skin along the abdominal midline exposing the intestine. For cannulation of the duodenal segment, the common bile duct was first tied off to avoid possible interference of bile with the spectrophotometric assay. It has been observed also that the presence of sodium deoxycholate increases phenol red absorption (Feldman et al. 1970). For the duodenal segment, the inlet cannula was inserted through a small incision in the wall of the stomach, pushed through the pyloric sphincter and tied in place. The outlet cannula was inserted through an incision in the anti-mesenteric side of the small intestine at the end of the duodenum, located at the ligament of Treitz, and tied in place. The jejunal segment was measured from the end of the duodenum to a point 10 cm distal from that. The duodenum was tied off and the inlet cannula inserted through an incision close to the ligament of Treitz. For the ileal segment, the distal end was located 1 cm proximal to the ileo-caecal junction, where the ileum was tied off and an incision made. The inlet cannula was inserted in an incision made at a point 10 cm proximal to the outlet. Since there was usually some food residue in the ileum, it was necessary to run the perfusion solution through the segment until the emerging solution was clear before tying in the outlet cannula. The segment was covered with laboratory film (Parafilm "M", American Can Company, Greenwich). The inlet and outlet cannulae were passed through a slit in the film. The laboratory film was then covered with cotton gauze moistened with warm physiological saline. The perfusion solution was warmed in a $37^\circ$C water bath before loading in the syringe. The proximal cannula was connected to a
syringe pump (Vickers 1P3, Basingstoke, England) with polyethylene tubing having a bore sufficient to give a firm fitting over a No. 18 syringe needle.

The solutions for perfusion were adjusted to 304 mOsm/kg with NaCl, making them iso-osmolar with tissue fluids, and phenol red (phenolsulphonphthalein) was used as a non-absorbable marker to allow correction for water movement. The osmolality of each solution was determined by freezing point osmometry (Osmometer Automatic, Knauer, Berlin). The osmometer was calibrated before each use with distilled water and a sodium chloride standard, 400 mOsm/kg.

Perfusion of a segment of intestine with a hyperosmolar solution, usually over 340 mOsm/kg has been shown to result in net fluid secretion to the gut (Binder 1983) and solutions of low osmolality will result in water uptake. Measurement of phenol red in the effluent solution allowed correction to be made for net water movement into or out of the perfusion solution (Schall 1966). Water uptake or loss from the tissues was corrected using the formula:

\[
\frac{[\text{phenol red}] \text{ in}}{[\text{phenol red}] \text{ out}} \times [\text{glucose}] \text{ uncorrected} = [\text{glucose}] \text{ corrected}
\]

Phenol red was determined spectrophotometrically at 550 nm in the presence of NaOH (50 mmol/L), using an auto-analyser (Roche Centrifichem). In experiments in which phenol red was determined in a spectrophotometer, the solution was made alkaline by addition of 0.90 mL of sodium carbonate (0.1 mol/L) to 0.10 mL of sample.

The experiments of Pons and Laralde (1951) indicate that the optimal pH of the intestinal contents for absorption is near neutrality and that buffers are rapidly neutralised by the secretions of the intestine. In these experiments therefore, the perfusing solution was unbuffered. It was considered desirable to allow the intra-luminal pH to be adjusted by the normal reaction of the gut tissues. In general, the pH of the emerging solution was slightly acid, in the range 6.2 to 6.5.
The perfusion was carried out with a motor syringe set to a flow rate of 0.125 mL/min which is in the range of rates of normal stomach emptying (Malik, A. and Read, R.S.D., unpublished).

Preliminary experiments were carried out to determine the time taken for the solution to traverse the segment. The fluid volume of a perfused 10 cm-segment of the gut was calculated as follows. The mean weight of two segments squeezed dry of liquid and mucus was 1.33 g and the mean weight for two segments tied off while being perfused then removed and weighed was 1.95 g. Subtraction gives a segment volume of 0.82 mL. When this volume is divided by the flow rate (0.125 mL/min) the time taken for the solution to traverse the segment (6.5 minutes) is confirmed.

2.4 PERFUSION SOLUTION

The composition of the solutions for perfusion of glucose at 10, 4 and 2.5 mmol/L was 1.8016 g, 0.7206 g and 0.4504 g glucose, 20 mg phenol red, made up to 1 L and the pH adjusted to 7.4 with NaOH. The composition of the solution for perfusion of maltose (5 mmol/L) was 1.716 g maltose, 8.8 g NaCl and 20 mg phenol red made up to 1 L with glass-distilled water and the pH adjusted to 7.4 with NaOH. The maltose solution with phlorizin added contained, in addition to the above, 0.436 g of phlorizin. The solutions contained NaCl to adjust osmolality. The glucose solution with phlorizin added to a concentration of 1 mmol/L contained, in addition to the above, 0.4364 g of phlorizin. Phlorizin at 0.25, 0.5 and 2 mmol/L contained 0.1091, 0.2182 and 0.8728 g of phlorizin respectively in 1 L of perfusion solution.

2.5 SAMPLE COLLECTION

The solution collected during the first 10 minutes of perfusion was routinely discarded and, in the usual method, a sample of the emerging solution was collected over the following 20 minutes. The perfusing solution was then changed to a solution containing phlorizin, or
otherwise altered according to the variable to be tested. The solution collected during the first 15 minutes after changing the perfusion solution was discarded, and a following 20-minute sample was collected as before. Samples were chilled immediately after collection and kept frozen until analysed.

2.6 GLUCOSE MEASUREMENT

Glucose in the perfusion solutions was determined by use of an autoanalyser (Roche Centrifichem) using the hexokinase method in which the rate of reduction of \( \text{NAD}^+ \) is measured spectrometrically at 340 nm (Noose et al. 1975). The hexokinase method involves the following reactions:

\[
\text{[hexokinase]}
\]

\[
\text{D-glucose + ATP} \rightarrow \text{glucose-6-phosphate + ATP}
\]

\[
\text{[glucose-6-phosphate dehydrogenase]}
\]

\[
\text{glucose-6-phosphate + NAD}^+ \rightarrow \text{gluconate-6-phosphate + NADH + H}^+
\]

The rate of increase in the NADH concentration is directly proportional to the glucose concentration. Measurement of glucose in the glucose solution of 10 mmol/L containing phlorizin at 1 mmol/L, showed the same result as obtained for the solution without phlorizin, indicating that phlorizin did not interfere with the hexokinase method.

Blood glucose measurement:

Rat blood was taken from the tail, collected in sodium fluoride EDTA tubes, and blood glucose was measured within several minutes by a glucose oxidase method, using a glucose analyser (YSI, model 27, Industrial Analyser, Yellow Springs, Ohio, U.S.A.).
2.7 MALTOSE MEASUREMENT

Maltose was measured by first hydrolysing the disaccharide with amyloglucosidase in acetate buffer pH 4.5, then using the hexokinase (Centrifichem) method to measure the glucose released. Virtually complete conversion of maltose to glucose occurred in 30 minutes at 55°C. In the routine procedure, hydrolysis was carried out under these conditions. At the conclusion of the perfusion, the animals were sacrificed by intra-cardiac injection of pentobarbitone solution and the gut segments removed. The segments were squeezed firmly from the centre to the ends to remove mucus and liquid, blotted dry and oven dried at 105°C before weighing. In these experiments uptake rate was measured with reference to dry weight of gut tissue. A variety of ways of expressing uptake with reference to the gut can be used, and gut length, surface area, and wet weight have all been used in previous studies. Gut length, however, is inappropriate because the tissue is elastic and its length alters when handled. Internal surface area cannot conveniently be calculated because of internal folds. Wet weight and dry weight appear to be equally satisfactory (Dryden, P. and Read, R.S.D., unpublished). Glucose uptake is therefore expressed as mmol/min/kg dry tissue.

2.8 ACCURACY, PRECISION AND QUALITY CONTROL

2.8.1 Glucose Measurement: Centrifichem Hexokinase Method

Accuracy:

Accuracy is defined as the extent to which the mean measurement is close to the true value. Determination of accuracy consists of comparing observed values with true values.

Interference by phenol red and phlorizin with the measurement of glucose by the Centrifichem hexokinase method is shown in Tables 2.1 and 2.2.
Table 2.1

Effect of phenol red (20 mg/L) and phlorizin (1 mmol/L) with measurement of glucose (10 mmol/L) in a solution of sodium chloride (pH 7.4) by the Centriferchem hexokinase method (mean ± SEM, n = 8).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose in NaCl</td>
<td>9.74 ± 0.05</td>
</tr>
<tr>
<td>Glucose in NaCl and phenol red</td>
<td>9.79 ± 0.05</td>
</tr>
<tr>
<td>Glucose in NaCl, phenol red and phlorizin</td>
<td>9.7 ± 0.05</td>
</tr>
</tbody>
</table>
Table 2.2

Interference by phlorizin at 1 and 2 nmol/L with measurement of glucose (2.5 nmol/L) in a solution containing sodium chloride and phenol red.

<table>
<thead>
<tr>
<th>Glucose (no phlorizin)</th>
<th>Glucose + Phlorizin (1 nmol/L)</th>
<th>Glucose + Phlorizin (2 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5^{a} ± 0.03</td>
<td>2.46^{b} ± 0.02</td>
<td>2.11^{c} ± 0.03</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 13)</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed; a > c, b > c (p < 0.005).
The results showed that phenol red (20 mg/L) and phlorizin (1 mmol/L) did not interfere with the Centrifichem hexokinase method for glucose measurement. However, phlorizin at 2 mmol/L reduced significantly the value obtained by this method (p < 0.005).

Quality Control:
The control of accuracy can be achieved by proper standardisation procedures, comparison with reference methods, submission of samples with known values, and proficiency testing (Reed and Henry 1974). Before running each set of unknown samples, the Centrifichem analyser was standardised against distilled water. The first and second cuvettes were always used for the glucose standard (5.55 mmol/L) during the analysis of unknown samples.

Unknown samples were routinely analysed in duplicate. Whiteby et al. (1967) recommend that duplicate controls be run whenever possible. Quality control was checked by routinely analysing glucose standard solutions of 2.78 and 11.10 mmol/L.

Precision:
Precision is defined as the random error or variation of results, obtained by a method when the same sample is analysed repeatedly under the same conditions. In other words, the reproducibility of what is observed (Tonks 1968, Reed and Henry 1974).

Precision is observed in terms of standard deviation (S.D.), and coefficient of variation (C.V.). S.D. is a mathematical measure of precision or reproducibility; or conversely, of scatter. C.V. is an expression for the standard deviation in terms of percentage, thus eliminating units of measurement.

The precision of the hexokinase method, using the Centrifichem analyser, was determined by analysing 28 identical samples for glucose. The standard glucose solution contained 5.55 mmol/L and the C.V. on analysis was found to be 1.09% with a mean value of 5.51 and a S.D. of 0.06 mmol/L.
2.8.2 Use of Radio-Labelled Glucose

Since phlorizin at 2 mmol/L interfered with the Centrifichen hexokinase method for glucose, radio-labelled glucose ($^{14}$C-, or $^3$H-) was used in uptake studies requiring phlorizin at this concentration. In some experiments $^{14}$C-polyethylene glycol (PEG) was used as a non-absorbed marker to allow correction for water movement.

Measurement of $^{14}$C- and $^3$H-isotopes:

$^{14}$C-PEG and $^3$H-glucose were measured in the original and perfusion solutions using a liquid scintillation spectrometer (Tri-Carb 2000 CA, United Technologies, Pickford, U.S.A.) using a scintillation mixture for aqueous samples (12 g PPO, 2 L toluene and 1 L Tetric).

The number of $^3$H-glucose and $^{14}$C-PEG-counts from the perfusion solution recorded per vial was approximately 4800 and 900 cpm per 0.25 mL sample respectively. Samples were normally counted twice for 10 minutes. Counts were corrected for background and spillage of $^{14}$C into the $^3$H-channel. Preliminary experiments were carried out to determine spillage of $^{14}$C into the tritium channel, when dual isotopes, $^{14}$C-PEG and $^3$H-glucose, were used in the perfusion solution.

The spillage of $^{14}$C into the $^3$H-channel = CPMB x 0.4516.

where CPMB = the $^{14}$C- count in B channel

0.4516 = constant factor (C)

$$C = \frac{^{14}
\text{C-count in A channel}}{\text{CPMB}}$$
Internal Standards:
An internal standard was used to check for variation in efficiency of counting of the $^{14}\text{C}$. $^{14}\text{C}$-PEG added to a series of vials ($n = 4$) was recovered with a coefficient of variation of 2.49%.

2.8.3 Measurement of Glucose: Glucose Oxidase Method

To test the accuracy, a glucose standard 100 mg/mL (5.5 mmol/L) or 180 mg/mL (9.99 mmol/L) was included as a calibration standard with each group of blood samples. Quality control was checked by analysing glucose standard solutions of 100 mg/mL (5.55 mmol/L) and 500 mg/mL (27.75 mmol/L). Blood samples were analysed in duplicate. A precision test was carried out by analysing 10 identical samples for glucose.

The standard glucose solution contained 5.55 mmol/L and the C.V. on analysis was 1.20% with a mean value of 5.555 mmol/L and a S.D. of 0.066 mmol/L.

2.9 STATISTICAL ANALYSIS

The rates of uptake are reported as means with the standard errors of means. Significance of the differences between means was determined using Student’s t-test which is appropriate for assessing the significance of differences between two independent means obtained from small samples ($n = 6$). The use for the single-tailed test is appropriate for a test of significance with respect to one mean being greater than another. Differences were considered significant if $p$ values were less than 0.05.
CHAPTER 3

PERFUSION STUDIES

SUMMARY

Initial experiments were carried out to characterise the in vivo, single pass perfusion system for the measurement of rate of glucose uptake. It was found that steady-state glucose uptake could be maintained for periods of at least 60 minutes. The problems of determining the substrate concentration available to the absorptive sites during steady-state uptake are discussed.

Phenol red was found to be superior to polyethylene glycol as a non-absorbable marker for calculation of net water movement to or from the gut.

Phlorizin was investigated as an inhibitor of active uptake. Inhibition was shown to be rapid and reversible, however it was not possible to show that active uptake could be completely suppressed with phlorizin. Residual phlorizin-resistant uptake may represent an alternative (phlorizin-resistant) pathway of uptake or possibly diffusion to a lower concentration inside the villous epithelial cell.
3.1 NON-ABSORBABLE MARKERS

3.1.1 INTRODUCTION

Non-absorbable reference substances are widely used to study intestinal water movement to and from the intestine. For perfusion studies of the absorption of nutrients, the two most commonly used markers have been phenol red (phenolsulphonphthalein) and polyethylene glycol (PEG). Phenol red has been used for the quantitative estimation of dilution (Pannen et al. 1940, Ivey and Scheld 1970), transit (Reynell and Spray 1956, Leeds et al. 1979) and absorption (Reynell and Spray 1956, French et al. 1960, Miller and Scheld 1970, 1972, Dryden et al. 1983, 1985) of test solutions and fed or infused meals.

Appearance time and quantitative urinary excretion of a small dose of phenol red (6 mg intramuscularly or intravenously) provides a renal function test which is still clinically useful (Rowntree and Geraghty 1910). Pannen (1931) demonstrated in dogs, that 3 to 5 per cent of phenol red is present in the urine after oral feeding. While Tidball (1964) showed that phenol red can move from the gut lumen to portal vein blood as a passive process.

Fordtran (1966) in his review, recommended that an ideal reference substance for use as nonabsorbable marker should:

1. be strictly nonabsorbable (slowly absorbed markers slightly underestimate absorption rates),
2. not be subject to degradation in the intestine,
3. not be trapped by mucus or other intestinal contents,
4. not influence intestinal motility,
5. be measurable with accuracy,
6. have the same solubility characteristics as the test substance,
7. not influence digestion of the test substance.

A marker meeting all these criteria has not been found, although literature reports suggest that PEG is adequate for the study of absorption of water-soluble and non-digestible test solutes. Chromium has also been used.
It has been shown that phenol red is poorly absorbed from the gut (McLeod et al. 1968) and recoveries of more than 90 per cent are usually obtained (Reynell and Spray 1956, Soergel et al. 1967, Miller and Schedl 1970, 1972). Hyden (1955) provided evidence to show that the observed losses of PEG may be partly due to destruction in the alimentary tract.

Wiggins and Dawson (1961) found that estimates of fat absorption were misleading if obtained using PEG as a reference marker, and this was suggested to be due to the heterogeneous distribution of PEG in the intestinal contents. However Jacobson et al. (1963) showed that PEG 4000 is a reliable indicator for estimating intestinal water volume in perfusion studies in vivo using rat intestine, even though there was a discrepancy between measured volumes and volumes estimated with PEG.

In their studies, Worsing and Andrup (1965) showed that calculation of total intestinal content based on the concentration of PEG in the aspirate may give values which are up to 50 per cent too high. However this result has been questioned, because pancreatic and biliary fluids emptied directly into the test segment used, were not taken into account (Jacobson 1966). Maddrey et al. (1967) found that PEG was completely recovered in the dog in perfusion experiments using Thiry-Volla loops. Alder and Stanley (1968) observed that PEG may significantly modify transport of both solutes and water.

Chadwick et al. (1977a) showed that the greatest absorption of PEG probably takes place in the jejunum. Allen et al. (1979) observed that the faecal recovery of PEG in human nutrition experiments averaged only 93 per cent of that consumed. Barnden et al. (1983) found that PEG appeared to be an unreliable marker of change of luminal volume in the rat jejunum. However, a change in luminal PEG concentration has been used and validated by other workers for experiments in the colon and ileum of the rat (Bunce and Sprag 1982), and in human jejunum (Wheeler and Barwell 1986).
Recently, Sundqvist and Magnusson (1985) observed that the variation between individuals in intestinal absorption of low molecular weight species of PEG between individuals can be assessed by urinary recovery of a multi-probe system made up of a mixture of PEG 400 and PEG 1000. Lifschitz et al. (1986) studied intestinal permeability in children by using a mixture of low molecular weight polymers of PEG. However, Ukabam and Cooper (1984) have claimed that PEG 400 cannot be recommended as a suitable marker for permeability studies of the small intestine.

The present study compares phenol red and PEG as non-absorbable markers by using them together in intestinal perfusion experiments.

3.1.2 METHODS

Preliminary experiments were carried out to determine the appropriate concentration of phenol red to be used in the perfusion solution. A concentration of 20 mg/L when diluted one in five in a solution of sodium carbonate (0.1 mmol/L) gave sufficient colour to be read easily at 550 nm, the absorption peak under alkaline conditions (Figure 3.1). Phenol red was measured using a spectrophotometer (Novaspec, LKB Biochrom Ltd., Cambridge, England) at a wavelength of 550 nm after alkalization of the sample (0.01 mL) with Na₂CO₃ (0.90 mL, 0.1 mmol/L, Figure 3.2).

