THE EFFECT OF SUGARS ON AMINO-ACID TRANSPORT

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for

the Degree of Doctor of Medicine

by

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To

My Wife

and

My Parents

"The vast stretches of the unknown and
the unanswered and the unfinished still
far outstrip our collective comprehension"

John Fitzgerald Kennedy.

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CHAPTER I

The Development of the Concept of Amino-acid Absorption from the Intestine.

At the turn of this century the form in which ingested protein was absorbed from the intestine was unknown. Voit[1] had postulated in 1867 that protein was absorbed as such, and passed through the blood to the tissues, where it was catabolised without becoming part of the "living protoplasm". This concept implied that "circulating protein" was in solution; that protein catabolism could take place only in that state; and that when living protoplasm "died", it was first dissolved, and subsequently became part of the circulating protein derived from the food. The evidence in favour of Voit's theory was the fact that large quantities of food protein were known to be more or less completely catabolised in the course of a few hours, and it appeared impossible that metabolic activity of such dimensions could take place in such a short time. While Pflüger[2] and Schöndorff[3] both accepted that protein was absorbed as such, they disagreed with Voit as to its metabolic fate. On inadequate evidence they favoured the theory that there was a chemical difference between circulating protein and living protoplasm. They believed that most of the ingested protein was catabolised and subsequently became an integral part of living tissue.

In his classic paper entitled "A Theory of Protein Metabolism"

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published in 1905, Folin pointed out that while direct evidence was still lacking, there were several indirect observations which suggested that proteins were in fact digested before being absorbed. Nencki and Zaleski[5] had shown that the blood coming from the intestine at the height of digestion contained two to three times as much ammonia as the blood on the other side of the liver, as had Folin himself[6]. Cohnheim[7], in searching for an enzyme which would be able to recombine peptides into albumin, found instead an enzyme which split peptides further into amino-acids. Likewise Kutscher[8] had demonstrated that trypsin was capable of completely splitting protein into amino-acid molecules, and Kossel and Dakin[9] had claimed that protein was broken down in the alimentary tract into proteoses, amino-acids, ammonia and possibly urea.

While many were beginning to accept that at least some protein digestion occurred in the intestine, the prevailing idea in the early 1900's was that the split products were reconstituted in the intestinal wall to form albumin, which subsequently entered the circulation. This erroneous conclusion arose from the fact that poor techniques were responsible for the failure to demonstrate amino-acids in the blood after a protein meal. Bergman and Langstein[10] did show that protein digestion in dogs was sometimes accompanied by an increase in "non-albuminous nitrogen" in the blood. Such a finding with the relatively crude techniques at their disposal, was very significant.

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In 1906 Howell[11] using the β naphthalinsulphochloride reaction, reported that in the dog, after the ingestion of a meal, the concentration of amino-acids in the portal venous blood was greater than that in the jugular vein. He also noted an increase in the concentration of amino-acids in the lymph from the thoracic duct under the same experimental circumstances.

In 1912 Buglia[12] demonstrated that the slow intravenous administration of amino-acids into dogs was harmless, and that only a small fraction of these amino-acids was subsequently excreted in the urine, indicating the physiological feasibility of absorbed amino-acids being utilized by the body.

In the same year Folin and Denis[13] fed cats large amounts of amino-acids and noted an increase in that fraction of blood nitrogen, calculated by subtracting urea nitrogen from total nitrogen.

However, it was Van Slyke and Meyer[14] who provided the first convincing evidence of the absorption of amino-acids, as such from the intestine into the portal blood. This resulted from the development of the nitrous acid method for amino nitrogen estimation. They found that the blood from a fasting dog normally contained 3 - 5mg./100ml. of amino-acid nitrogen. They showed that, after a loop of the dog's small intestine had been perfused with 10g. of alanine, the amino-acid nitrogen of the mesenteric venous blood rose from 3.9mg./100ml. to 6.3mg./100ml. In addition blood from both the femoral artery and the mesenteric vein showed

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a significant increase in amino-acid content after the dog had been given a meal rich in meat. In another experiment Van Slyke and his co-worker demonstrated how rapidly the absorbed amino-acids were cleared from the blood. After injecting 12g. of alanine intravenously over 13 minutes into a dog, only 1.5g. could be detected in the blood after 5 minutes, and only 0.4g. after 35 minutes.