The concentration of phenol red was measured in the effluent solution in samples taken at 10-minute intervals, after perfusion with glucose 4 mmol/L for an initial 20 minutes to establish a steady state in a 10 cm-length of proximal jejunum. In another set of studies, the segment was perfused with a solution containing ³H-glucose (4 mmol/L), together with sufficient sodium chloride to make the solution iso-osmolar (304 mOsm/kg). Phenol red (10 mg/L) and ¹⁴C-PEG 4000, together with PEG 6000 (1 mmol/L) as a carrier, were included as non-absorbable markers.
Figure 3.1

Absorbance of phenol red (phenolsulphonphthalein, 20 mg/L) versus wavelength at 550 nm in perfusion solution (0.10 mL) made alkaline by the addition of sodium carbonate (0.1 mol/L, 0.90 mL).
Figure 3.2

Concentration of phenol red (phenolsulphonphthalein) versus absorbance at 550 nm. Phenol red was dissolved in the perfusion solution and samples (100 uL) made alkaline by the addition of sodium carbonate (0.1 mol/L, 0.90 mL).
The solution was perfused for 20 minutes to establish a steady state, and the effluent solution was collected over a further 20 minutes. Net water movement to or from the gut lumen was determined by three methods; by measuring the ratio of the concentrations of either phenol red or $^{14}$C-PEG in the solutions entering and leaving the cannulated segments, and by direct weighing of the solution delivered to and recovered from the segment.

$^{14}$C- and $^3$H- isotope measurements:

$^{14}$C-PEG and $^3$H-glucose were measured in the original and effluent solutions using a liquid scintillation counter (Liquid Scintillation Analyser, Tri-Carb 2000 CA series, United Technologies, Pickford, U.S.A.) and a scintillation mixture for aqueous samples (12 g PPO, 2 L toluene and 1 L Toluic).

Counts were corrected for background and spillage of $^{14}$C into the tritium channel. The number of $^3$H-glucose and $^{14}$C-PEG counts measured in the perfusate solutions per vial was approximately 4800 and 900 cpm per 0.25 mL sample respectively. The coefficient of variation for counting of a $^{14}$C-standard was found to be 2.49%.

Recovery study:

In a separate study, total recovery of phenol red and $^{14}$C-PEG from a perfused segment was measured. The segment was perfused first with the glucose solution, but without indicators, for 10 minutes, followed by the same glucose solution with indicators for 30 minutes, and finally by the glucose solution without indicators for 15 minutes to wash the remaining indicator out of the segment. The syringe and all containers were weighed before and after perfusion to determine the exact amounts of perfused and collected solutions.

Recovery of the dye from jejunal segments by washing was calculated from the spectrophotometric reading.
Calculations
1. Net water absorption

\[ (1.00 - \frac{C_i}{C_f}) \times 100 = \% \text{ net water flux.} \]

2. Total indicator recovery

\[ A = \text{total amount perfused} = C_i \times \text{mL perfused.} \]

\[ B = \text{total amount collected} = C_f \times \text{mL collected.} \]

Thus, total recovery, (\%) = \frac{B \times 100}{A}

where \( C_i \) and \( C_f \) are the initial and final indicator concentrations.

3.1.3 RESULTS

Maintenance of a steady state in vivo:
When a glucose solution (4 mmol/L) was perfused through a 10 cm-length of proximal jejunum at a flow rate of 0.125 mL/minute, the concentration of phenol red in samples of the effluent was maintained at a plateau level for at least 60 minutes (Figure 3.3).

Phenol red and \(^{14}\text{C}-\text{PEG} \) as non-absorbable markers:
Water uptake determined by the direct method was slightly more than that calculated from measurement of phenol red. Calculated water movement based on measurements of \(^{14}\text{C}-\text{PEG} \) showed greater variability and indicated an efflux of water, thus giving poor agreement with the two other methods. Measured glucose uptake was affected according to which marker was used to correct for water movement but differences were not significant (Table 3.1).

Washing procedure and recovery of phenol red:
In another set of experiments, the efficiency of recovery of the dye from the segment was tested. Recovery of the dye from gut segments by washing was calculated from sample volume and absorbance at 550 nm.
Figure 3.3

Phenol red (●●●) in the eluate solution after perfusion through a 10-cm segment of proximal jejunum, expressed as per cent concentration in the original solution, together with rate of glucose uptake (○○○) from a glucose solution (4 mmol/L) showing establishment of a steady state (mean ± SEM, n = 5).
Table 3.1

Rates of water movement and rates of glucose uptake, corrected for water movement, using each of three methods, direct measurement, recovery of phenol red and recovery of $^{14}C$-PEG (mean ± SEM, n = 6)

<table>
<thead>
<tr>
<th>MARKER</th>
<th>UPTAKE (mL/min/kg dry tissue)</th>
<th>Glucose (mmol/min/kg dry tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol red</td>
<td>$81.7 ± 6.8^a$</td>
<td>$1.86 ± 0.18$</td>
</tr>
<tr>
<td>$^{14}C$-PEG</td>
<td>$- 66.4 ± 54.5^b$</td>
<td>$1.58 ± 0.23$</td>
</tr>
<tr>
<td>Direct measurement</td>
<td>$107.8 ± 7.2^c$</td>
<td>$1.91 ± 0.17$</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed;

$c > b, p < 0.01; a > b, c > a, p < 0.05.$
Possible interference by turbidity from the effluent solution was checked by reading the absorbance at 700 nm, which was found to be not greater than 0.003. There was therefore no significant interference due to turbidity in the samples.

The procedure gave satisfactory recovery of the phenol red (99.1 ± 1.2%, mean ± SEM, n = 4) but recovery of the $^{14}$C-PEG was overestimated and was more variable (104.9 ± 4.4%, mean ± SEM, n = 4).

3.1.4. DISCUSSION

During 60 minutes of perfusion, there was an initial equilibration (15–20 min) following which glucose and phenol red measured in the effluent solution did not differ significantly between samples. This confirms that a steady state was maintained over that 40 minute period. In further work an equilibration (15 min) was generally used and experiments were usually terminated within 60 minutes.

In the present study, water uptake determined by the direct method was comparable with that calculated using measurement of phenol red concentration. However, the measured $^{14}$C-PEG (4000) concentration varied significantly between samples. Thus $^{14}$C-PEG appears to be an unreliable marker for measuring the change in volume of the effluent solutions from perfusions of rat jejunum, and this finding agrees with that of Ramaeda et al. (1983).

In this study, uptake of glucose was demonstrated under conditions in which the intra-luminal glucose concentration was similar to that in the blood of fasting rats. The calculated rate of glucose uptake was affected by the choice of indicator used for calculation of net water movement to or from the gut. Almost all of the phenol red was recovered in the effluent solutions in this study. The efficiency of recovery (mean 99.1%) was comparable with that reported in previous studies. Reynell and Spray (1956) found a recovery of 91 to 94 per cent in perfusion studies of rat small intestine, while Miller and Schedl (1970) recovered 96.6 per cent of phenol red in one study and 99.96 per cent in a later single pass perfusion study (Miller and Schedl 1972). Soergel and Hogan (1967) found a recovery of 91.8 ± 12% in perfusions of the human gastrointestinal tract.
3.2 PHLORizin AS AN INHIBITOR OF ACTIVE TRANSPORT

3.2.1 INTRODUCTION

Phlorizin has been widely used as an inhibitor of the active transport of glucose (Chapter 1). In this study phlorizin was used to inhibit active transport in order to study separately the processes of active uptake and diffusion. It was necessary therefore to study the characteristics of phlorizin as an inhibitor of active transport under the steady state conditions used in these experiments. Several experiments were therefore carried out.

The first was to determine the time required to achieve a steady state after the addition of phlorizin to inhibit active uptake. To determine this, jejunal segments were perfused first with a glucose solution for 30 minutes to establish steady state uptake and from 30 minutes onwards with a similar glucose solution containing phlorizin (1 mmol/L) to inhibit active uptake. From 30 minutes onwards the effluent solution was sampled at 5 minute intervals to determine the degree of inhibition of uptake. In this experiment the glucose concentration of the perfusion solution was made approximately equal to that of blood glucose in order to minimise glucose uptake by diffusion.

Blood glucose in male Sprague-Dawley rats of 250 - 300 g body weight, which had been fasted for 18 - 23 hours was found to be $3.93 \pm 0.18$ mmol/L (mean $\pm$ SEM, $n = 14$).

The second experiment was to determine the concentration of phlorizin required to give maximum inhibition of active transport. Previous reports (Chapter 1) have suggested that a concentration in the range 0.5 to 1 mmol/L was required to achieve maximum inhibition. In this experiment the rate of glucose uptake from glucose (2.5 mmol/L) containing phlorizin (0.5 mmol/L) was compared with uptake from the same glucose solution containing phlorizin at twice the concentration (1 mmol/L). Glucose concentrations in the effluent solution in this experiment were measured by a hexokinase method in a Centrifichem analyser.
Other experiments in this series showed that phlorizin at greater than 1 mmol/L interfered with the measurement of glucose in the Centrificom analyser. As it seemed desirable to test phlorizin at a concentration of 2 mmol/L, it was necessary to change from glucose analysis by a hexokinase (Centrificom) method to an isotopic measurement (Chapter 2).

A third experiment was carried out to compare inhibition of transport by phlorizin at 0.25, 0.5, 1 and 2 mmol/L using an isotopic method for measurement of glucose. In this experiment the glucose concentration of the perfusion solution was 4 mmol/L. The experiment was also carried out to compare inhibition of transport by phlorizin at 2 mmol/L with that at 1 mmol/L using a glucose concentration of 10 mmol/L for the perfusion.

The fourth experiment was to show that inhibition of active transport by phlorizin was reversible under the steady state conditions used in this study, and that phlorizin had no permanent effect on the transport capacity of the villous epithelium. In this experiment jejunal segments were perfused first with glucose (2.5 mmol/L) and then with the same glucose solution containing phlorizin (1 mmol/L). These results were compared with those obtained by perfusing the glucose solutions with and without phlorizin in the opposite order; first with phlorizin and then without, enabling recovery of active transport activity to be observed.

3.2.2 METHODS

Perfusions of isolated jejunal segments were performed as described in Chapter 2 (General Methods).

3.2.3 RESULTS

In the experiment to determine the time required to achieve a steady state after the addition of phlorizin to inhibit active transport, it was found that inhibition was established within 20 minutes of
the change-over to the phlorizin-containing solution. This is shown in Figure 3.4 which shows the rate of glucose uptake during successive 5-minute intervals after phlorizin was introduced.

In the second experiment, to determine the concentration of phlorizin which gave maximum inhibition of active transport, it was found that phlorizin gave significantly greater inhibition at 1 mmol/L than at 0.5 mmol/L (p < 0.005, Table 3.2).

In the third experiment, after changing to isotopically-labelled glucose for the measurement of glucose uptake, inhibition of active uptake by phlorizin at 0.25, 0.5, 1 and 2 mmol/L was tested in the presence of glucose at 4 mmol/L.

It was found that phlorizin gave significantly greater inhibition at 0.5 mmol/L than at 0.25 mmol/L (p < 0.005), and phlorizin at 1 mmol/L gave greater inhibition than at 0.5 mmol/L (p < 0.05). Phlorizin at 2 mmol/L showed a slight tendency to give greater inhibition of glucose uptake than at 1 mmol/L (Figure 3.5). At a glucose concentration of 10 mmol/L, phlorizin at 2 mmol/L also showed a tendency to give greater inhibition of glucose uptake than at 1 mmol/L (Table 3.3).

Perfusion of the solutions in opposite order, first with the inhibitor and then without, showed that inhibition by phlorizin was reversible, and that the inhibitor had no lasting effect on the transport activity of the epithelium (Table 3.4).

3.2.4 DISCUSSION

Full inhibition of glucose uptake by phlorizin occurred within about 15 minutes of the change-over to the phlorizin-containing solution (Figure 3.4). Since the time taken for the solution to traverse the gut segment and to come into contact with all of the villous epithelium is approximately six minutes, inhibition of active transport occurs within approximately 10 minutes of contact of phlorizin with the glucose-transport system.
Figure 3.4

Rate of glucose uptake in the jejunum from a perfusion solution containing glucose (4 mmol/L) followed by a solution containing glucose (4 mmol/L) with phlorizin (1 mmol/L, mean ± SEM, n = 6).
Comparison of the rates of glucose uptake in the jejunum (mmol/min/kg dry tissue) from glucose (2.5 mmol/L) with phlorizin (0.5 mmol/L and 1 mmol/L, mean ± SEM, n = 8)

<table>
<thead>
<tr>
<th>Substrate perfused</th>
<th>Phlorizin-resistant glucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose + phlorizin (0.5 mmol/L)</td>
<td>$0.43 \pm 0.03^a$</td>
</tr>
<tr>
<td>glucose + phlorizin (1 mmol/L)</td>
<td>$0.25 \pm 0.05^b$</td>
</tr>
</tbody>
</table>

Significant differences; Student’s t-test, single-tailed

\[ a > b \ (p < 0.005) \]
Figure 3.5
Rate of glucose uptake in the jejunum from a perfusion solution containing glucose (4 mmol/L), in the presence of increasing concentrations of phlorizin (mean ± SEM, n = 6 at zero phlorizin, n = 4 at other points). Significant differences; Student's t-test, single tailed; a > b, a > c, c > d (p < 0.05); a > d, a > e, b > c, b > d, b > e, c > e, (p < 0.005).
Table 3.3

Rate of glucose uptake in the jejunum (mmol/min/kg dry tissue) from $^{14}$C-glucose (10 mmol/L) followed by $^{14}$C-glucose (10 mmol/L) + phlorizin (1 mmol/L) compared with phlorizin (2 mmol/L, mean ± SEM, n = 5).

<table>
<thead>
<tr>
<th>Substrate perfused</th>
<th>Total uptake</th>
<th>Phlorizin-resistant uptake</th>
<th>Difference (active uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose followed by glucose + phlorizin 1 mmol/L</td>
<td>4.25 ± 0.26</td>
<td>1.66 ± 0.32 *</td>
<td>2.59 ± 0.54</td>
</tr>
<tr>
<td>Glucose followed by glucose + phlorizin 2 mmol/L</td>
<td>4.37 ± 0.24</td>
<td>1.13 ± 0.12 *</td>
<td>3.24 ± 0.17</td>
</tr>
</tbody>
</table>

Differences not significant; Student's t-test, single-tailed.
Table 3.4

Rate of glucose uptake in the jejunum (mmol/min/kg dry tissue) from glucose (2.5 mmol/L) with and without phlorizin (1 mmol/L) (mean ± SEM, n = 4).

<table>
<thead>
<tr>
<th>Substrate perfused</th>
<th>Uptake from glucose + phlorizin</th>
<th>Uptake from glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + phlorizin followed by glucose</td>
<td>0.08 ± 0.05*</td>
<td>1.03 ± 0.12**</td>
</tr>
<tr>
<td>Glucose followed by glucose + phlorizin</td>
<td>0.20 ± 0.08*</td>
<td>1.15 ± 0.12**</td>
</tr>
</tbody>
</table>

Differences, * and **, not significant; Student's t-test, single-tailed.
A series of experiments was undertaken in order to determine the concentration of phlorizin giving maximum inhibition of active transport. The intention was to subsequently measure the passive processes of uptake under a variety of conditions in the presence of phlorizin at a concentration sufficient to block active transport.

It was found initially that phlorizin gave greater inhibition of uptake at 1 mmol/L than at 0.5 mmol/L, however the concentration of phlorizin could not be further increased while using the hexokinase (Contrifichem) method for measuring glucose because phlorizin at 2 mmol/L was found to interfere with the assay.

This necessitated a changeover to an isotopic method for the measurement of rate of glucose uptake. The use of phlorizin at more than 1 mmol/L introduced a problem of solubility of the inhibitor. The limit of solubility of phlorizin is about 1 g in 1 L of water at 22°C (Merck Index 1976) which gives a concentration of approximately 2.3 mmol/L.

Poor solubility of phlorizin in Ringer's solution at concentrations above 1 mmol/L has been reported by Ferraris et al. (1986a). It was found that phlorizin at 2 mmol/L dissolved completely after approximately 2 hours stirring at 70°C.

Perfusion experiments using this solution revealed a tendency toward greater inhibition by phlorizin at 2 mmol/L than at 1 mmol/L, at glucose concentrations of both 4 mmol/L and 10 mmol/L, but the differences were not significant. Since glucose at 4 mmol/L is approximately equal to blood glucose in the fasting rat, it would be expected that net uptake by diffusion would be zero at this concentration.

The residual net uptake of glucose from a perfusion solution with a glucose concentration of 4 mmol/L and a phlorizin concentration of 2 mmol/L therefore appears to be active uptake which is resistant to phlorizin at the highest concentration at which the inhibitor can be used. This result suggests that it is
not possible to suppress active uptake completely with phlorizin in order to allow measurement of the passive processes of uptake.

An alternative explanation is possible in that the concentration of glucose in the enterocyte is unknown, and that, at an intra-luminal glucose concentration of 4 mmol/L, approximately equal to the concentration in blood, there may be diffusion of glucose into the enterocyte from both the luminal side and the serosal side. This explanation is made more likely by a report (Nicholls et al. 1983) showing that glucose taken up from the lumen at low intra-luminal concentration, is not exported to the blood but is metabolized within the enterocytes.

Debnam and Levin (1972, 1975) have shown that phlorizin at 0.5 mmol/L is sufficient to abolish the transfer potentials generated by the circulating sugars, and this suggests that, at that concentration, active transport should be virtually completely blocked.

Inhibition of sugar-evoked increases in transmural potentials and currents in the small intestine by phlorizin was also observed by Schultz and Zalusky (1964), Roso and Schultz (1971) and Hoshi et al. (1986). Hopfer et al. (1973) showed that phlorizin inhibited D-glucose efflux from vesicles isolated from intestinal brush border membranes. Toggenburger et al. (1978, 1982), reported that phlorizin inhibition of D-glucose in rabbit intestinal vesicles is mainly due to its binding with the glucose binding sites.

The inhibition of active uptake of glucose by phlorizin is reversible, as has been reported by Ponz and Lalarde (1952) and Ponz and Lluch (1955). Alvarado and Crane (1962, 1964), Alvarado (1967) and Rodriguez et al. (1982) have shown that phlorizin is a competitive inhibitor of the active transport of glucose.

In this study it was found that phlorizin at a concentration of 2 mmol/L did not completely block the active transport of glucose. Stevens et al. (1984) have concluded that diffusion, both simple and facilitated, accounts for glucose exit across the basolateral membrane to the portal blood. Other research workers have found
that glucose transport by preparations of microvillous brush border membranes is much more sensitive to phlorizin inhibition than is that of the basolateral plasma membrane (Murer et al. 1974, Hopfer et al. 1976).

Another possibility is that the residual phlorizin-resistant uptake in the presence of phlorizin at 1 or 2 mmol/L is a phlorizin resistant alternative enzyme.

One problem in kinetic studies of nutrient uptake from perfused segments of intestine concerns the 'true' substrate concentration which is presented to the absorptive sites on the villous epithelium. Active transport of nutrients from the gut lumen to the tissues generally conforms to the Michaelis-Menten model in which the rate of active uptake (v) is given by:

\[
v = \frac{V \cdot s}{K_m + s}
\]

where\( V \) = maximum rate of active uptake
\( s \) = substrate or nutrient concentration, and
\( K_m \) = 'Michaelis constant' or nutrient concentration giving half the maximum rate of active uptake.

The concentration of substrate used for the perfusion (s) is the highest concentration available to the villous epithelial tissues at the beginning of the segment (\( s_{\text{entry}} \)). As the solution travels through the segment, a portion of the substrate is taken up and the concentration available to the absorptive surface is reduced. If s is less than a concentration sufficient to saturate the carrier, v is reduced below the maximum rate of uptake (V); the magnitude of the reduction depending on s in relation to \( K_m \).

The concentration of s at the exit end of the perfused segment (s_{exit}) can be varied by altering the flow rate. If the flow rate is increased, s_{exit} is increased and vice versa.
It is desirable that $S_{\text{exit}}$ be reduced sufficiently to allow a reliable measurement of total uptake ($S_{\text{entry}} - S_{\text{exit}}$). But if $S_{\text{exit}}$ is too low in relation to $S_{\text{entry}}$, the villous epithelium from the entry to the exit of the segment encounters a wide range of substrate concentrations and the data are then more difficult to interpret.

Atkins (1981) has discussed the problem of determining what is the appropriate substrate concentration to use in analysing kinetic data from perfusion experiments. A number of alternatives was considered and tested and it was concluded that the 'integrated average' concentration of Fisher and Parsons (1953) was appropriate.

In this model the fall in substrate concentration is assumed to follow a first order decay;

$$S_{\text{exit}} = S_{\text{entry}} e^{-kx}$$

where $k$ is a constant related to rate of uptake and $x$ is distance along the segment.

Integration then yields the integrated average of Fisher and Parsons (1953);

$$S_i = S_{\text{entry}} - S_{\text{exit}}/\ln (S_{\text{entry}}/S_{\text{exit}})$$

The table below shows the calculated integrated average substrate concentrations where $S_{\text{entry}}$ is set at 100% and $S_{\text{exit}}$ is given values from 70% down to 30%.
<table>
<thead>
<tr>
<th>$S_{\text{entry}}(%)$</th>
<th>$S_{\text{exit}}(%)$</th>
<th>$S_i(%)$</th>
<th>$S_{\text{arithmetic mean}}(%)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>70</td>
<td>84.1</td>
<td>85</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>78.3</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>72.1</td>
<td>75</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>65.5</td>
<td>70</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>58.1</td>
<td>65</td>
</tr>
</tbody>
</table>

The value for $S_i$ becomes progressively lower than the arithmetic mean of the values for $S_{\text{entry}}$ and $S_{\text{exit}}$ as a higher proportion of the substrate is taken up during passage of the solution through the gut segment.

A second problem concerns the effect of the mucous layer which overlies the villous epithelium. In Chapter 1 we have referred to the effect of the unistirred water layer on the rate of nutrient absorption. This effect is caused by the depletion of substrate in the solution immediately adjacent to the substrate binding sites. There is then a diffusion gradient set up between the bulk of the solution in the lumen and solution adjacent to the binding sites.

The effect of the layer of mucus which overlies the villous epithelium must also be considered. The mucus layer forms part but not necessarily all of the unstirred water layer and the properties of the mucus may affect the diffusion of substrate to the absorptive sites. In the case of a molecule with minimal charge the effect is probably small.

The overall effect of the mucous and unstirred water layers is to provide a lower concentration at the absorptive site than is present in the lumen. Therefore, while it is satisfying to use an integrated average to arrive at an appropriate mean concentration of substrate in the perfused segment, the concentration at the absorptive site will be less than this.

A third complicating factor concerns the effect of the distribution of absorptive sites on the villi on substrate concentration. The concentration of substrate close to the brush border on cells near
to tips of the villi could be expected to be greater than that near the base of the villi and close to the crypts. The difference in concentration of substrate at these different sites is not known.

In summary two points are important; first, the concentration of substrate at the absorptive site is not precisely known, but is less than that in the lumen and, second, the absorptive sites throughout the gut segment will be subject to a range of substrate concentrations.

For these reasons we have chosen to report the concentrations of substrate used in these experiments as $s_{entry}$, the concentration perfused into the segment.

The values for glucose concentrations in solutions entering and leaving the perfused segments in a set of experiments using different substrate concentrations (glucose), with and without phlorizin, are shown below (data from Table 5.1). The integrated average ($S_i$ is also shown).

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>Substrate absorbed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose (mmol/L)</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>$s_{entry}$</td>
<td>$s_{exit}$</td>
<td>$S_i$</td>
</tr>
<tr>
<td>Without phlorizin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.99</td>
<td>1.63</td>
</tr>
<tr>
<td>4.0</td>
<td>1.92</td>
<td>2.84</td>
</tr>
<tr>
<td>10.0</td>
<td>4.91</td>
<td>7.15</td>
</tr>
<tr>
<td>With phlorizin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.01</td>
<td>2.25</td>
</tr>
<tr>
<td>4.0</td>
<td>3.29</td>
<td>3.63</td>
</tr>
<tr>
<td>10.0</td>
<td>8.16</td>
<td>9.05</td>
</tr>
</tbody>
</table>

Where the substrate concentration is low (2.5 mmol/L), 60 per cent of the substrate was absorbed in the single pass and the integrated average substrate concentration ($S_i$) was 65 per cent of the concentration at entry. In the presence of phlorizin only 20 per
cent of the substrate was taken up and \( S_i \) was 90 per cent of \( S_{\text{entry}} \). The use of phlorizin to block active uptake therefore raises the effective substrate concentration available for diffusion. When a higher substrate concentration was used (10 mmol/L), \( S_i \) was 72 per cent of \( S_{\text{entry}} \) without phlorizin and 91 per cent with phlorizin, so the difference between \( S_{\text{entry}} \) and \( S_i \) persists but is less.

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CHAPTER 4

UPTAKE OF GLUCOSE FROM MALTOSE SOLUTIONS COMPARED TO THE
UPTAKE OF GLUCOSE FROM A GLUCOSE SOLUTION

SUMMARY

As part of a study of the effects of vitamin A deficiency on gut function, it was necessary to see whether the kinetics of glucose uptake from a maltose solution were different from the kinetics of glucose uptake from an equivalent glucose solution. If there is no difference, the effects of vitamin A deficiency on energy uptake from carbohydrate sources could be explored using glucose solutions only.

Rats were anaesthetized and segments of the duodenum, jejunum and ileum cannulated. Iso-osmolar solutions containing maltose (5 mmol/L) with and without phlorizin (1 mmol/L) were perfused through the segments. Glucose uptake from maltose (5 mmol/L) was compared to glucose uptake from an equivalent glucose solution (10 mmol/L). The rate of phlorizin-resistant uptake indicated the proportion of uptake accounted for by diffusion.

The pattern of glucose uptake from maltose (5 mmol/L) was found to be similar to that of glucose uptake from an equivalent glucose solution. Thus uptake of glucose from maltose appeared to have no kinetic advantage over uptake of glucose from an equivalent glucose solution. The rate of active uptake decreased significantly with distance down the intestine, while diffusion was found to be significantly more rapid in the jejunum. The necessity for the hydrolysis of maltose before the uptake of glucose did not significantly reduce the rate of uptake. During passage through the intestinal segment almost all of the maltose was hydrolysed, even in the presence of phlorizin.
4.1 INTRODUCTION

Vitamin A deficiency is a problem in many developing countries (Appendix 1). It is characteristic of many of these countries that the populations are reliant on a single starchy staple such as rice or corn. Starch is broken down, mainly by pancreatic alpha-amylase, to maltose and lesser quantities of isomaltose and limit dextrins. The final process of breakdown to monosaccharide is brought about by maltase and isomaltase activities which form part of the membrane of the brush border or microvilli of the villous epithelial cells. The final product is glucose which is absorbed by both active transport and diffusion, into the epithelial cells, and from there moves into the portal blood. Maltose is therefore one of the major substrates provided to the wall of the gut for hydrolysis and absorption.

Glucose, potentially available for absorption, is therefore presented to the absorptive surface as maltose. Thus, if maltose has any kinetic advantage over glucose as a substrate for the absorptive site, the possible effects of vitamin A deficiency on absorptive function would need to be investigated using maltose as substrate rather than glucose.

4.1.1 Disaccharidase Activity

Several disaccharidases are located on the outer membrane of the intestinal epithelial cells. These include maltase-glucoamylase (E.C. 3.2.1.20), sucrase-isomaltase (E.C. 3.2.1.48-10), trehalase (E.C. 3.2.1.28), and lactase-phlorizin hydrolase (E.C. 3.2.23-62). Both maltase-glucoamylase and sucrase-isomaltase are complexes of two enzymes and both have maltase activity (Sjostrom et al. 1983). Glucose produced from maltose is transported into the epithelial cells by carrier processes also located in the brush border membrane, thus the processes for disaccharide hydrolysis and for transport of the products are located close together. Despite high activity of the brush border disaccharidases, large amounts of monosaccharides do not accumulate in the intestinal lumen suggesting that there may be some interaction between the surface digestive process and the transport mechanism (Gray 1981).
4.1.2 Disaccharidase-Linked Transport

It has been suggested that disaccharide hydrolysis at the mucosal membrane may be closely associated with monosaccharide transport and that the monosaccharide products of a disaccharidase may have a kinetic advantage in transport (Crane 1966, Ramaswamy et al. 1974). Evidence for this was based on apparently separate routes of transport for glucose from sucrose and for free intra-luminal glucose. The experiment was based on initial rate studies using everted segments of hamster jejunum. In addition, it was found that the established $V_{\text{max}}$ for glucose transport was substantially exceeded when sucrose or maltose was added to a glucose solution which apparently was already saturating glucose transport. Supporting evidence has come from studies on the rates of transport of $^3$H-labelled glucose liberated by phlorizin hydrolase compared to the rate of transport of $^{14}$C-labelled glucose.

Gray and Ingelfinger (1966) however, in uptake studies in the human gut, observed that the absorption of sugar from sucrose (73 mmol/L) was the same as absorption from a mixture of glucose (73 mmol/L) and maltose (73 mmol/L). This suggests that, at that concentration, rates of uptake were equal. Sandle et al. (1983) investigated disaccharidase-linked transport of glucose in human subjects by perfusing maltose (112 mmol/L) in the presence of free glucose (112 mmol/L), the glucose concentration being sufficient to saturate the active component of glucose transport. Glucose transport from the maltose plus glucose solution was less than the sum of the transport rates for the maltose and glucose solutions perfused separately, thus failing to provide support for the existence of disaccharidase-linked transport.

These experiments however, were carried out at a relatively high concentration of substrate. Various values for the $K_m$ of active transport of glucose have been reported, and the value appears to be in the range 4 - 15 mmol/L for both rat and man (Fordtran 1975,
Rodriguez et al. 1982). Therefore, if there is a kinetic advantage for the transport of glucose produced by disaccharidase action over the free monosaccharide, it should be most easily detected at low substrate concentration where the diffusion component of transport is minimal.

4.1.3 Aims

In this chapter we propose to describe the characteristics of glucose absorption from maltose under steady state conditions in the anaesthetized rat, and to test the hypothesis that the kinetics of uptake of glucose from maltose are the same as those governing the uptake of glucose from an equivalent glucose solution. Uptake was studied in the steady state situation to mimic post-prandial conditions. It also seemed desirable to study uptake at a low concentration of substrate so that active uptake, defined as phlorizin-sensitive uptake, would provide the major part of total uptake.

In previous studies it has been shown that, with glucose at 5 mmol/L in the lumen, phlorizin-sensitive uptake is 95 per cent of total uptake in the duodenum, 65 per cent in the jejunum and 68 per cent in the ileum (Dryden, P. and Read, R.S.D., unpublished). Since the absorptive characteristics of the intestine vary with distance distal from the stomach (Booth 1968), it appeared desirable to test the uptake of glucose in three separate regions of the intestine.

The hypothesis that the kinetics of glucose uptake from maltose and from glucose solutions are the same, would be tested by looking for significant differences in the rates of uptake under defined conditions. In the experiments described below it was found that there were, in general, only minor differences between glucose uptake from maltose as compared to that from an equivalent glucose solution. Therefore at the substrate concentration tested, glucose released by disaccharidase action does not appear to have a kinetic advantage with regard to uptake.
4.2 METHODS

The method for perfusion is as described in Chapter 2, General Methods.

4.3 RESULTS

4.3.1 Inhibition of Glucose Uptake from Maltose by Phlorizin

In these experiments the rate of uptake of glucose from maltose was investigated in perfused sections of the gut in the anaesthetised rat. When a maltose solution (5 mmol/L) was perfused through a duodenal segment at a flow rate of 0.125 mL/minute, the rate of glucose uptake increased to a plateau level at 20 minutes and continued at that rate for at least 70 minutes (Figure 4.1). This figure also shows glucose uptake from maltose (5 mmol/L) in the presence of phlorizin (1 mmol/L), an inhibitor of active transport of glucose. A low level of uptake of glucose continued in the presence of phlorizin and this phlorizin-resistant transport represents uptake by diffusion.

The active transport of glucose from maltose was fully inhibited within approximately 20 minutes of the start of perfusion with the phlorizin-containing maltose solution. This is demonstrated in Figure 4.2 which shows the rate of glucose uptake during successive 5-minute intervals after the change-over to the phlorizin-containing solution.

The time taken for a solution to traverse the gut segment was observed to be approximately six minutes. Thus the phlorizin-containing solution would require about six minutes to come into contact with all of the villous epithelium. Inhibition of active transport occurs, therefore, within approximately 15 minutes of contact of phlorizin with the glucose transport system.
Figure 4.1

Rate of glucose uptake in the duodenum from a perfused solution of maltose (5 mmol/L) (○—○) and from a solution of maltose (5 mmol/L with phlorizin (1 mmol/L, •••) (mean ± SEM, n = 3).
Figure 4.2

Rate of glucose uptake in the jejunum from a perfusion solution containing maltose (5 mmol/L, ○○○) followed by maltose (5 mmol/L) with phlorizin (1 mmol/L, ●●●) (mean ± SEM, n = 3).
4.3.2 Rates of Uptake of Glucose from Maltose and Glucose Solutions

The rate of active uptake of glucose from maltose became progressively less with distance down the small intestine. Table 4.1 shows the rates of uptake of glucose from the maltose and glucose solutions in the duodenum, jejunum and ileum, both in the presence and absence of phlorizin.

The significances of the differences observed in the rates of glucose transport from the maltose solution in the different segments of the gut, assessed by Student's t-test (one-tailed) were:

- **Active transport (phlorizin sensitive)**
  - duodenum > jejunum, jejunum > ileum (p < 0.005)
- **Diffusion (phlorizin resistant)**
  - jejunum > duodenum (p < 0.05)

And for the rates of glucose transport from the glucose solution:

- **Active transport**
  - duodenum > jejunum (p < 0.005); jejunum > ileum (p < 0.05).
- **Diffusion**
  - jejunum > duodenum (p < 0.05)
  - jejunum > ileum (p < 0.005)

Comparison of rates of glucose uptake from the maltose solutions with that from the glucose solutions (Figure 4.3) shows that the rates of total uptake in the three successive intestinal segments were generally similar for the two sugars. The rates of active transport in the duodenal segments as well as in the jejunal and ileal segments, were not significantly different (p > 0.05).

Diffusion was significantly greater in the duodenum for glucose from the glucose solution as compared to glucose from maltose (p < 0.05). A similar tendency was evident for the ileum but the difference was not significant.
<table>
<thead>
<tr>
<th>Substrate Perfused</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>5.36 ± 0.35</td>
<td>3.60 ± 0.39</td>
<td>2.12 ± 0.19</td>
</tr>
<tr>
<td>(total uptake)</td>
<td>0.53 ± 0.14</td>
<td>1.43 ± 0.31</td>
<td>0.85 ± 0.19</td>
</tr>
<tr>
<td>Glucose + phlorizin (diffusion)</td>
<td>4.23 ± 0.38</td>
<td>(n = 6)</td>
<td>2.17 ± 0.25</td>
</tr>
<tr>
<td>difference: (active uptake)</td>
<td>6.66 ± 0.50</td>
<td>(n = 6)</td>
<td>3.94 ± 0.46</td>
</tr>
<tr>
<td>Glucose + phlorizin (diffusion)</td>
<td>5.72 ± 0.56</td>
<td>(n = 6)</td>
<td>2.33 ± 0.57</td>
</tr>
<tr>
<td>difference: (active uptake)</td>
<td>6.04 ± 0.16</td>
<td>(n = 6)</td>
<td>1.51 ± 0.25</td>
</tr>
</tbody>
</table>

Table 4.1: Rates of glucose uptake from maltose (5 mol/L) with and without phlorizin (1 mol/L), and from glucose (10 mol/L) with and without phlorizin (1 mol/L), mean ± SEM.

**Significant differences; Student’s t-test; single-tailed.**

- c > d, e > f, g > h, i > j, p < 0.005
- b > a, g > f, j > k, f > a, p < 0.05
Comparison of rate of glucose uptake from maltose (5 mmol/L) with that from glucose (10 mmol/L), with and without phlorizin (1 mmol/L), where T represents rate of total uptake; D, rate of uptake by diffusion (phlorizin-resistant); and A, rate of active uptake (phlorizin-sensitive). Significant differences; Student's t-test, single-tailed; b > a (p < 0.05)
4.3.3 Hydrolysis of Maltose During Passage Through the Gut

Analysis of the maltose perfusion solutions, with and without phlorizin, after passage through the gut, showed that 80 per cent or more of the maltose emerging from the segment had been hydrolysed to glucose, both in the presence and absence of phlorizin (Figure 4.4).

4.4 DISCUSSION

Glucose is absorbed from a maltose solution perfused through the gut of the anaesthetized rat. In these experiments the rate of uptake of glucose from a maltose solution of 5 mmol/L in three segments of the gut was found to be generally similar to the rate of uptake of glucose from an equivalent solution of glucose (10 mmol/L) (Oenzil et al. 1985).

For both glucose uptake from maltose and for glucose uptake from the glucose solution, the activity of the transport process expressed as mmol/min/kg dry weight of tissue, decreased with distance down the intestine.

The decrease in rate of uptake with distance distal from the stomach is in general agreement with previous studies, though some determinations in rats have shown more rapid transport in the jejunum than the duodenum (Booth 1968). Illundain et al. (1979) have studied glucose uptake in the rat and found that uptake by diffusion from a 10 mmol/L glucose solution in the presence of 0.5 mmol/L phlorizin was approximately 35 per cent.