In his Harvey lecture in 1929, London(15) claimed that large quantities of peptides appeared in the portal blood after a meal, but the techniques he employed were unsatisfactory and the question of whether significant quantities of these larger molecules are absorbed as such was only answered in recent years. The next major contribution was in 1949 when Dent and Schilling(16) applied the technique of paper partition chromatography to the analysis of portal blood obtained from dogs, after they had digested protein. They found large increases in amino-acid concentrations in portal blood after the ingestion of heterologous proteins (casein, casein hydrolysate, i.e. amigen, ground beef and human serum albumin). The jugular blood showed quantitatively smaller, but qualitatively similar changes. They were unable to demonstrate appreciable amounts of peptides in the portal blood chromatographically. In an addendum to their paper, Christensen(17) reported that in blood samples from the same dogs used by Dent and Schilling, he had found bound alpha amino nitrogen in amounts of 1mg./100ml. in fasting animals, and that this level increased after

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protein feeding. He could not be certain of the bound nitrogen which might or might not have represented a small amount of peptides.

Dent and Schilling[16] also showed that the amino-acids which decreased in amount in the portal blood after feeding protein to dogs, seemed to fall into three groups : -

(i) Those which rose and fell characteristically after a protein meal and consisted roughly of the amino-acids commonly found in a protein hydrolysate. They regarded these amino-acids as the most important and possibly the only compounds by which the original protein was transferred from the gut to the portal blood. In fact they were able to show, in the case of casein that, with the exception of glutamic acid, the rise in concentration in each amino-acid corresponded roughly to what would be expected if the appropriate amount of hydrolysate had been added to the blood. It is not always possible to show such a close correlation experimentally.

(ii) The amino-acids in the second group tended, if anything, to rise slightly throughout the five hour period after feeding protein, and consisted of alpha and beta amino-isobutyric acid and perhaps glutamine. These were thought to be released by the tissues after the further metabolism of the common amino-acids.

(iii) The third group, which included taurine and citrulline, consisted of amino-acids, the concentration of which did not change appreciably during the experiment. They regarded this group as playing no part in the processes occurring during protein digestion and absorption.

From this excellent work it was clear that the bulk of digested protein was absorbed in the form of amino-acids and that little, if any, peptides were absorbed as such.

With the development of quantitative column chromatography

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of amino-acids by Moore and Stein[18], more precise data could be obtained, and Levenson, Rosen and Upjohn[19], using this technique, were able to confirm the work of Dent and Schilling. In addition, they made a direct search for peptides by ultrafiltration and ion exchange fractionation of all the low molecular plasma amino compounds, and were unable to detect the presence of any peptides in the portal blood after a protein meal. They did isolate an amino-acid conjugate, but its changing structure, and failure to respond in an obvious way to meals, made them conclude that it was non-dietary in origin. Whipple[20] had also provided evidence that only amino-acids were absorbed by feeding C^{14}-lysine to dogs, and demonstrating that it was metabolised in the same way regardless of whether it was free or bound in protein.

Using <u>in vitro</u> experiments, Love confirmed the poor absorption of peptides. Utilising the perfusion method of Fisher and Parsons[21], Agar, Hird and Sidhu[22] were able to find only small amounts of peptides on the serosal side after leucylglycine, glycylglycine or glycylglycylglycine were placed in the fluid perfusing the gut lumen in rats. They suggested that peptides might undergo hydrolysis, not only in the lumen, but also in the wall of the small bowel.

Using everted rat gut sacs and two other <u>in vitro</u> methods, as well as an <u>in vivo</u> one, Newey and Smyth[23] confirmed that only very small amounts of the dipeptides glycyl-L-leucine, glycyl-L-

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tyrosine and glycylglycine appeared on the serosal side as such. Wiggans and Johnston reported that a peptidase was present on the serosal as well as on the mucosal side of an everted rat gut preparation[24], and, further that hydrolysis of peptides on the serosal side might account for the failure to demonstrate their absorption as peptides in some of the in vitro experiments[25]. They did not believe that this was an important factor as β-alanyl-DL-phenylalanine, which is slowly hydrolysed, also failed to appear in the serosal medium.