In this study we found that, under slightly different conditions, (1 mmol/L phlorizin and a slower flow rate) diffusion accounted for 26 per cent of uptake, giving a generally similar result. Comparison of the rates of active uptake, defined as phlorizin-sensitive uptake, for glucose from maltose as compared to glucose from a glucose solution, showed no significant difference for the duodenum, jejunum and ileum. Thus, there is no evidence for the existence of a kinetic advantage for the transport of glucose from maltose over the transport of glucose from an equivalent glucose
Figure 4.4

Concentration of maltose and glucose in maltose perfusion solutions (initially 5 mmol/L), with and without phlorizin (1 mmol/L) after passage through the respective gut segments (mean ± SEM, n = 6).

G: glucose; M: maltose.
solution in these segments of the gut, at the substrate concentration tested. This supports the hypothesis that glucose is transported similarly whether it is derived from maltose or glucose and agrees with the results of Gray and Inglefinger (1966) and Sandie et al. (1983) who investigated glucose transport from maltose at higher substrate concentrations, 73 to 112 mM/L.

Uptake by diffusion, defined as phlorizin-resistant uptake, was highest in the jejunum for both glucose from maltose and glucose from the equivalent glucose solution. This is in agreement with the observation that the jejunum appears to have a more leaky membrane than either the duodenum or ileum (Grim 1962).

An interesting observation was that in the duodenum uptake by diffusion was greater from the glucose solution than from the maltose solution. An explanation for this is not readily apparent. It might be suggested that the necessity for hydrolysis of maltose keeps the glucose concentration at the membrane slightly lower than is the case for the glucose solution. However, it has been shown that maltase activity is sufficient to hydrolyse maltose more rapidly than the glucose can be absorbed (Figure 4.4), so glucose derived from maltose might be expected to be at much the same concentration at the membrane as glucose from the glucose solution. A possible explanation is that the rate of diffusion of maltose through the unstirred layer to the membrane would be slower for the larger maltose molecules than for glucose, thus leading to a lower concentration of glucose from maltose. Effective mixing reduces the thickness of the unstirred layer (Dietschy and Westergaard 1975, Wilson and Dietschy 1974) so that an increased flow rate in the gut segment would reduce the effect of molecular size on access to the membrane and could be used to test this possibility. If it was shown that the unstirred layer reduces access of maltose to the membrane more than that of glucose, it would require a reassessment of the question of kinetic advantage in the transport of glucose derived from maltose.
The results obtained in these experiments suggest that the rate of hydrolysis of maltose is unlikely to be rate limiting in the absorption of glucose from maltose. The high level of brush border maltase activity is shown by the fact that the residual sugar emerging unabsorbed from the distal end of the intestinal segment was substantially hydrolysed to glucose. Only 5 - 20 per cent of the unabsorbed maltose remained in the disaccharide form (Figure 4.4). Misch et al. (1980) have demonstrated the formation of membrane bodies from the microvilli. It is suggested that these microbodies could carry active membrane-bound maltase and other enzymes into the lumen. This is consistent with the findings of Dahlqvist and Borgstrom (1961) that the lumen of the intestine may contain a substantial proportion of the total maltase activity. However, it has been found (Reed, R.S.D. and Dryden, P.A., unpublished) that there is no maltase activity in eluate solutions.

There appears to have been no previous systematic study of the transport of glucose from maltose in different regions of the gut of the rat using an in vivo steady state perfusion technique. In these experiments we have shown that the rate of uptake of glucose from maltose is generally similar to the rate of uptake of glucose from a glucose solution of equivalent total glucose concentration, in each of the gut segments studied. Active uptake is highest in the duodenum while diffusion is most rapid in the jejunum, and the necessity for the hydrolysis step prior to glucose uptake from maltose appears to have little affect on the uptake rate. On the evidence available, glucose uptake from maltose appears to have no kinetic advantage over glucose uptake from an equivalent glucose solution.
CHAPTER 5

GLUCOSE UPTAKE BY DIFFUSION AND SOLVENT DRAG

SUMMARY

As part of a study of the effects of vitamin A deficiency on gut function, a series of experiments was undertaken to clarify the processes that contributed to total passive (non-energy dependent) glucose uptake. Rates of uptake of glucose were measured using a single-pass perfusion of jejunal segments in anaesthetized rats. Inhibition of uptake by phlorizin was used to differentiate active from passive uptake.

As expected, the rate of water uptake was shown to increase when the osmotic pressure of the perfusing solution was decreased. It was also found that the rate of uptake of water from the gut was significantly increased as the glucose concentration was increased in a perfusion solution which was maintained iso-osmotic. The rate of exsorption of glucose from the intestinal tissues to the lumen was found to be insignificant in normal animals.
5.1 INTRODUCTION

Glucose is absorbed from the lumen of the gut by both active uptake and passive (non-energy dependent) processes. Passive uptake apparently involves two components, true diffusion and uptake by solvent drag. Net uptake of glucose by diffusion can only occur down a concentration gradient, thus net transport of glucose from the lumen to the blood by diffusion occurs only when the luminal concentration of glucose is higher than that in the blood.

The lateral space between the villous epithelial cells normally contains a sodium-rich hyperosmolar solution. The high sodium concentration is maintained by the ATP-driven sodium pump which moves sodium across the basolateral membrane of the epithelial cells into the intercellular spaces. Sodium is able to diffuse outwards from the intercellular spaces to the lumen through the tight junctions between the epithelial cells, resulting in a supply of sodium ions to the lumen from the intercellular hyperosmolar solution. Water molecules are able to pass through the tight junctions as well as the cell membrane, and the maintenance of the hyperosmolar solution in the intercellular space leads to a net absorption of water by diffusion.

The passive movement of non-electrolytes across membranes such as that of the villous epithelium cannot always be described by simple diffusion expressions because, where there are transmembrane hydrostatic or osmotic pressure gradients, there can be coupling of solute and solvent flow. The net inwards movement of water through the tight junctions is believed to assist the inwards movement of low molecular weight solute molecules from the lumen to the intercellular space, providing what is termed solvent drag.

Glucose stimulation of water uptake:
The active uptake of glucose is coupled to that of sodium (Chapter 1) so that the presence of glucose in the lumen increases the rate of entry to the tissues of both glucose and sodium. The resulting osmotic shift facilitates the uptake of water from the lumen.
Ricklis and Quastel (1958) showed that the presence of glucose in the lumen of perfused small intestine in the guinea pig markedly increased the absorption of sodium ions and water. This was subsequently shown to be true for most mammalian species including the human. In both humans and experimental animals, increasing luminal glucose concentrations have been shown to increase the rate of sodium and water absorption, reaching a plateau of stimulation of transport at glucose concentrations above 50 mmol/L in the jejunum (Fordtran et al. 1968, Modigliani and Bernier 1971, Sladen and Dawson 1969).

Exosorption of glucose:
Glucose may pass from the blood stream into the intestinal lumen, a process termed exosorption. Non-actively absorbed sugars are exsorbed at a rate which is proportional to plasma concentration (Axon 1971). The intracellular glucose concentration of the villous epithelium of the gut in the rat during absorption is influenced more by the concentration of sugar in the plasma than that in the lumen (Leese 1974). It has been shown that there is a linear correlation between the concentration of glucose in the blood and the rate of glucose exosorption, a factor which may be of significance in the pathogenesis of diabetic diarrhoea (Levine et al. 1980).

More recently, Fischer and Lauterbach (1984) demonstrated that permeation of 3-O-methyl-D-glucose and 6-methyl-D-glucoside in the direction blood-to-lumen was mainly peracellular in isolated mucosa from guinea-pig small intestine. Diamond and Bossert (1967) suggested that isotonic transport might arise from hypertonicity within the lateral intercellular spaces. Weinstein and Stephenson (1981) have modified their standing gradient model to take account of a possible secondary route of fluid entry to the lateral intercellular spaces via the tight-junctions.

In this section two experiments were conducted. The first investigated the relationship between the glucose concentration of the intraluminal solution and the rate of water uptake from the lumen. Solutions of normal osmotic pressure (304 mOsm/kg), containing varying concentrations of glucose were perfused through
jejunal segments as described in Methods (Chapter 2). Net water uptake was determined from the change in concentration of the non-absorbable marker, phenol red.

The rate of egress of glucose from the intestinal tissue to the lumen was also determined in this experiment by measuring glucose in the glucose-free solution which had been perfused through the gut segment.

The second experiment was aimed at determining whether uptake by solvent drag made a significant contribution to total uptake. Segments were perfused with a glucose solution (2.5 mmol/L) containing phenol red. At this concentration there will be no passive diffusion into the blood; fasting blood glucose being approximately 4 mmol/L. The osmolality of the solutions was adjusted with sodium chloride. The osmotic pressures of the perfusion solutions used were 304 mOsm/kg (iso-osmolar solution) and 240 and 188 mOsm/kg (hypo-osmolar solutions). Solutions were perfused, first without, then with phlorizin (1 mmol/L), allowing separate determinations of total and passive uptake. The rate of active uptake was calculated by subtracting the rate of phlorizin-resistant uptake from total uptake. The uptake of glucose by solvent drag was demonstrated under conditions in which a low intra-luminal concentration of glucose was used to prevent net uptake by simple diffusion, and active uptake was blocked with phlorizin.

5.2 METHODS

The methods used for the perfusion studies were generally as described in Chapter 2. Where solutions of different osmolalities were used, the solutions contained phenol red together with glucose, at the concentrations given in the tables, and the osmolality was adjusted by varying the quantity of sodium chloride.
5.3 RESULTS

5.3.1 Glucose Stimulation of Water Uptake

In the first experiment it was found that the presence of glucose (2.5 mmol/L) in the perfusion solution significantly increased the rate of water uptake (p < 0.05, Table 5.1). Increasing the concentration of glucose to 4 and to 10 mmol/L gave further progressive increases in the rate of water uptake (p < 0.005).

Glucose Exsorption:
Perfusion with a solution of sodium chloride in phenol red (glucose-free solution) showed no measurable rate of glucose exsorption in 5 animals out of 7, and exsorption rates of 0.012 and 0.002 mmol/min/kg dry tissue in two animals. It was concluded that the rate of exsorption of glucose in normal animals was very low, and in fact, was too low to be measurable in the present system.

5.3.2 Glucose Uptake by Solvent Drag

In the experiment to investigate the contribution of uptake by solvent drag to the total rate of uptake, jejunal segments of normal rats were perfused with solutions having osmolalities adjusted to 304, 240 and 188 mOsm/kg. As might be expected, there was a marked increase in the rate of water uptake when the osmotic pressure was reduced from 304 to 240 and to 188 mOsm/kg in the absence of phlorizin (Table 5.2). However, also as might be expected, there was no significant increase in the rate of active (phlorizin-sensitive) uptake with the reduction in osmotic pressure.

With the perfusion of the iso-osmotic solution, the presence of phlorizin caused a significant reduction in the rate of water uptake (p < 0.01). When the osmotic pressure of the perfusing solution was lower (240 and 188 mOsm/kg) the rate of water uptake was high and the presence of phlorizin had no significant effect on the rate of water uptake (Table 5.2).
Table 5.1

Water uptake (mL/min/kg dry tissue) from solutions of varying glucose concentration, and constant osmotic pressure (304 mOsm/kg) perfused through a jejunal segment (mean ± SEM).

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Substrate perfused</th>
<th>Water uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose-free solution (NaCl + phenol red)</td>
<td>17.2 ± 5.0&lt;sup&gt;a&lt;/sup&gt; (n = 7)</td>
</tr>
<tr>
<td>2</td>
<td>Glucose 2.5 mmol/L</td>
<td>38.5 ± 7.5&lt;sup&gt;b&lt;/sup&gt; (n = 7)</td>
</tr>
<tr>
<td>3</td>
<td>Glucose 4 mmol/L</td>
<td>51.8 ± 7.8&lt;sup&gt;c&lt;/sup&gt; (n = 12)</td>
</tr>
<tr>
<td>4</td>
<td>Glucose 10 mmol/L</td>
<td>79.6 ± 5.3&lt;sup&gt;d&lt;/sup&gt; (n = 5)</td>
</tr>
</tbody>
</table>

Significant differences: Student’s t-test, single-tailed;

a < b, c < d (p < 0.05); a < c, a < d, b < d (p < 0.005).
Table 5.2

Effect of changing osmotic pressure on the rate of glucose uptake from a perfusion solution containing glucose (2.5 mmol/L), with and without phlorizin (1 mmol/L), at three osmotic pressures, 304 (iso-osmotic) 240 and 188 mOsm/kg (mean ± SEM, n = 7)

<table>
<thead>
<tr>
<th>OSMOLALITY mOsm/kg</th>
<th>Glucose mmol/min/kg</th>
<th>Water mL/min/kg</th>
<th>Glucose mmol/min/kg</th>
<th>Water mL/min/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>304</td>
<td>1.25 ± 0.07</td>
<td>38.5c ± 7.5</td>
<td>0.31a ± 0.03</td>
<td>13.1f ± 5.3</td>
</tr>
<tr>
<td>240</td>
<td>1.28 ± 0.06</td>
<td>98.4d ± 4.7</td>
<td>0.37 ± 0.05</td>
<td>111.4g ± 9.6</td>
</tr>
<tr>
<td>188</td>
<td>1.41 ± 0.09</td>
<td>213.3e ±12.4</td>
<td>0.40b ± 0.03</td>
<td>188.5h ± 9.1</td>
</tr>
</tbody>
</table>

Significant differences; Student’s t-test, single-tailed;
- b > a (p < 0.05); c > f (p < 0.01)
- c < d, d < e, f < g, g < h (p < 0.005).
Reduction in the osmotic pressure of the perfusing solution was found to give a small but significant increase in the rate of phlorizin-resistant uptake (p < 0.05), thus providing evidence for the existence of uptake by solvent drag.

5.4 DISCUSSION

Results obtained in these experiments show that under steady state conditions, there is significant absorption of water even when the perfusion solution is iso-osmolar. This is presumably brought about by the existence of a hyperosmolar solution in the intercellular spaces. There is a marked increase in the rate of absorption of water from a perfused jejunal segment when glucose is present. Perfusion of glucose at 10 mmol/L increased water absorption as much as 4.6 fold as compared with the glucose-free solution (Table 5.1). This is in accord with the current model (Chapter 1) in which glucose assists the uptake of Na⁺, leading to a more rapid rise in the sodium concentration in the intercellular spaces and a more rapid uptake of water.

Other research workers have found that, at an intra-luminal glucose concentration of 50 mmol/L in the jejunal segment, glucose increased sodium absorption by a factor of four, and water absorption as much as six-fold (Fordtran et al. 1968, Modigliani and Bernier 1971, Sladen and Dowson 1969); values which are comparable with this study. They demonstrated that at glucose concentrations above 50 mmol/L a plateau of stimulation of absorption of sodium and water was achieved. Brown and Ammon (1981) demonstrated that in jejunal perfusion studies on 12 healthy volunteers, glucose absorption can stimulate water absorption directly without sodium being present in the medium and that water movement follows glucose at a rate which maintains approximate isotonicity. Sodium for the transport process is presumably provided by leakage outwards through the tight junctions from the hyperosmotic solution in the intercellular space.
Suppression of water uptake by phlorizin indicates that the active transport of glucose mediates an osmotic shift which favours water uptake. Phlorizin at 1 mmol/L was found to suppress water uptake by 66 per cent. This compares with the findings of Smyth and Taylor (1955) who observed an 80 per cent inhibition of water transport when using phlorizin at a concentration of 0.25 mmol/L in an in vitro intestinal preparation.

In this study we were unable to detect a significant rate of exsorption of glucose from the tissues to the lumen in normal animals. Other investigators found that hyperglycaemia increases the efflux of glucose into the intestinal lumen (Axon 1971, Axon et al. 1975, Levine et al. 1980), and in experiments on rats made hyperglycaemic as a result of streptozotocin-induced diabetes mellitus we were able to show significant exsorption of glucose (Chapter 6).

Uptake of glucose by solvent drag was demonstrated but only from a solution which was markedly hypo-osmotic. Csaky and Autenrieth (1975), and Vinardell and Bolufer (1983) have proposed that water entry to the villous epithelial tissue may be substantially paracellular. This paracellular uptake could therefore provide the driving force for transport by solvent drag. Prior studies (Visscher et al. 1945, Curran and Solomon 1957, Nalin et al. 1972), suggest that the osmolality of either hypo- or hyperosmotic intestinal contents is adjusted rapidly to be iso-osmotic with plasma. Miller et al. (1979) showed that both hypo- and hyperosmotic solutions of sodium chloride are adjusted toward iso-osmolality with plasma by the dual mechanisms of water and solute movement.

Work in the early 1960's by Curran (1960) and later by Diamond (1979) established that transporting epithelia, like that of the small intestine can transport fluid from the luminal (mucosal) to the serosal side, against considerable osmotic pressure gradients (up to between 50 and 200 milliosmoles). In rabbit ileum, Naftalin and Tripethy (1986) showed transport of water against gradients of approximately 75 milliosmoles per kg of sucrose, sodium chloride or mannitol.
Whittenbury et al. (1980) demonstrated that solvent transport of large solutes (such as sucrose, inulin and dextran) by solvent drag indicates paracellular water flow in leaky epithelia such as guinea-pig gall bladder. Mullen et al. (1985) have recently demonstrated that both solvent drag (solvent flow) and solute drag (solute flow) can play significant roles in the transepithelial movement of solute, as well as affecting permeability to solute in the rat ileum in vivo.

The low rate of glucose uptake observed in these experiments suggests that solvent drag does not provide a quantitatively important mechanism of uptake, at least at low intra-luminal glucose concentrations.

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CHAPTER 6

JEJUNAL UPTAKE OF GLUCOSE BY SOLVENT DRAG IN VIVO
IN STREPTOZOTOCIN-DIABETIC RATS

SUMMARY

As part of a study on the effect of vitamin A deficiency on glucose uptake, using a rat model, experiments were carried out to determine whether uptake by solvent drag made a significant contribution to total uptake. Streptozotocin was used to establish diabetes mellitus in rats, providing a situation of sustained high blood glucose and hyperosmolality. Comparison of uptake in hyperosmotic animals with that in control animals enabled the study of uptake by solvent drag.

Streptozotocin-treated rats developed the classic signs of type I diabetes mellitus and also showed an increased weight of both small intestine and intestinal mucus. A single pass perfusion technique was used to determine the rate of glucose uptake in the jejunum of streptozotocin-diabetic rats under conditions in which a low intraluminal glucose concentration was used to prevent net uptake by diffusion, and active uptake was blocked with phlorizin.

The rate of both active uptake and total uptake of glucose per unit length of fresh tissue in diabetic rats was higher than that in control animals. A change in the osmotic pressure of the intraluminal solution had no effect on active transport. The rate of water uptake was suppressed by phlorizin, indicating, as in the case of normal animals, that the active transport of glucose mediates an osmotic shift which favours water uptake. A significant increase in phlorizin-resistant uptake of glucose in hyperglycaemic diabetic rats when the osmotic pressure of the perfusion solution was decreased from 318 to 290 mOsm/kg confirmed that uptake of glucose by solvent drag does occur. A back-diffusion of glucose from the blood into the jejunal lumen of diabetic animals was also observed.
6.1 INTRODUCTION

As part of a study investigating the effect of vitamin A deficiency on glucose uptake, it was necessary to investigate the contribution of passive uptake processes to overall uptake. Passive uptake includes both diffusion and uptake by solvent drag. Diffusion is bi-directional and the rate of diffusion is affected by the characteristics of the cell membrane through which it occurs. Net uptake by diffusion can only occur down a concentration gradient, from a region of high solute concentration to one of low concentration.

Uptake by solvent drag has been proposed to occur as a process in which solute molecules are drawn into the epithelial tissue as a result of a net inflow of water molecules to a hypotonic solution in the inter-cellular space. Sodium which enters cells by diffusion or as a result of active uptake processes, is actively pumped out of the cell by an ATP-driven pump located in the basolateral membrane. There is a net movement of sodium ions outwards from the intercellular spaces to the lumen across the tight junctions and a net movement of water inwards. If the net inwards movement of water contributes to the total passive uptake of glucose (uptake by solvent drag), it should be possible to demonstrate this process under conditions where net movement of glucose by conventional (free) diffusion is minimised.

The diabetic rat appeared to be a useful model in which to investigate uptake by diffusion and solvent drag. The advantage provided by this model is the maintenance of a high level of glucose in the plasma, together with elevated plasma osmotic pressure, increased intestinal permeability and the opportunity to minimize net inwards diffusion of glucose at a higher intra-luminal concentration of glucose. The model is not without disadvantages since insulin affects numerous interrelated processes in the body, making some results difficult to interpret.
Previous in vitro studies have demonstrated an increased rate of intestinal uptake of nutrients such as sugars in streptozotocin-diabetic rats, but the effect of diabetes on the in vivo absorption of glucose remains controversial.

Thomson and Rajotte (1985) found that glucose uptake in vitro was greater in untreated diabetic rats than in control animals due to a higher maximal transport rate, a higher passive permeability of the jejunum, and a lower value of the apparent Michaelis constant. The rate of glucose absorption in vivo is slightly increased in the isolated small intestine of STZ-diabetic rats (Csaky and Fischer 1981), and it was proposed that sustained high blood sugar induces the synthesis of new carrier sites which are most likely located in the basolateral membrane. However, failure to account for the resistance of the unstirred water layer leads to gross errors in the estimation of the kinetics of active and passive transport (Thomson 1981, Hotke et al. 1985). So the kinetic basis of enhanced intestinal absorption of glucose is disputed.

Streptozotocin can be used to produce irreversible diabetes mellitus in rats, resulting in a marked decrease or complete absence of granulos in the beta cells of the islets of Langerhans (Pakieton 1963, Whiting et al. 1982).

6.2 METHODS

6.2.1 Induction of Diabetes Mellitus

Male Sprague-Dawley rats (body weight 160 - 200 g) were injected intraperitoneally with streptozotocin (STZ), 75 mg/kg body weight, dissolved in citrate buffer, pH 4.5, immediately before use. Each control animal was injected with an equivalent volume of the buffer (1 mL/kg body weight).

Animals were housed in Nalgene plastic metabolism cages and provided with tap water and a pelleted diet ad libitum (GR2+, Clark King & Co., Melbourne). Body weight gain, food consumed, water intake and urine output of each animal were measured every morning between 8.00
and 10.00 am. Rats which showed poor weight gain, polyphagia, polyuria and polydipsia were defined as having diabetes mellitus and were used in perfusion experiments.

Seven to eight days after injection, the rats were fasted on the morning of the experiment (one to six hours). A blood sample was taken from the tail, collected in sodium fluoride-EDTA tubes and blood glucose measured within ten minutes by a glucose oxidase method, using a glucose analyser (YSI, model 27, Industrial Analyzer, Yellow Springs, Ohio, U.S.A.).

6.2.2 Perfusion System

In vivo perfusions of isolated jejunal segments were performed as described in Chapter 2 (General Methods).

Preliminary experiments were carried out to determine the osmolality of rat blood. Osmolalities were determined using a freezing point osmometer (Osmometer Automatic, Knauer). Sodium chloride was added to adjust the osmolalities of the perfusion solutions to the required level. On the seventh day, STZ-injected (diabetic) rats had a random blood osmolality, in blood samples drawn by cardiac puncture, of 318 ± 2 mOsm/kg \( (n = 5) \), with a blood glucose reading of 20.29 ± 2.03 mmol/L, while in healthy control rats, random blood osmolality \( (n = 3) \) was 304 ± 3 mOsm/kg, with a blood glucose value of 6.77 ± 0.31 mmol/L.

At the conclusion of the perfusion, the animals were sacrificed by intracardiac injection of pentobarbitone solution and the 10 cm-length jejunal segment as well as the remainder of the small intestine removed. Each total small intestine together with the segment was squeezed firmly from the centre to the ends to remove mucus. The mucus was collected onto paper and weighed. The intestines, segments and mucus were dried at 105°C overnight and weighed. Glucose uptake is expressed both as \( \mu \text{mol/min/m} \) small intestine length and \( \text{mmol/min/kg} \) dry tissue, while water uptake is as expressed as \( \text{mL/min/kg} \) dry tissue.
6.3 RESULTS

6.3.1 Body Weight

Control rats gained weight steadily after injection with citrate buffer (pH 4.5) until the end of the experimental period (seven to eight days). However, the STZ-injected group showed a reduction in body weight on the first day after injection. These animals gained weight more slowly than the controls (Figure 6.1). On day 7, the body weight of the STZ-injected rats was significantly less than that of the controls (p < 0.01).

6.3.2 Food Intake

On the first day after injection of STZ, the intake of food by the diabetic rats was significantly less than that of the controls (p < 0.005). However, during the six days, there was a steady increase in food intake in the STZ-group (Figure 6.1). Finally (on day 7), the food intake of the STZ-injected group was significantly higher than that of the controls (p < 0.005). The food intake of the STZ-group was 60 per cent greater than that of the control group.

6.3.3 Fluid Balance

Throughout the experiment, water intake and urine production remained relatively constant in the control group. On the first four days after injection of STZ, water intake and urine production increased markedly to reach a plateau level which was maintained until the end of the 7 to 8-day experimental period. At day 7 to 8, the water intake in the STZ-group was approximately seven times that of the controls, while urine production in the STZ-rats was 25 times that of control rats (Figure 6.2).
Figure 6.1

Food intake and body weight gain of streptozotocin-diabetic (●●●) and control rats (○○○) (mean ± SEM). Significant differences; Student's t-test, single-tailed; a > b, c > d (p < 0.005); e > f (p < 0.01).
Figure 6.2

Water intake (○—○) and urine production (●—●) of control and streptozotocin-diabetic rats.
6.3.4 Weight of Small Intestine and Mucus Content

The effect of STZ-injection on the weight of the small intestine and mucus is shown in Table 6.1. The weight of the intestine, both fresh and dry, as a percentage of body weight, was significantly greater in the STZ-injected rats as compared to the controls (p < 0.005). The fresh and dry weights of mucus in the STZ-group was significantly higher than in the controls (p < 0.005 and p < 0.05 respectively).

6.3.5 Blood Glucose

Rats with poor weight gain, polyphagia, polyuria and polydipsia developed hyperglycaemia (Table 6.2). Perfusion with an iso-osmolar or hypo-osmolar glucose solution (10 mmol/L) in the STZ-group had no effect on the blood glucose level, while perfusion of the solution in the control rats produced a significant reduction in blood glucose (p < 0.005).

6.3.6 Glucose and Water Uptake

Perfusion with a solution of lower osmolality in STZ-injected rats resulted in a significant increase in the rate of water uptake, both in the presence and absence of phlorizin (p < 0.01, Table 6.3). There was a tendency for an increase in the rate of water uptake in STZ-diabetic animals as compared with controls. With regard to water uptake from a hypo-osmolar solution (290 mOsm/kg), there was a significant increase both with and without phlorizin (p < 0.005). In the presence of phlorizin, water uptake was significantly suppressed in control rats (p < 0.005). In STZ-diabetic rats, phlorizin showed a tendency to suppress water uptake, but the effect was not significant.

The rate of total uptake of glucose from a hypo-osmolar solution (10 mmol/L) was significantly higher in the STZ-diabetic group than in controls (p < 0.005), while there was a tendency for the rate of total uptake from an iso-osmolar solution to be higher in the STZ-diabetic group than in controls (Table 6.4).
Table 6.1

Weight of small intestine and weight of intestinal mucus in control and STZ-diabetic rats (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>STZ- diabetic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small intestine</strong> (per cent of bodyweight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>$4.48^{a} ± 0.20$ (n = 9)</td>
<td>$2.55^{b} ± 0.06$ (n=6)</td>
</tr>
<tr>
<td>dry</td>
<td>$0.97^{c} ± 0.04$ (n=7)</td>
<td>$0.59^{d} ± 0.01$ (n=6)</td>
</tr>
<tr>
<td><strong>Mucus (mg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fresh</td>
<td>$1.03^{e} ± 0.08$ (n=5)</td>
<td>$0.57^{f} ± 0.09$ (n=4)</td>
</tr>
<tr>
<td>dry</td>
<td>$0.16^{g} ± 0.02$ (n=5)</td>
<td>$0.12^{h} ± 0.02$ (n=4)</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed;

- $a > b$, $c > d$, $e > f$ ($p < 0.005$);
- $g > h$ ($p < 0.05$).
Table 6.2

Blood glucose concentration (mmol/L) after perfusion of a solution of glucose (10 mmol/L) through a jejunal segment for 30 minutes, in rats which had been fasted for several hours (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Before perfusion</th>
<th>After perfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>318 mOsm/kg (n=5)</td>
<td>15.46 ± 0.37</td>
<td>15.35 ± 0.74</td>
</tr>
<tr>
<td>290 mOsm/kg (n=5)</td>
<td>16.85 ± 1.13</td>
<td>17.04 ± 0.57</td>
</tr>
<tr>
<td>Control rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsm/kg (n=6)</td>
<td>6.48 ± 0.18^a</td>
<td>4.94 ± 0.42^b</td>
</tr>
</tbody>
</table>

Significant differences; Student’s t-test, single-tailed;
  a > b (p < 0.005).
Table 6.3

Rate of water uptake in the jejunum (mL/min/kg dry tissue) from glucose (10 mmol/L) with and without phlorizin (1 mmol/L, mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>From glucose</th>
<th>From glucose + phlorizin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetic rat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>318 mOsm/kg (n = 5)</td>
<td>91.7 ± 15.6^a</td>
<td>77.2 ± 19.3^d</td>
</tr>
<tr>
<td>(iso-osmolar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsm/kg (n = 6)</td>
<td>180.4 ± 24.9^b</td>
<td>149.7 ± 13.6^e</td>
</tr>
<tr>
<td>(hypo-osmolar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control rat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsmol (n = 6)</td>
<td>72.4 ± 3.8^c</td>
<td>46.0 ± 6.9^f</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed;

b > a, e > d (p < 0.01); b > c, e > f, c > f (p < 0.005).
Table 6.4

Rate of glucose uptake in the jejunum (μmol/min/m fresh tissue) from glucose (10 mmol/L) with and without phlorizin (1 mmol/L, mean ± SEM) in STZ-diabetic and control rats.

<table>
<thead>
<tr>
<th></th>
<th>From glucose</th>
<th>From glucose + phlorizin</th>
<th>Active uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(total uptake)</td>
<td>(phlorizin-resistant)</td>
<td>(phlorizin-sensitive)</td>
</tr>
</tbody>
</table>

**STZ-diabetic rats**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>318 mOsm/kg (n=5)</td>
<td>7.82 ± 0.87</td>
<td>2.97 ± 0.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.85 ± 0.67</td>
</tr>
<tr>
<td>(iso-osmolal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsm/kg (n=6)</td>
<td>8.67 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.80 ± 0.26&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.87 ± 0.60</td>
</tr>
<tr>
<td>(hypo-osmolal)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Control rats**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>290 mOsm/kg (n=5)</td>
<td>6.05 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.99 ± 0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.05 ± 0.36</td>
</tr>
<tr>
<td>(approx. iso-osmolal)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant differences; Student's *t*-test, single-tailed;

- *c* > *e* (*p* < 0.05), *a* > *b*, *d* > *e* (*p* < 0.005).
Phlorizin-resistant uptake of glucose from iso-osmolar and hypo-osmolar solutions was also significantly greater in the STZ-diabetic group than in controls (p < 0.05 and p < 0.005 respectively). There was a tendency for an increase in the rate of phlorizin-sensitive uptake (active uptake) of glucose in STZ-diabetic rats per unit length of fresh tissue. In the STZ-diabetic group, with the perfusion of a hypo-osmolar solution (290 mOsm/kg), there was a tendency towards an increased rate of total glucose uptake with the increasing water uptake, but the difference was not significant. For phlorizin-resistant uptake, there was a tendency towards an increased rate of glucose uptake at 290 mOsm/kg.

In STZ-diabetic rats, phlorizin suppressed glucose uptake at iso-osmotic and hypo-osmotic pressure, by 62 and 56 per cent respectively. Phlorizin suppressed water uptake as well as glucose uptake. The rate of glucose uptake per unit dry weight of tissue for phlorizin-resistant uptake in the STZ-diabetic group is shown in Table 6.5. There was a significant increase in rate of uptake when the osmolality was reduced from 318 mOsm/kg to 290 mOsm/kg (p < 0.05), thus providing evidence for the existence of glucose uptake by solvent drag in diabetic animals. The rate of phlorizin-resistant uptake from the hypo-osmolar solution was significantly greater in the STZ-diabetic group than in the controls (p < 0.05). But the rate of total uptake and phlorizin-sensitive (active) uptake expressed per unit dry weight of jejunum was found to be similar to that of the control animals.

6.3.7 Exsorption of Glucose from Plasma to Lumen

Perfusion of the jejunal segment with a solution of sodium chloride in phenol red (a glucose-free solution) revealed a release of glucose from intestinal tissue to the intestinal lumen. Perfusion with an iso-osmolar solution showed a tendency toward a higher rate of glucose efflux to the lumen than occurred with a hypo-osmolar solution (290 mOsm/kg, Table 6.6). The results in Table 6.6 also show that there was no significant increase in the rate of water uptake when the osmotic pressure was decreased from 318 to 290 mOsm/kg.
Table 6.5

Rate of glucose uptake in the jejunum of control and STZ-diabetic rats (mmol/min/kg dry tissue) from glucose (10 mmol/L) followed by glucose (10 mmol/L) with phlorizin (1 mmol/L, mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>From glucose (total uptake)</th>
<th>From glucose with phlorizin (phlorizin-resistant)</th>
<th>Difference (phlorizin-sensitive)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetic rats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>318 mOsm/kg</td>
<td>3.33 ± 0.25</td>
<td>1.27 ± 0.19</td>
<td>2.05 ± 0.23</td>
</tr>
<tr>
<td>(iso-osmolar, n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsm/kg</td>
<td>3.80 ± 0.26</td>
<td>1.66 ± 0.13</td>
<td>2.14 ± 0.27</td>
</tr>
<tr>
<td>(hypo-osmolar, n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control rats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290 mOsm/kg</td>
<td>3.77 ± 0.16</td>
<td>1.23 ± 0.14</td>
<td>2.53 ± 0.25</td>
</tr>
<tr>
<td>(approximately iso-osmolar, n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant differences; Students t-test, single-tailed;

b > a, b > c (p < 0.05)
Table 5.6

Glucose release into the jejunum as compared with blood glucose and water uptake after perfusion from a glucose-free solution (sodium chloride and phenol red, 318 mOsm/kg) followed by a similar solution of lower osmotic pressure (290 mOsm/kg) in STZ-diabetic rats (mean ± SEM, n = 4).

<table>
<thead>
<tr>
<th>Perfusion solution</th>
<th>Glucose release mmol/min/kg dry tissue</th>
<th>Blood glucose mmol/L</th>
<th>Water uptake mL/min/kg dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>318 m Osmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iso-osmolar)</td>
<td>0.37* ± 0.17</td>
<td>21.13 ± 1.54</td>
<td>107.85 ± 15.55</td>
</tr>
<tr>
<td>290 m Osmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(hypo-osmolar)</td>
<td>0.07* ± 0.02</td>
<td>18.89 ± 0.99</td>
<td>109.49 ± 23.36</td>
</tr>
</tbody>
</table>

* Differences not significant; Student's t-test, single-tailed.
6.4 DISCUSSION

Diabetes mellitus can be produced in experimental animals by injection of streptozotocin (STZ), the diabetic animal then provides a useful model in which to study particular aspects of glucose absorption.

Seven to eight days after intra-peritoneal injection of a single, high dose of STZ (75 mg/kg body weight), the classical signs of type I diabetes mellitus develop, including poor weight gain, polyphagia, polydipsia, polyuria and hyperglycaemia, together with a blood glucose level more than two and a half times the level in controls.

Rakieten (1963) found that, in the rat, polydipsia, polyuria and glycosuria but no ketonuria accompanied the hyperglycaemia resulting from the administration of STZ (50 mg/kg body weight). The hyperglycaemia was present in the rat from 2 to 206 days after treatment. Whiting et al. (1982) found that a single i.p. injection of STZ (35 mg/kg body weight), maintained a stable, mild diabetes in rats, with a hyperglycaemia of over 30 mmol/L. Sandler and Jansson (1985) reported that a single intravenous dose of 160 mg/kg body weight or multiple low dose daily injections of 40 mg/kg body weight, for up to five consecutive days, induced increased vascular permeability indicating lesions in the pancreatic islets, which were essential for the development of hyperglycaemia.

In the present experiments, the daily food intake and body weight of the diabetic rats was reduced significantly during the first day after the STZ injection. But after the second day, the daily food intake increased, so that at the end of the experimental period (7 days), food intake was significantly greater than that of the control animals. By the seventh day, body weight was significantly lower than that of the control animals. Water intake and urine output were increased after the first day injection with STZ, which agrees with the findings of Hebdon et al. (1986), who used STZ at 60 mg/kg body weight (i.p.) in the rat.
In these experiments the osmolality of the blood was found to be higher in the diabetic animals, principally because of a higher blood glucose concentration. Habden et al. (1986) found that plasma glucose, urea and osmolality were significantly higher in STZ-diabetic rats than in controls, while plasma sodium concentration was significantly lower in diabetic rats than in controls, though there was no significant difference between the plasma potassium values. Habden et al. (1986) also observed a difference between the measured osmolality and the osmolality calculated from the values for the identified, osmotically active constituents of plasma; i.e., $2(\text{Na}^+ + \text{K}^+)$ + urea + glucose, probably due to the presence of unidentified solutes.