Of great interest is the ability of the newborn mammal's small intestine to absorb protein as such. It has been assumed that this is based upon a phagocytic or pinocytic mechanism. Voit and Bauer[26] had first proposed that intact proteins might be absorbed unchanged in adults in 1869. Dent and Schilling[16] presented data in adult dogs suggesting that homologous plasma protein was absorbed intact or as large particles. Their evidence for this was largely negative in character. While the homologous protein disappeared from the gut, no trace of it or its derived amino-acids could be found in the portal or systemic blood. Levenson, Rosen and Upjohn [19] however were able to demonstrate amino-acids in mesenteric blood after ingestion of autologous plasma proteins.

It seems likely that the adult mammal probably can absorb intact protein molecules in the alimentary tract under certain

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circumstances, but the amount involved must be very small indeed. It is probably important in some antigenic processes.

Thus it is clear from the accumulated evidence that ingested protein is absorbed almost entirely as amino-acids. The nature of this process of absorption will be discussed in the next chapter.

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CHAPTER II

The Mechanism of Amino-acid Absorption from the Small Intestine.

Section 1 - Definition of Transport Processes

Transport may be defined as the process by which a solute is transferred from one phase to another, being in the same initial and final states in both phases[27].

The three most important ways for transport to take place across cell membranes are : -

(i) Diffusion

When diffusion occurs, the net movement of molecules bearing no net charge depends upon the concentration gradient, and the flux will be dependent upon the concentration in the phase of origin. The flux is defined as the amount of a substance which passes through a unit area of the membrane in a given direction per unit time. With increasing concentration in the phase of origin, a linear relationship between flux and concentration will be observed until such high concentrations are reached that some change in the membrane probably takes place[27]. While such a linear relationship between the absorption rate and the concentration gradient is characteristic of diffusion, its presence does not exclude absorption by enzymatic mechanisms or by one involving some type of absorption

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reaction. For example, if the substrate concentration was well below the Km (Michaelis - Menten constant) a similar result could be obtained in a transport process mediated by enzymes. The velocity of diffusion per unit membrane area may be expressed by the equation : -

$$V = K_D (S_1 - S_2)$$

Where K_D represents the diffusion constant and is governed by the permeability of the membrane concerned. $S_1 - S_2$ is the concentration gradient and V is the velocity of diffusion.

The cell membrane is now believed to consist of phospholipids and other fatty acid esters lying between two protein layers[28]. The lipid present creates a considerable obstacle to the transport of non-lipophilic substances. Indeed, in 1937, Collander[29] confirmed the hypothesis postulated much earlier by Overton[30] that the capacity for molecules to cross cell membranes was related to their oil-water distribution coefficients. This emphasises the fact that in considering diffusion one must recognise the role played by the polarity of the substance concerned. In addition, if the molecules bear a net charge, the electromotive gradient will also play a part.

A low temperature coefficient is characteristic of diffusion. Of interest is the discovery that this is not always so[28], and that therefore the finding of a high temperature coefficient does not exclude the presence of diffusion.

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The intestinal epithelium constitutes an effective barrier to the diffusion of many substances, particularly those that are charged, or of high molecular weight. In the latter instance the pore size of the epithelium is probably important especially in the case of substances which are insoluble in the lipid. Estimations of the size of the pores have varied from 36A° to 4A° (31)(32). Hydrogen ion concentration may also play an important role in diffusion. A good example of this is the role of pH in the non-ionic diffusion of weak acids and bases(33). One might also point out here that the movement of water across membranes can be an independent process(34) and may influence other transport phenomena.

One might comment with some justification that "simple" diffusion might ultimately prove to be very complex indeed.

(ii) Facilitated Diffusion.