Csaky and Fischer (1981) found that blood glucose started rising as soon as two hours after the injection of STZ (65 mg/kg body weight) into the tail vein, and reached a maximum value of about 400 mg/dL (22.2 mmol/L) at approximately 48 hours. The weight of the small intestine as a percentage of body weight, and the weight of mucus, both fresh and dry, was significantly greater in diabetic rats than in control animals.

Lorenz-Meyer et al. (1977) showed that villous hyperplasia developed in the intestines of diabetic rats having free access to food. The hyperplasia was characterised by an increase in the area of the villous surface, caused by increased mitotic activity in the crypts. The positive correlation between daily food intake and the villous height suggests that hyperphagia and increased nutrient supply to the epithelium had a role in the development of the hyperplasia.

Several studies have shown increased intestinal mass in animals diabetic for 14 days or longer (Karasov and Diamond 1983). The effect of diabetes mellitus of most relevance to intestinal absorption is reduced uptake and metabolism of glucose by the tissues and the consequent loss of glucose in the urine, resulting in increased dietary energy intake to compensate. Non-specifically increased nutrient absorption through an increased mass (or surface area) of intestinal mucosa might therefore be expected.
Ballman and Conlon (1985) observed a significant increase in the total weight of the small intestine in STZ-induced diabetes in the rat, and Chatamura et al. (1983) reported an increase in the weight of both the intestine and contents in rats with STZ-induced diabetes. Earlier work (Jervis and Levin 1966, Schedl and Wilson 1971, Nakayama et al. 1974) showed that experimentally induced diabetes resulted in appreciable increases in the weights of the digestive organs compared with those of control animals. Schedl and Wilson (1971) showed that growth is localised primarily in the mucosa. Nakayama et al. (1974) observed a decrease in DNA synthesis in the intestine of diabetic animals on a restricted diet and that this was reversed following hyperphagia in animals fed ad libitum.

During the seven to eight days after STZ administration, at the time when the in vivo study of glucose absorption was carried out, the rates of total uptake of glucose, phlorizin-resistant uptake and phlorizin-sensitive (active) uptake per unit length of fresh tissue were higher in diabetic rats as compared to controls. However, the rates of glucose absorption per unit dry weight of jejunum were found to be similar to those of control animals indicating that the increased rate per unit length of intestine is due to epithelial hyperplasia.

In vitro studies have generally demonstrated increased glucose absorption by intestines of diabetic animals (Csaky and Fischer 1981, Lorenz-Moyer et al. 1977), but in vivo studies have been equivocal.

Bandyopadhyay et al. (1981) observed that the intestinal absorption of glucose did not increase in alloxan-diabetic rats or rabbits. Arvanitakis and Olsen (1976) showed that the rate of sucrose hydrolysis and absorption of monosaccharide products (fructose and glucose) were not significantly different in diabetic and control rats. But Duffy et al. (1980) found that the influx of glucose was enhanced in an in vivo study of jejunal uptake in diabetic animals.
Hotke et al. (1985) found that the effective resistance of the un-stirred water layer in vivo was higher in diabetic than in non-diabetic control rats. There was a tendency for an increase in the rate of active uptake in diabetic rats as compared to controls, which agree with the kinetic studies by Thomson (1981).

In the present study, uptake of glucose by solvent drag in diabetic rat jejunum was demonstrated under conditions in which a low intraluminal glucose concentration was used to prevent net uptake by diffusion, and active uptake was blocked with phlorizin.

The significant increase in phlorizin-resistant uptake of glucose in diabetic rats when the osmotic pressure of the perfusion solution was decreased from 318 to 290 mOsm/kg, confirms that uptake of glucose by solvent drag does occur. There was a tendency towards an increase in the total uptake of glucose from hypo-osmolar solution. A change in the osmotic pressure of the intraluminal contents has no effect on active transport.

The rate of glucose uptake is expressed both per unit length of fresh tissue and per unit dry weight of tissue. The differences between diabetic rats and control animals may relate to hypertrophy of the diabetic gut, in which case it may be more appropriate to express uptake rate in terms of length rather than dry weight. However it was found to be impossible to accurately measure the length of fresh tissue because of the elasticity of the small intestine.

Suppression of water uptake by phlorizin indicates that the active transport of glucose mediates an osmotic shift which favours water uptake.

It is probable that the increased rate of water uptake in diabetic animals related to higher intestinal permeability.
Finally, there is a significant leak of glucose in the lumen which can be detected in diabetic rats. In agreement with Axon (1971), Levine et al. (1980) observed that hyperglycaemia increases the efflux of glucose into the lumen.

These observations show that the net glucose absorption that is measured experimentally is the result of a bidirectional process which is influenced by the level of glucose in the blood. Uptake occurs by active uptake, solvent drag and diffusion. Solvent drag is more significant in the diabetic rat, as is glucose efflux, presumably due both to a more leaky intestinal epithelium and the higher level of blood glucose.

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CHAPTER 7

EFFECT OF VITAMIN A DEFICIENCY ON GLUCOSE TRANSPORT IN THE JEJUNUM

SUMMARY

The effect of vitamin A deficiency on villous epithelial structure and glucose uptake from the small intestine was studied in rats. Feeding of a vitamin A-deficient diet led to clear signs of vitamin A deficiency within 6 to 7 weeks from weaning. Treatment of the deficient animals with vitamin A as retinoic acid (5 μg/g diet), cured the signs of deficiency within three days.

The rate of glucose uptake in control and vitamin A-deficient rats was investigated. The rate of glucose uptake by diffusion was found to be significantly greater in the vitamin A-deficient animals as compared with controls (p < 0.05). In addition, there was a tendency for a higher rate of total uptake and active uptake in the vitamin A-deficient animals. The body weight of the vitamin A-deficient rats was significantly less than that of the controls (p < 0.005). There was no significant difference in the mean weight of the small intestine, fresh or dried, between the control and vitamin A-deficient groups. There was also a tendency towards decreased intestinal mucus content in the vitamin A-deficient rats.

Histological studies showed that the number of goblet cells per 50 cell nuclei around the tips of the villi in jejunal sections was fewer in the vitamin A-deficient animals than in comparison with the controls (p < 0.05). In other respects the histological appearance of jejunal sections was not altered by acute vitamin A deficiency.
7.1 INTRODUCTION

Vitamin A is known to be involved with a range of physiological processes including vision, growth, reproduction, immune function and maintenance of epithelial tissues.

Only its role in vision has been explained in detail (Chapter 1). Its involvement with the immune system and in the maintenance of mucus-secreting epithelial tissues suggests that a deficiency may make the individual more susceptible to a range of infections. Much less is known about the effect of vitamin A deficiency on other organs in the body.

Two sites where mucous membranes are of particular importance are the gut and the lung. If vitamin A affects the gut in the same way that the eye is affected, it might be expected that the mucus-secreting epithelium which covers the intestinal villi would begin to be replaced by a keratinized epithelium. This would be expected to be reflected in a progressive loss of the capacity to absorb nutrients from the intestine.

Recently, Sommer et al. (1984) carried out a study on Indonesian children which showed that vitamin A deficiency was associated with increased risk of respiratory disease and diarrhoea. Other research workers have found that the number of goblet cells in the intestinal epithelium is reduced (DeLuca et al. 1969, Rojanapo et al. 1980, Olson et al. 1981). However, Zile et al. (1981) reported that the cells of the intestinal epithelium appear to remain normal in mild vitamin A deficiency. Recently, Gwochinskii et al. (1987) have reported that, in rats, vitamin A deficiency changes the permeability of the small intestinal mucosa for macromolecules.

The aim of the present study was therefore, to study the effect of an acute deficiency of vitamin A on the characteristics of glucose uptake in the rat.
7.2 METHODS

7.2.1 Induction of Vitamin A Deficiency

Animals:
Male Sprague-Dawley rats, just after weaning at approximately 50 g weight were used. Rats were housed in individual wire cages in an environment controlled for temperature (25°C), humidity (40 - 50 per cent), and with a 12 hours light-dark cycle. The suggested optimum temperature for rats and mice is 21 ± 2°C, and a range of humidity from 40 to 70 per cent saturation is acceptable for most animal houses (Harkness and Wagner 1983). A light-dark cycle of 12 hours is satisfactory for most species (Blackshaw and Allan 1987).

Diets:
There are two principal difficulties in producing vitamin A deficiency in rats. First, there is considerable reserve of vitamin A in normally nourished animals, and second, there are difficulties in obtaining a satisfactory protein source which is free of the vitamin. In order to create a deficiency in reasonable time, weanling rats of approximately 50 g body weight were transferred to a vitamin A-deficient diet. The low body weight and rapid growth of weanling animals assists in achieving deficiency in minimum time.

The composition of the vitamin A-deficient diet is shown in Table 7.1.

Dietary constituents:
The dietary constituents and their sources are shown below.

Starch
Starch was obtained from Silver Star Starch Pty. Ltd., Melbourne. This starch is prepared from wheat in a coarse granular form for laundry use. It was ground sufficiently fine to pass a 0.3 mm sieve. The ground starch retained a slightly gritty texture and, when made up in the final diet, gave a slightly adhesive texture. A diet with this texture was found to be advantageous for
Table 7.1

Composition of the vitamin A-deficient diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>58</td>
</tr>
<tr>
<td>casein (washed with hot alcohol 3x)</td>
<td>25</td>
</tr>
<tr>
<td>vitamin mix (vitamin A free)</td>
<td>1</td>
</tr>
<tr>
<td>cellulose</td>
<td>1</td>
</tr>
<tr>
<td>mineral mix</td>
<td>5</td>
</tr>
<tr>
<td>coconut oil (deodorised)</td>
<td>10</td>
</tr>
</tbody>
</table>

Details of the ingredients are discussed in the text.
measuring food intake by the rats. Although finely powdered starch was commercially available, it produced a diet with a fine dry powdered texture. This texture was less suitable since more of it was wasted by the rats.

Casein

Casein was used as the sole protein source in the diet because of its high biological value. In initial trials commercially available vitamin-free casein (ICN Biochemicals Inc., Cleveland, Ohio) was used, however the animals were slow to develop signs of vitamin A deficiency. In view of this and because of the expense, it was decided to prepare casein for the diet.

The casein used was obtained from a local source (Colac Butter Factory, Colac, Victoria). In order to remove vitamin A and pro-vitamin A, the casein was washed four times with anhydrous ethanol (absolute alcohol, CSR Ltd., Yarraville, Melbourne). A separate batch of casein was washed twice with chloroform: methanol (2:1) and then twice with methanol. The washed casein was dried in a vacuum oven at 30°C overnight, at which time the residue of ethanol had been removed, as judged by smell.

Vitamin mixture

The vitamin mixture used was USP XVII without vitamin A (ICN Biochemicals Inc., Cleveland, Ohio). It contained (g/kg) vitamin D concentrate (850,000 IU per gram) 0.125; alpha-tocopherol (250 IU per gram) 22.0; ascorbic acid 45.0; inositol 5.0; choline chloride 75.0; menadione 2.25; p-Aminobenzoic acid 5.0; niacin 4.25; riboflavin 1.0; pyridoxine hydrochloride 1.0; thiamine hydrochloride 1.0; calcium pantothenate 3.0; biotin 0.020; folic acid 0.090; vitamin B-12 0.00135.

Mineral mixture

The mineral mixture used was USP XVII (ICN Biochemicals Inc., Cleveland, Ohio) which contained (g/kg) sodium chloride 139.3; potassium biphosphate 389.0; magnesium sulphate (anhydrous) 57.3; calcium carbonate 381.4; ferrous sulphate 27.0; manganese
sulphate 4.01; potassium iodide 0.78; zinc sulphate 0.548; cupric sulphate 0.477; cobaltous chloride 0.023.

Oil

Edible oil in the form of deodorised coconut oil (BOI Pty. Ltd., Melbourne) was added to the diet. A personal communication from the company indicated that this oil was nearly free of provitamin A. A diet with a slightly greasy texture is more acceptable to the animals than a dry diet. Coconut oil is quite low in essential fatty acids, containing about 1.8 g per 100 g fatty acids (Paul and Southgate 1978). In the final diet the level of essential fatty acids was therefore 0.4 per cent of energy. This is lower than desirable in the rat but not low enough to give rise to signs of deficiency (Pudelkewicz et al., 1968).

Dietary Fibre

Dietary fibre in the form of cellulose powder (Whatman, W & R Balston Ltd., England) was added.

Energy

The energy content of the diet was calculated to be 17 kJ/g (4.1 kcal/g). The National Research Council recommends 4 kcal/g of dry diet and a net protein content of at least 12 per cent for growth (Warner and Breuer 1972). The energy, protein, vitamin and mineral contents of the diet met the recommendations specified. The Laboratory Animal Science Association (1969), has made recommendations for an energy content between 3.6 and 3.8 kcal/g (15 - 16 kJ/g) and a total lipid content of at least 4 per cent of energy.

The vitamin A-free and control diets (Table 7.1) and water were provided ad libitum. The control group was fed an identical diet except that it contained vitamin A.

Body weight was measured twice a week, in the morning between 8.00 a.m. and 10.00 a.m.
When growth of the vitamin A-deficient animals reached a plateau, some animals were used in studies of glucose uptake, while the diet of two of the animals was supplemented with retinoic acid (5 μg/g diet) in order to confirm that the cessation of growth was due to vitamin A deficiency.

Retinoic acid (all-trans) was purchased from Sigma Chemical Company (St. Louis, U.S.A). Retinoic acid is reputed to be much less stable than vitamin A in the animal body but is stable when incorporated into tablets or capsules (Roche Products Pty. Ltd., Australia, personal communication).

Preparation, mixing and storage of the diet mixture:
The powdered starch and washed casein, which represent the major proportion of the diet, were placed in a mixer and the melted coconut oil added and mixed slowly until the oil was uniformly dispersed. The mineral mixture was then added and incorporated thoroughly. The vitamins were first mixed with cellulose then added to the other diet constituents after the mineral mixture had been thoroughly dispersed.

It was necessary to mix the oil thoroughly with the dry starch and casein prior to the addition of the minerals and vitamins in order to avoid the formation of oil balls containing higher concentrations of particular nutrients. These conglomerates might be discriminated against by the animals if the taste was different. The diet mixture was finally moistened with distilled water (50 mL/kg dry mix) and stored in closed plastic containers at approximately 4°C in a cool room. The diet mixture was freshly prepared each week to avoid nutrient losses.

When retinoic acid was added to the diet, it was dissolved in coconut oil and added to the diet mixture in a dark room. The diet mixture containing retinoic acid was prepared as close as possible to the time of feeding and for short term storage was wrapped in aluminium foil to exclude light and was refrigerated.
7.2.2 Perfusion Studies

Perfusion studies were carried out using 10 cm segments of proximal jejunum as described in General Methods (Chapter 2).

7.2.3 Histological Studies

At the conclusion of the period of perfusion, the rats were sacrificed by injecting an overdose of pentobarbitone (60 mg) intracardially. Immediately, a 1 cm-length of the proximal jejunum, distal to the perfused segment, was removed and fixed in 10 per cent neutral buffered formalin at room temperature for at least 24 hours. Fixed tissue was dehydrated in increasing concentrations of alcohol, cleared in xylene and embedded in paraffin wax. Transverse sections 4 μm thick were cut on a microtome (American Optical, model 820, H.B. Selby and Co., Melbourne). One set of sections was stained with haematoxylin and eosin, and a second set with periodic acid Schiff reagent (PAS) and counterstained with haematoxylin. The outline of the procedure for staining paraffin sections is attached (Appendix 4). The tissue sections were coded and were examined blind by both the author and a skilled hospital pathologist.

7.3 RESULTS

In an initial trial it was observed that after six weeks on the diets, the rats fed on a diet made up with casein washed with hot ethanol and to which vitamin A had been added had a significantly (p < 0.01) greater body weight than those fed on a similar diet made up with casein which had been washed with a chloroform-methanol mixture. There was a similar difference (p < 0.05) between the weights of the groups of rats fed the vitamin A-deficient diets based on ethanol-washed and chloroform-methanol-washed casein (Figure 7.1).
Figure 7.1

Growth of rats on a control diet (○○○) and a vitamin A-deficient diet (••••), each based in casein washed with hot ethanol, compared with growth of rats on a control diet (△△△) and a vitamin A-deficient diet (××××) each based in casein washed with chloroform:methanol (2:1), (mean ± SEM). Significant differences; Student's t-test, single-tailed; a > b, b > d, c > d (p < 0.05); a > c (p < 0.01).
This suggested that there was a solvent residue or some other toxic factor in the casein prepared by this method, despite the fact that the chloroform-methanol washed casein was washed finally with methanol and dried extensively to remove solvent residue. It was decided not to pursue the cause of this unexpected result but instead the extraction procedure based on chloroform-methanol was abandoned and serial extractions with hot ethanol used instead.

When fed to rats, vitamin A-free diets prepared from casein washed with hot ethanol produced growth restriction and signs of vitamin A deficiency in approximately six weeks. When the same experiment was undertaken with diets based on commercially available vitamin-free casein, growth restriction and signs of vitamin deficiency did not become apparent until week 8 (Figure 7.2). It appeared that the procedure used in our laboratory achieved a more thorough removal of vitamin A and provitamin A, and it was therefore decided that we would use vitamin A-free casein prepared in our own laboratory.

Using diets based on casein washed with hot ethanol, both the control and vitamin A-deficient groups of weanling rats showed an initial steady weight gain for several weeks after being transferred to the control and deficient diets. After the second week the rate of weight gain of the rats on the deficient diet became noticeably less than that of the controls. At 4 weeks the mean weight of the vitamin A-deficient group was consistently less than that of the control groups and after week 5 the growth rate of the vitamin A-deficient rats entered a relative plateau phase with the growth rate slowing to approximately 1.76 g/day. The control rats at that stage were growing at a rate of approximately 4 g/day.

By weeks 6 to 7 the animals showed classic signs of vitamin A deficiency, including swelling and porphyrin encrustation of the eyelids and retarded growth. Body weight was significantly less; the mean body weight of the deficient group being about 95 per cent of that of the control group.
Growth of rats on a control diet (△△) and a vitamin A-deficient diet (○○) both based on commercially supplied vitamin-free casein (ethanol-washed).

Two vitamin A-deficient rats were continued on the diet until 10.5 weeks then supplemented with retinoic acid (mean values).
In order to confirm that the inhibition of growth and the observed signs of deficiency were due to a deficiency of vitamin A, two of the animals were maintained on the vitamin A-deficient diet until week 8, by which time growth had ceased. Retinoic acid was added to the previously deficient diet (5 mg/g of diet) and it was observed that eye signs normalized and growth was restored within three days of feeding the supplemented diet (Figures 7.2 and 7.3).

Experiments on the rate of glucose uptake in the vitamin A-deficient animals showed that phlorizin-resistant uptake (diffusion) was significantly greater than in the controls (p < 0.05, Table 7.2). There also was a tendency toward a higher rate of total uptake and active uptake (phlorizin-sensitive) in the vitamin A-deficient animals. There was no significant difference between the mean weights of the small intestines, fresh or dried, of the control and vitamin A-deficient groups. There was also no difference in the dry weights of the mucus obtained from the small intestines of the two groups (Table 7.3).

No significant differences in appearance were observed between sections taken from the jejunum of vitamin A-deficient rats and sections from control animals (Figure 7.4). However, a count of the number of goblet cells per 50 cell nuclei around the tips of the villi on the sections showed vitamin A-deficient animals to have significantly fewer goblet cells than the controls (p < 0.05, Table 7.4).
Figure 7.3
Growth of rats on a control diet (●—●) and a vitamin A-deficient diet (○—○) both based on casein washed with hot ethanol. Two vitamin A-deficient rats were continued on the diet to 8 weeks and then supplemented with retinoic acid (mean ± SEM).
Table 7.2

Rates of glucose uptake in the jejunum (nmol/min/kg dry tissue) from glucose (10 mmol/L) followed by glucose (10 mmol/L) with phlorizin (1 mmol/L, mean ± SEM).

<table>
<thead>
<tr>
<th>Substrate perfused</th>
<th>Vitamin A-deficient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose (total uptake)</td>
<td>4.57 ± 0.56 (n=5)</td>
<td>3.61 ± 0.37 (n=6)</td>
</tr>
<tr>
<td>glucose + phlorizin (phlorizin-resistant uptake)</td>
<td>1.76 ± 0.18 (n=5)</td>
<td>1.20 ± 0.16 (n=6)</td>
</tr>
<tr>
<td>difference (active uptake)</td>
<td>2.82 ± 0.65 (n=5)</td>
<td>2.42 ± 0.46 (n=6)</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed; 
a > b (p < 0.05).
### Table 7.3

**Body weight and weight of small intestine and mucus of vitamin A-deficient and control rats.**

<table>
<thead>
<tr>
<th></th>
<th>Vitamin A-deficiency</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>255.7(^a) ± 7.4 (n=6)</td>
<td>298.5(^b) ± 9.3 (n=6)</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fresh (g)</td>
<td>4.88 ± 0.14 (n=6)</td>
<td>4.71 ± 0.05 (n=6)</td>
</tr>
<tr>
<td>dry (g)</td>
<td>1.18 ± 0.03 (n=6)</td>
<td>1.21 ± 0.04 (n=6)</td>
</tr>
<tr>
<td>Mucus dry (mg)</td>
<td>65.0 ± 7.6 (n=5)</td>
<td>74.6 ± 12.6 (n=6)</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed;  
\( a > b \) ( \( p < 0.005 \)).
Figure 7.4

Transverse section of rat jejunum on a control diet (above) and a vitamin A-deficient diet (below). Sections stained with PAS; magnification 150.
Table 7.4

Number of goblet cells per 50 cell nuclei around the tips of the villi of sections taken from the jejunum (mean ± SEM, n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin A-deficient</th>
<th>Control Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of goblet cells/ 50 cell nuclei</td>
<td>11.4\textsuperscript{a}± 0.7</td>
<td>13.2\textsuperscript{b}± 0.5</td>
</tr>
</tbody>
</table>

Significant differences; Student's t-test, single-tailed;
\( a < b \) (\( p < 0.05 \))
7.4 DISCUSSION

There is considerable evidence that vitamin A is involved in the development and maintenance of mucus-secreting epithelial tissues (Chapter 1). The experiments reported here support that role.

Weanling rats maintained on a diet deficient in vitamin A for 6 - 7 weeks developed signs of vitamin A deficiency, particularly involving the eyes, and growth was inhibited. The addition of retinoic acid to the previously deficient diet (5 μg/g) restored apparent health as well as normal growth to the rat, confirming that the deficiency signs were the result of vitamin A deficiency. Retinoic acid is able to meet vitamin A requirements with regard to growth and the maintenance of mucus-secreting epithelium but not for vision (Dowling and Wald 1960) or for reproduction (Thompson et al. 1964).

It was appropriate to add retinoic acid back to the diet because only external eye signs and restriction of growth were observed in these experiments, and because retinoic acid is not stored by the liver but is metabolized rapidly. Swanson et al. (1981) showed that 34 per cent of biliary metabolites of retinoic acid were reabsorbed in the intestine, thus demonstrating enterohepatic circulation.

It was found that there was a significant increase in the rate of passive (phlorizin-resistant) uptake of glucose in the vitamin A-deficient rats as compared to controls. There was a slight but non-significant increase in the rate of active uptake (phlorizin-sensitive). It is possible that the increased rate of passive uptake is due to a reduced production of mucus in the deficient animals. The mucus gel which overlies the villi, and forms part of the unstirred layer, markedly alters the kinetics of both active and passive absorption (Westergaard and Dietschy 1972).

There was a tendency towards decreased mucus production in the vitamin A-deficient rats but the level did not reach significance at the p < 0.05 level. The method used to measure mucus present in the gut was simple and not specific. The fresh weight measurement is
mostly of water retained in the gut lumen, and the dry weight measurement would be mainly mucus, but would include also salts and bacterial cells.

The possibility of involvement of reduced mucus production in the increased rate of passive uptake of glucose is strengthened by the finding of a small but significant reduction (13.6 per cent) in the number of mucus-secreting goblet cells on the villi of the vitamin A-deficient rats.

Visual inspection of the sections of tissue obtained from the vitamin A-deficient animals showed no obvious differences from equivalent sections obtained from control animals. It may be that a more prolonged period of deficiency would produce more marked effects, but at least in these experiments, the acute deficiency imposed on weanling animals had comparatively mild effects on the villous epithelium as compared to the effects on growth and on the eye.

The influence of the gut mucous layer on intestinal transport is far from established. The existence of an 'unstirred water layer' overlying the surface of the villi has been recognized and its effect on the kinetic parameters of active transport processes studied (Wilson and Dietschy 1974, Winne 1977, Thomson 1979b, Thomson and Dietschy 1980). The gut mucous layer forms part but not necessarily all of the unstirred water layer.

The findings of this study are in agreement with those of DaLuca et al. (1969) and Rojanapo et al. (1980) suggesting that vitamin A is required for the maintenance of intestinal mucus-secreting goblet cells in the rat. Rojanapo et al. (1980) found that in vitamin A deficiency, differentiation of sensitive goblet cells from oligomucous cells and other precursor cells appeared to be blocked. Other histological characteristics of the jejunum were not altered by vitamin A deficiency. Zile et al. (1981) have also reported that the morphology of rat jejunal epithelium is not significantly affected by mild vitamin A deficiency.
It has been suggested that increased intestinal permeability will lead to increased passive transport (Cooper 1986). The jejunal epithelial membrane in vitamin A-deficient rats, when compared with control animals, is relatively more leaky and this would be consistent with a more rapid uptake of glucose.

Recently, Gmoshinskii et al. (1987) found an effect of vitamin A deficiency on permeability of the small intestinal mucosa for macromolecules in adult rats. It is probable that a more permeable small intestine would allow increased absorption of antigens, toxins, carcinogens and other macromolecules which are normally excluded.

In the present experiments there was a tendency for the rate of active uptake to be increased in vitamin A-deficient animals. Anzano et al. (1979) noted that vitamin A deficiency depressed appetite which reduced food intake. It has been known since the early sixties that the small intestine of semi-starved rats possesses a greater transport capacity for sugars, amino acids and sodium ions as compared to animals fed ad libitum (Esposito et al. 1967, Hindmarsh et al. 1967, Kershaw et al. 1960, Wright and Barber 1969). Also the effect of partial food deprivation, resulting in 20 - 22 per cent loss of body weight has been reported to enhance net D-glucose transport by increasing the rate of sugar entry across the apical membranes of the enterocytes (Marciani et al. 1987).

In the present study, the body weight of vitamin A-deficient rats was approximately 14 per cent less than that of the controls, so that reduced food intake could have contributed to a possible increase in the rate of active transport.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

Vitamin A deficiency is a major problem in the developing world. It affects children particularly, and is expressed most obviously in the prevalence of xerophthalmia in particular areas of many developing countries (Appendix 1).

The physiological roles of vitamin A are as yet poorly understood. It is involved in vision, cell differentiation and probably acts as a basic regulator of gene expression (Chapter 1). Deficiency affects a range of body systems, and, whereas early work tended to concentrate on ocular manifestations of vitamin A deficiency, more recent work has emphasized that deficiency produces wide-ranging effects. The more recent demonstration of the association of vitamin A deficiency with impairment of immunological function are of obvious importance. Nutrient deficiencies seldom occur singly and under field conditions it is frequently observed that vitamin A deficiency is associated with protein-energy malnutrition, as well as deficiencies of several vitamins and minerals.

Protein-energy malnutrition is usually associated with infection and malabsorption, and the effects of nutrient deficiencies on gut function are therefore of interest. The effects of vitamin A deficiency on gut function have received only minimal attention and the studies reported in this thesis have aimed at investigating such effects. Such an association might be expected because of the known relationship between vitamin A status and the maintenance of the mucus-secreting epithelium. An association between vitamin A deficiency and absorptive dysfunction would be of obvious importance in relation to the diarrhoea which often accompanies protein-energy malnutrition.

The studies in this thesis have therefore been directed towards testing the hypothesis that vitamin A deficiency affects gut function as might be shown by glucose uptake from the gut. In the
testing of this hypothesis it has been necessary to carry out a number of other experiments. These have been directed mainly towards gaining a better understanding of the processes involved in the uptake of glucose under steady-state conditions.

A single pass perfusion technique was established which could be maintained for periods in excess of an hour, and it was possible to demonstrate steady state uptake over this period (Chapter 3). A non-absorbable marker was used to correct for net water movement, allowing glucose uptake to be calculated from the difference in glucose concentration entering and leaving a gut segment. Phenol red (phenolsulphophthalein) and \(^{14}C\)-polyethylene glycol (PEG) were tested as markers and it was found that phenol red gave more reproducible results. Phenol red as a marker also gave a better correlation with a measure of net water movement based on direct measurement of the rate of delivery of the perfusion solution into the gut segment and weighing of the total solution recovered as effluent.

Phlorizin (phloretin-2'-beta-D-glucoside) was used as an inhibitor of the active uptake of glucose to study the contributions of active and passive processes to the total rate of uptake of glucose. Previous work, reviewed in Chapter 1, has suggested that the active uptake of glucose should be almost completely inhibited by phlorizin at a concentration of approximately 0.5 mmol/L.

In these experiments however, it was found that phlorizin at 2 mmol/L gave a lower rate of uptake than at 1 mmol/L (Chapter 3). Because of the limited solubility of phlorizin it was not possible to test higher concentrations of the inhibitor, and therefore not possible to determine the concentration which would give maximum inhibition. The true value for the rate of passive uptake is therefore less, but probably only slightly less, than the rate in the presence of phlorizin at 1 mmol/L, since the rate in the presence of phlorizin at twice this concentration was only slightly less.
It was demonstrated that phlorizin acts rapidly in the in vivo perfusion, producing maximum inhibition within 15 minutes of being introduced in the perfusion solution. The inhibitor produces no apparent lasting effect on the transport capacity of the gut, as shown by the ready reversibility of the inhibition when phlorizin was removed.

Experiments designed to clarify the major factors affecting steady-state uptake were most usually carried out using perfused jejunal segments. The characteristics of the gut transport processes change, to a degree, with distance distal to the duodenum (Chapter 4). However in terms of the rates of active and passive uptake of glucose, the change in uptake capacity is not large. The rate of active uptake was found to be reduced with distance distal to the duodenum, while the rate of passive uptake was greatest in the jejunum. Similar observations have been made previously and the jejunum has been shown to be more 'leaky' than the duodenum or ileum.

Although the major part of the dietary carbohydrate supply is taken in as starch which, as a result of the activity of pancreatic amylase, is presented to the villous epithelium predominantly as maltose, it was shown to be valid to study carbohydrate uptake using perfused glucose rather than maltose solutions (Chapter 4).

When the rate of uptake of glucose from maltose solutions was compared with the uptake of glucose from solutions of glucose of equivalent concentration, the rates of uptake did not differ in the duodenum or jejunum. In the duodenum, uptake from glucose was found to be slightly but significantly more rapid from glucose than from maltose. While the reason for this was not established it could be due to more rapid diffusion of the smaller glucose molecule through the mucous (or unstirred) layer to the absorptive surface.

There are a number of aspects of an in vivo perfusion system which are either not easily controlled or are poorly understood. These complicate the interpretation of results. The effect of anaesthetic, for example, has not been explored.
In this study, uptake was determined using a steady state gut perfusion in the anaesthetized rat to mimic post-prandial conditions. Rendering the animal unconscious with anaesthetic could change the intestinal blood flow and motility, and it is apparent that intestinal motility can influence mesenteric blood flow. A range of intrinsic and extrinsic factors affect the relationship between intestinal motility and blood flow (see Appendix 3). Physiologically, an adequate blood supply is necessary for the optimal performance of absorptive function in the gut. Studies by Lee (1983) showed that a relaxation of the intestinal muscle favours water absorption, and blood flow would be higher through relaxed muscle. Olsen et al. (1971) found that anoxia, did not affect binding of D-glucose to the intestinal brush border in the rat; glucose binding however is not equivalent to steady state uptake.

The transport of sugars by the small intestinal mucosa has been investigated largely by studies on excised tissue in vitro, but the relevance of the mechanisms which have been characterized by in vitro techniques, to the overall process of absorption in vivo is not always clear. For example, one specific property of sugar transport in vitro, namely the absolute dependence of transport on the presence of sodium ions (Kimmich 1973) has not been satisfactorily reproduced in vivo (Vinardell and Bolufer 1984).

In this study, the perfusion solution was administered at a constant flow rate of 0.125 mL/min, a rate which is in the range of rates of normal stomach emptying (Malik, A. and Read, R.S.D., unpublished). Ortiz et al. (1982) found that the effective thickness of the unstirred water layer becomes less as the perfusion rate is increased. Kerlin et al. (1982) found that the flow rate of intestinal contents increases promptly after a meal and fluctuates markedly during the post-prandial period in the human.

In these experiments animals were routinely fasted for 18 to 22 hours before perfusion. Murray and Wild (1980) have found that fasting for 17 to 48 hours was associated with consistent decreases in the specific and total activity of Na-K-ATPase in the jejunum, while the level tended to rise in the more distal region in the
ileum. The Na-K-ATPase is intimately associated with active sodium transport. Thus ATP-ase activity in the animals used in the present experiments may be reduced, however we do not know if this affects transport rate. If only the sodium gradient is affected, transport rate might not be affected because sodium is provided in the perfusion solution. It should be noted, however, that the fasted animal is not necessarily equivalent, with respect to gut characteristics, to the animal allowed food freely.

Achieving a practical definition of active uptake also poses difficulties. In this study phlorizin sensitivity was used to define active uptake, however because of the limited solubility of phlorizin it was difficult to demonstrate a plateau of inhibition. It could not be shown satisfactorily that phlorizin-resistant uptake was all passive uptake. The residual phlorizin-resistant uptake of glucose in the presence of phlorizin at 1 or 2 mmol/L could be residual active uptake, uptake by a phlorizin-resistant alternative pathway, or true diffusion. It should be emphasized though, that this uncertainty applies to only a small proportion of the total uptake.

The present study showed that phlorizin at 2 mmol/L gives almost maximal inhibition of glucose uptake as evidenced by only a slight tendency towards a decreased rate of uptake at 2 mmol/L as compared to 1 mmol/L. Evans and Diedrich (1980) estimated that the apparent \( K_m \) value for phlorizin in hamster intestine was 0.6 mmol/L. Recently, Dobran (1985) showed that the concentration of phlorizin used (2 mmol/L) was the minimum necessary to produce a reversible inhibition of the glucose-induced transmural potential difference \( \text{in vivo} \) in rat intestine.

The jejunum appears to have a more permeable membrane than either the duodenum or ileum. Recently, Jones et al. (1987) found that glucose absorption from maltotriose and an oligosaccharide mixture exceeded that from paired free glucose solutions. They suggest that the stoichiometric relationship of the active hydrolysis sites for sucrose to the glucose transport system is less advantageous than that of active sites for maltose hydrolysis.
Water absorption from the small intestine is linked to uptake of intra-luminal glucose and sodium. It is readily shown that water uptake is significantly increased with decreasing osmotic pressure of the perfusion solution. In this study it was found that uptake of glucose by solvent drag does occur. This supports current recommendations that hypo-osmolar glucose-containing electrolyte solutions be used for oral rehydration in the treatment of acute watery diarrhoea. Gracey (1984) has recommended that rehydration solutions with a lower sodium content (hypo-osmolar) be used as the solutions of choice for oral therapy of patients with acute diarrhoea in Australia.

In other studies, it has been found that an efflux of glucose into the gut lumen can occur and that the rate is proportional to plasma glucose concentration. In this study this was confirmed in that significant efflux was observed in diabetic rats (hyperglycaemic) but efflux was not detectable in normal animals. The jejunum of diabetic rats also appears to have a more permeable membrane than that of control animals, since the rate of water uptake was significantly increased in diabetic animals. Levine et al. (1980) showed that glucose secretion across rat jejunum is a passive process. In men, diabetic diarrhoea is a well recognised entity, the pathogenesis of which has not previously been defined but which may be due, at least in part, to a leakage of glucose into the gut lumen.

The present study also showed that the rate of both active uptake and total uptake of glucose per unit length of fresh tissue in diabetic rats in vivo was higher than that in control animals. It is well known that glucose transport in vitro is increased in diabetic rats (Lorenz-Meyer et al. 1977, Csaky et al. 1981, Thomson and Rajotte 1985). Results obtained in this study suggest that the increased absorption is probably due to the increased mucosal surface known to occur in diabetes.

Robinson and Antonioli (1980) suggest that passage across the tight junction is facilitated by the opening and shutting of the junctional complex, perhaps in response to the peristaltic movements
of the gut. Wright and Petras (1974) put forward the hypothesis that the diffusive pathway is more important in vivo than in vitro and that the paracellular pathway for non-electrolyte movement is more prominent in leaky than in tight epithelia where grooves and ridges interlock more firmly. In this study uptake by solvent drag was shown to occur but this could only be demonstrated unequivocally in diabetic animals. The process however, is likely to be important at higher glucose concentrations; and it is not possible to separate uptake by solvent drag from uptake by diffusion at luminal glucose concentrations higher than those existing in the blood.

The central hypothesis to be tested in this study was that deficiency of vitamin A adversely affects small intestine function as shown by effects on glucose absorption. By use of a diet which included casein washed in hot ethanol to extract retinoids and carotenoids, it was possible to produce vitamin A deficiency in rats.

Perfusion studies on vitamin A-deficient rats showed that the rate of glucose uptake by diffusion was significantly increased over that in control animals. There was as well, a tendency towards a higher rate of active uptake and total uptake in the vitamin A deficient animals. The hypothesis was therefore found to be untrue; there being no indication that acute vitamin A deficiency has an adverse effect on the processes of glucose absorption from the small intestine in the rat.