Some substances, particularly hydrophilic ones, diffuse by a process which can be saturated and is to a degree temperature dependent. The fact that with an increase in solute concentration, the flux eventually ceases to increase, implies the presence of a chemical site or structure which is essential for transport and only present in limited amounts. The carrier mediates transport and, when transfer occurs from an area of high concentration of

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solute to an area of lower concentration, facilitated diffusion (a term coined by Danielli[35]) is said to occur.

(iii) Active Transport.

By the term "active transport" we mean a migration of molecules across a cell membrane against an electrochemical gradient[36][37]. Energy is always required and there is a quantitative relationship between the energy supplied and the transport work performed.

In essence two stages are involved : -

(a) The combination with a highly specific carrier in order to pass through the lipid-rich cell membrane; and

(b) The concentration of the substance in the cell against a concentration gradient by the so-called "biological pump".

The latter mechanism is usually sodium dependent, probably due to the fact that membrane adenosine triphosphatase is sodium activated. In all likelihood this enzyme is responsible for the cleavage of adenosine triphosphatase (ATP) in the cell. This releases the energy necessary for the activity of the "biological pump"[38][39][40].

It is worth emphasising that while the demonstration of a high temperature coefficient, dependency on energy-yielding metabolism, saturation phenomena and competitive inhibition by

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similar compounds having a common pathway, is strong evidence of active transport, by themselves these phenomena do not prove that this process is involved. In addition the demonstration that a cell accumulates a substance, also does not prove that active transport has occurred, as solutes may become bound to cellular components. However such an accumulation nearly always does mean that active transport has taken place. Finally it should be noted that in a process as complex as active transport, other factors may play a role under special circumstances, for example, "flow driven by counter-flow". Christensen[236] has also postulated that methionine may mediate to produce stronger uphill transport of certain other amino-acids. Unfolding and folding of protein molecules may also be important in transfer involving a carrier[41].

Section 2 - Terminology of Amino-acids and Imino-acids.

Amino-acids are substances with the chemical formula R. $CH(NH_2)$. COOH., R being an organic radical. Amino-acids are derived from proteins by hydrolysis.

The rotation of plane-polarised light by a solution of a natural amino-acid was first observed by Louis Pasteur in 1851[42]. Subsequently, with only one exception, all the amino-acids occurring commonly as the structural units of proteins, have been shown to have at least one centre of asymmetry, and therefore to exhibit optical activity. Although the degree and direction of rotation differ widely, it is generally true that the spatial

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configuration of the groups attached to the asymmetric carbon atoms is relatively the same. Therefore designation by the direction of rotation has been superseded by designation according to configurational interrelationships. The commonly occurring natural amino-acids having the same configuration as the laevorotatory serine found in proteins, are designated L-amino-acids. Their antipodes are known as the D-amino-acids. Detailed rules of nomenclature have been published(44)(45)(46).

The imino-acids include proline, and hydroxyproline and also demonstrate stereo-isomerism.

Section 3 - Intestinal Transport of Amino-acids

(i) Diffusion of Amino-acids.

Mehl and Schmidt(47) found that the diffusion coefficient of amino-acids in aqueous solution was related to the volume and shape of the molecules rather than to their molecular weights. In 1944 Kratzer(48) applied this to the absorption of amino-acids from the intestinal tract of the chick. He concluded that the rate of absorption of an amino-acid under the experimental conditions used, varied inversely with its apparent molal volume and that this represented diffusion of the amino-acid into and through the intestinal cell. Similarly, Bolton and Wright(49) in 1937, had come to the conclusion that amino-acids were absorbed according to the physical law of diffusion, while Chase and

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Lewis[50] were unable to find any evidence of stereospecifity in the absorption of valine or leucine in the rat.