The increased rate of both passive and active absorption of glucose in jejunum of vitamin A-deficient rats could be caused by a decreased thickness of the mucus layer or by increased intestinal permeability. It should also be noted that reduced food intake in the vitamin A-deficient animals could also affect glucose uptake.

Histological studies showed that the acute vitamin A deficiency produced a slight but significant reduction in the number of goblet cells around the tips of the villi, but otherwise there was no discernable effect. The reduction in number of goblet cells suggests that reduced mucus production in the gut would most likely account for the altered characteristics of glucose absorption.
The steady state perfusion system in the rat was found to be a suitable model for the study of the effects of vitamin A deficiency. However, in this study we used the situation of an acute deficiency, whereas in children in developing countries, a situation of chronic deficiency is more likely. Studies of the effects of vitamin A deficiency in an animal model could therefore usefully be extended by looking at the effects of chronic deficiency. It would also be useful to look at combined marginal deficiencies of other nutrients as might be expected to occur in protein-energy malnutrition. Because of the range of nutrients possibly involved, such a study would be complex.
REFERENCES


Laboratory Animal Science Association (1969). 'Dietary Standards For Laboratory Rats and Mice'. Laboratory Animal Handbooks 2. (Laboratory Animals Ltd.: London).


*****
APPENDIX 1

Prevalence of Vitamin A Deficiency

In 1985 the World Health Organization (WHO) listed 34 countries in Africa, Asia, scattered areas of Latin America and the Caribbean where vitamin A deficiency is, or is strongly suspected to be, a significant health problem. More than 500,000 children in these countries are estimated to become blind every year because of vitamin A deficiency, and more than two thirds of them die within a few weeks of becoming blind. Another 6 - 7 million have signs of moderate vitamin A deficiency. (Figure A1.1, Table A1.1 and Appendix 2).
FIGURE A1.1

THE GEOGRAPHICAL DISTRIBUTION OF VITAMIN A DEFICIENCY AND XEROPHTHALMIA IN THE WORLD IN 1984, BY COUNTRY CATEGORY

Control of Vitamin A Deficiency in Indonesia

The vitamin A deficiency problem in Indonesia is significant. Surveys in the early 1980s estimated that about 375,000 children under 5 years of age were suffering vitamin A deficiency, of which about one third could be expected to become blind. The problem areas identified were particularly, West Java, Central Java and North Sumatra. From a number of surveys the average prevalence of active corneal disease in the rural population was found to be 6.4 per 10,000 (Kodyat et al. 1988, footnote ref.1). Where the prevalence of xerophthalmia was 0.5 - 2.0 percent, the proportion of children with low levels of vitamin A was about 50 percent, suggesting that all of the children in that community should be treated with vitamin A.

Active corneal disease is associated with stunting and wasting, and 72 percent of cases had a history of diarrhoea in the recent past (Kodyat et al. 1988, footnote ref.1). There is thus an association of xerophthalmia with protein energy malnutrition. Serum albumin levels have been found to be inversely related to the severity of xerophthalmia, and oedema is also associated with xerophthalmia, the prevalence increasing with the severity of the disease (Sommer 1982, footnote ref.2). Severe corneal scarring and blindness also have a strong association with the occurrence of measles in the proceeding 1 - 4 weeks.

Footnotes


Nutritional strategies to control vitamin A deficiency are based most specifically on nutrition education, the use of massive oral doses of vitamin A, and the use of vitamin A-fortified foods.

In terms of nutrition education, the most important measure is to educate mothers to increase the consumption of green leafy vegetables by children of less than five years of age. The general adequacy of supply of food energy and high quality protein is also important.

Oral massive dosing with vitamin A is an important short-term measure for the control of vitamin A deficiency. A program of oral dosing twice a year with 200 000 I.U. vitamin A has been in use since 1974. A recent community based field trial in Aceh (Sommer et al. 1986, footnote ref.1) showed that periodic massive dosing with vitamin A was associated with a 35 - 70 percent lower mortality rate. Since the coverage by dosing may still not be adequate, social marketing of capsules is being tried in Central Java.

Fortification of foods with vitamin A has advantages in that the foods to be fortified can be chosen with a view to their acceptance and use by children, the target group. A national survey showed that four substances, sugar, flour, monosodium glutamate (MSG) and salt, reached the target group, and MSG was chosen as the most appropriate vehicle for fortification on the basis of the average consumption (0.23 g/day), and penetration to the target group.

A trial of vitamin A fortification was carried out from 1984; MSG being fortified to a level of approximately 3000 I.U./g, so that the mixture should deliver about 50 percent of the RDA to school children.

Footnote:
children. A survey of the villagers involved in the trial showed a significant rise in serum vitamin A at 5 and 11 months. The prevalence of Bitot's spots declined by 73 percent at 5 months and by 81 percent by 11 months, and mortality also declined. Haemoglobin was increased and linear growth, but not weight, was significantly increased (Muhilal 1988, footnote ref.1)

The problem of vitamin A deficiency is soluble and substantial progress is being made. Massive oral dosing may be required for some time but fortification of key foods and particularly nutrition education will be most effective and economical in the long term.

Footnote:

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### TABLE A1.1

**PREVALENCE CRITERIA FOR DETERMINING THE PUBLIC HEALTH SIGNIFICANCE OF XEROPTHALMIA AND VITAMIN A DEFICIENCY**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Population at risk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night blindness (XN)</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>Bitot's spot (S1B)</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Corneal xerosis/corneal ulceration/keratomalacia (X2/X3A/X3B)</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Corneal scar (XS)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Plasma vitamin A level &gt;100 µg/l</td>
<td>&gt;5.0</td>
</tr>
</tbody>
</table>


The xerophthalmia classification is as follows: night blindness, XN; conjunctival xerosis, X1A; Bitot's spot, X1B; corneal xerosis, X2; corneal ulceration/keratomalacia <1/3 corneal surface, X3A; corneal ulceration/keratomalacia >1/3 corneal surface, X3B; corneal scar, XS; xerophthalmia fundus, XF.

*Children aged between 6 months and 6 years.*

Reproduced from: *World Health Organization (WHO 1985a). Prevention and control of vitamin A deficiency xerophthalmia and nutritional blindness; proposal for a ten-year programme of support to countries (WHO 1985a).*
### APPENDIX 2

**Surveys Demonstrating Vitamin A Deficiency in Several Countries**

<table>
<thead>
<tr>
<th>Country</th>
<th>Population group</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asia:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia</td>
<td>children 0 - 6 years</td>
<td>Incidence rate 2.7 per 1000 per year, at least 63,000 Indonesian children become xerophthalnic each year.</td>
<td>Tarwotjo et al. (1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sommer et al. (1981)</td>
</tr>
<tr>
<td>India</td>
<td>children</td>
<td>An estimated 52,500 children become blind while 110,000 to 132,000 become partially blind each year. Prevalence of xerophthalmia is &gt;8%.</td>
<td>WHO (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>18,660 children</td>
<td>About 30,000 pre-school children become blind each year.</td>
<td>Cohen et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>3 months to 6 years</td>
<td></td>
<td>Helen Keller International and the Bangladesh Institute of Public Health Nutrition (1985).</td>
</tr>
<tr>
<td>Nepal</td>
<td>7,580 children under 6 years</td>
<td>Bitot’s spot (X1B) was 0.64%. Xerophthalmia-related corneal scars was 0.2%.</td>
<td>Brilliant et al. (1985)</td>
</tr>
<tr>
<td>Country</td>
<td>Population group</td>
<td>Comment</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>13,450 children</td>
<td>Public health problem in two out of 15 health areas.</td>
<td>Brink et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>6 to 71 months.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Pacific:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phillipines</td>
<td>1,715 children</td>
<td>4.5% with clinical and 47% with biochemical signs of deficiency.</td>
<td>Solon et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>1 to 16 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African region:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benin</td>
<td>1,965 children</td>
<td>0.55% with Bitot's spots; 2.6% with xerosis of the cornea; 0.2% with keratomalacia.</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malawi</td>
<td>5,400 children under 6 years</td>
<td>Overall prevalence of xerophthalmia 3.9%</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>Mauritania</td>
<td></td>
<td>2.3% with xerophthalmia (X1B and XN)</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>United Republic of Tanzania</td>
<td>7,431 children</td>
<td>0.3% with night blindness, 0.4% with xerosis of the conjunctiva, 0.9% with active corneal lesions including keratomalacia.</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td></td>
<td>between 0-10 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethiopia</td>
<td>560 children</td>
<td>2% with signs of xerophthalmia, 0.75% with corneal scars.</td>
<td>Pizzarello (1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>Population group</td>
<td>Comment</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
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<td>-------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Sudan</td>
<td>1,660 children less than 12 years</td>
<td>4.7% showed signs of xerophthalmia.</td>
<td>Pizzarello (1985)</td>
</tr>
<tr>
<td>Zambia</td>
<td></td>
<td>Night blindness was found to be endemic in Eastern, North Western, Luapula and Northern provinces (in June 1980).</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>Region of the Americas:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>10,922 children aged 0 - 12 years old in north-eastern part of Brazil</td>
<td>Population from 6 localities representative of the 3 different ecological regions of Paraiba. The regional distribution was markedly different. In the semi-arid region (Sertao) the prevalence suggested a public health problem in that area.</td>
<td>Santos et al. (1983)</td>
</tr>
<tr>
<td>Haiti</td>
<td></td>
<td>A public health problem, based on corneal scars, in the north but not in the south.</td>
<td>Sommer et al. (1976)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In the late 1970's, prevalence of corneal scars 0.81% in the northern part and 0.12% in the South.</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>Country</td>
<td>Population group</td>
<td>Comment</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>El Salvador</td>
<td>9,508 children</td>
<td>Prevalence of Bitot's spot and vitamin A-related corneal opacities was 5.3 and 3.2 per 10,000 respectively.</td>
<td>Sommer et al. (1975)</td>
</tr>
<tr>
<td>Eastern Mediterranean Region:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oman</td>
<td>1,078 children</td>
<td>Age 0 – 6 years: 5.3% had xerosis of the conjunctiva, 1.5% showed Bitot's spots; Over 6 years: 9.5% had xerosis of the conjunctiva and 4.4% conjunctival xerosis associated with Bitot's spots. Bitot's spots is higher than criteria set by WHO.</td>
<td>WHO (1985a)</td>
</tr>
<tr>
<td>Sudan</td>
<td>2,709 children 0 – 6 years</td>
<td>Bitot's spots associated with conjunctival xerosis among the younger children are</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,031 children 6 – 12 years</td>
<td>1.62%</td>
<td>WHO (1985a)</td>
</tr>
</tbody>
</table>

APPENDIX 3

Factors Affecting Intestinal Blood Flow

In summary, several factors are believed to play a role in the intrinsic regulation of intestinal blood flow. These factors include neural, myogenic, metabolic, and humoral influences (Figure A3.1).

Figure A3.2 shows several factors which play a role in the extrinsic control of intestinal blood flow. Of these, the influence of the sympathetic nervous system appears to be the most important (Granger et al. 1980).
Figure A3.1  Summary of the intrinsic mechanism contributing to the control of the intestinal blood flow. The major groups of influences are: (1) neural, (2) myogenic, (3) metabolic and (4) locally produced vasoactive agents.

(Reproduced from Granger et al. 1980).
Figure A3.2 Summary of the extrinsic mechanisms contributing to the control of intestinal blood flow. The major influences are: (1) neural - mainly sympathetic, (2) circulating vasoactive agents - released from the gastrointestinal tract and related organs or from unrelated endocrine sources, and (3) systemic hemodynamic changes which affect the intestinal circulation.

(Reproduced from Granger et al., 1980).
APPENDIX 4

Outline of Procedure for Staining Paraffin Sections

Before staining certain preparatory treatment of sections is necessary and involves the following steps. The reagents are either poured from drop bottles over the sections, or the slides are placed in jars or dishes of the reagents.

1. Removal of Wax
Paraffin wax being poorly permeable to stains, its removal with a solvent is necessary and xylol is used for the purpose. One or two min. in each of two changes of xylol is sufficient.

2. Removal of Xylol with Absolute Alcohol
Xylol is not miscible with aqueous solutions and low grade alcohols and it is therefore necessary to remove this with absolute alcohol. One or two min. in each of two changes of absolute alcohol is adequate for the purpose.

3. Treatment with Descending Grades of Alcohol
To avoid the possibility of diffusion currents causing damage and possible detachment of the section, it is standard practice to follow absolute alcohol with treatment with 90% alcohol and then 70% alcohol each for a minute or two.

4. Water
It is desirable to pass sections from a reagent in the sequence which is the same as, or similar to, the solvent or the stain to be used first. This is commonly distilled water, in the case of alcoholic stains, e.g. Weigert's Elastic Stain, transfer from the grade nearest to that of the stain solvent, in this case, absolute alcohol.

5. Staining
This may involve the treatment with a single (simple or compound) stain solution, or the use of two or more separate stains, with washing and differentiation between them.
6. **Dehydration**

In the majority of cases, paraffin sections are mounted in media miscible with xylol. It is necessary therefore, for sections to be dehydrated in alcohol before passing to xylol. Furthermore, xylol will not clear, or render sections transparent, unless completely dehydrated and opaque areas in the section, or droplets of water around the section, indicate lack of dehydration. Some stains however, are soluble in alcohol and judgement must be exercised in obtaining dehydration, without excessive extraction of the stain.

7. **Clearing**

At least two changes of xylol are required to ensure that all alcohol has been removed from the section. A clean (third) xylol change should be reserved for receiving sections prior to mounting. If transparency of the section is not achieved, then it is likely that the section has not been properly dehydrated.

8. **Mounting**

A glass coverslip is applied to protect and preserve the section. Surplus xylol is carefully wiped from around the section with a fluff-free cloth when, without allowing the section to dry, mounting media is applied as a drop or streak, the size of which will be governed by the shape and size of the section.
Routine haematoxylin and eosin method.

Method:
1. Dewax slides in xylol 1 for 3-5 min.
2. Dewax slides in xylol 2 for 3-5 min.
3. Rehydrate in absolute alcohol for 3-5 min.
4. Rehydrate in 90% alcohol for 3-5 min.
5. Rehydrate in 70% alcohol for 3-5 min.
6. Transfer slides to running tap water for 5-10 min.
   Rinse in distilled water for 1-2 min.
7. Stain in Mayer's HX 1891 for 10 min.
8. Wash in running tap water for 3-5 min (until blue colour gone).
9. Blue up in Scott's solution for 2 min.
10. Wash in running tap water for 3-5 min.
11. Rinse in 95% alcohol for 1-2 min.
12. Stain in buffered alcohol eosin for 3 min.
13. Rinse off excess eosin in three changes of absolute alcohol,
    each of approximately 1-2 min.
14. Clear in three (3) changes of xylol each of 3-5 min.
15. Mount in Depex.

Scott's solution pH approximately 8.2
  sodium bicarbonate 3.5 g
  magnesium sulfate 20 g
  distilled water 1000 mL

Dissolve the salts separately and then mix. Filter before use.

Nuclear stains - haematoxylin.
Mayer's haematoxylin 1891

Solution 1: haematoxylin 1 g
  distilled water 750 mL

Dissolve the haematoxylin in increasing amounts of the
above distilled water.
Then add, potassium aluminium sulfate 50 g.
Dissolve completely by stirring at room temperature.
Ensure that the alum is completely dissolved before
proceeding.
Solution 2: sodium iodate 0.2 g
distilled water 200 mL
Prepare separately. Add solution 2 to solution 1 with constant stirring. Allow to stand overnight. Then add in the following order, ChloraL hydrate 50 g, dissolve completely, citric acid 1 g. Filter through Whatman no. 1 filter paper. Make up to one (1) litre with distilled water.

Cytoplasmic stains: Buffered alcoholic eosin solution.
Stock solutions: 1. Saturated eosin in absolute alcohol (about 2%).
2. 0.2 mol/L sodium acetate in 95% alcohol (27.22 g/L).
3. 1 mol/L glacial acetic acid in 95% alcohol (60.05 mL/L).
4. 95% alcohol.

Working stain solutions.
166 mL saturated eosin in absolute alcohol.
83 mL 0.2 mol/L sodium acetate in 95% alcohol
140 mL 1 mol/L glacial acetic acid in 95% alcohol
8 mL 95% alcohol.

The solutions are compounded volumetrically. The resultant pH of the stain is approximately 4 - 5. The working life of the stain is 4 weeks, depending upon the quantity of material stained in that period.
PAS and haematoxyllin

Method.
1. Dewax slides in xylol 1 for 3-5 min.
2. Dewax slides in xylol 2 for 3-5 min.
3. Rehydrate in absolute alcohol for 3-5 min.
4. Rehydrate in 90% alcohol for 3-5 min.
5. Rehydrate in 70% alcohol for 3-5 min.
6. Transfer slides to running tap water for 5-10 min.
   Rinse in distilled water for 1-2 min.
7. Oxidise in 1% periodic acid for 5 min.
8. Rinse in distilled water.
9. Cover with Schiff’s reagent for 20-30 min.
10. Wash in running tap water for 10 min.
11. Stain nuclei in Mayer’s Hx 1891 for 10 min.
12. Wash in running tap water for 3-5 min (until blue colour gone).
13. Blue up in Scott’s solution for 2 min.
14. Wash in running tap water for 3-5 min.
15. Rinse in 95% alcohol for 1-2 min.
16. Rinse in three changes of absolute alcohol, each of
   approximately 1-2 min.
17. Clear in three (3) changes of xylol each of 3-5 min.
18. Mount with Depex.

PAS – ‘Cold Schiff’ method – Lillie

Solution: Basic fuchsin 1 g
          Anhydrous sodium metabisulfite 1.9 g
          N HCl acid 15 mL
          Distilled water 85 mL

Method: Add the N HCl acid to the distilled water and mix well (0.15 N acid solution). Then add the basic fuchsin and mix well. Finally add the sodium metabisulfite and mix well on a magnetic stirrer for 1 - 2 hours. Allow to stand in the dark overnight. The solution should now be clear and yellow to light brown in colour. Add approximately 0.5 g of fresh activated charcoal and mix on magnetic stirrer for about 5 - 10 minutes. Filter through Watman No. 1 and
keep filtrate. Make up to original volume by washing the residue with a small quantity of distilled water. The solution should be water white. Store at 4°C. pH of the solution is approximately 2.2. Life of reagent - 3 months under refrigeration.

Test for reagent: To 2 - 3 mL of reagent add a few drops of 40% formalin. The solution should turn a reddish/purple colour immediately. If the reaction is slow or colour muddy the solution is on the point of breaking down and will not give optimum results.

Sulfite rinse: 10% potassium metabisulfite 36 mL
N HCl acid 36 mL
Distilled water make up to 600 mL

Store at 4°C.