In 1955, Fridhandler and Quastel, perfusing the isolated surviving intestine of the guinea pig, thought that at high concentrations of amino-acids most transport took place by diffusion.[51]

More recently, Booth[52] has re-explored the role of diffusion in intestinal amino-acid transport. Using the everted rat gut-loop technique, he has shown that while H^3-labelled L-tryptophan can be transported from the mucosal to the serosal fluid against a concentration gradient, it can also pass across the mucosa in another way. This was demonstrated by putting tryptophan on the outside of the sac only and none within. Under aerobic conditions transport against a concentration gradient occurred, but when the sacs were incubated anaerobically, some transfer of the amino-acid did occur "downhill" from the phase of greater solute concentration. At the same time he was unable to demonstrate active transport for H^3-labelled pyridoxine hydrochloride, but this compound was transferred across the gut in a comparable manner to L-tryptophan when added to the mucosal medium only and incubated under anaerobic conditions. Of additional interest was the fact that pyridoxine hydrochloride and L-tryptophan have very similar molecular weights. To elucidate the relative importance of active transport and diffusion in the intestinal transfer of L-tryptophan, Booth fed H^3-labelled

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L-tryptophan or pyridoxine hydrochloride to rats and measured the amount of radioactivity remaining in the gastrointestinal tract. The rates of absorption of both substances were virtually identical. In addition, by feeding increasing oral doses of labelled tryptophan he was unable to saturate the absorptive capacity of the jejunal mucosa and there was a linear relationship between the amount absorbed and the amount given(53). Booth concluded that "one must seriously consider whether or not L-tryptophan (when fed in simple solution) is predominantly absorbed in the same way (as pyridoxine i.e. diffusion), active transport being only partially important". He postulated a dual concept of intestinal absorption where in the case of L-tryptophan, active transport acted as a booster to the transfer resulting from diffusion.

(ii) Active Transport of Amino-acids.

Hober and Hober(54) found that the rate of transport of glycine, alanine and valine was not proportional to their concentration in the gut and, in addition, that these amino-acids were absorbed faster than polyhydric alcohols of comparable volume. These two facts, plus the observation that unlike the alcohols, the amino-acids showed saturation phenomena at high concentrations, indicated that some type of selective absorption process was involved.

However, the credit for the unequivocal demonstration of the active transport of amino-acids by the intestine must go to

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Wiseman and his collaborators. The first clue came from the demonstration that the L-isomers were much more rapidly absorbed than the D-isomers. This became possible through the use of more precise methods of analysis using D and L amino oxidase from Neurospora crassa, and D amino oxidase from pigs' kidneys.

In 1950, Elsden, Gibson and Wiseman[55] tested the absorption of a racemic mixture of a number of amino-acids from the washed loop of intestine of an anaesthetised rat, and found that the L form was always preferentially absorbed. In some cases, (glutamate, histidine) the rate of absorption of the naturally occurring isomer was 6 times as great as that of the unnatural isomer.

In the following year, Gibson and Wiseman[56] reported in more detail on their studies of 13 amino-acids with essentially the same results. They studied alanine, valine, phenylalanine and lysine among others; in all of these amino-acids the L form was preferentially absorbed. (They are among the amino-acids studied in this thesis). This phenomenon was also shown to occur in the guinea pig[51], the dog[57], the cat[58] and in man[59]. While these results suggested that an active transport process was operating, the techniques used did not eliminate the possibility of metabolism of the L form preferentially in the intestine itself.

The first unequivocal demonstration of the active transport

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of amino-acids was an <u>in vitro</u> experiment by Wiseman in 1951[60]. He circulated the racemic mixtures of amino-acids through isolated loops of intestine in rats, and was able to show that the L stereo-isomers of alanine, phenylalanine, isoleucine and valine moved across the wall of the intestine against a concentration gradient while the D-isomers did not. His report in 1953[61] indicated that while this was also the case with L-histidine, there was no active transport of L aspartic acid or L-glutamic acid in the intestine.

Agar, Hird and Sidhu[22], using a technique in which the upper part of the small intestine of the rat was perfused, confirmed the stereospecifity of amino-acid transport against a concentration gradient. The next year the same authors[62] introduced a new technique and showed that tissue segments, incubated in solutions of amino-acids, were able to concentrate the amino-acids to a level much higher than that in the medium. This concentration was also stereospecific and was inhibited by metabolic inhibitors. While the uptake of L-histidine could be blocked by dinitrophenol, release of this amino-acid from tissue segments was not affected. Agar, Hird and Sidhu concluded that, while active transport is necessary for the transfer of amino-acids from the lumen into the intestinal epithelial cell, the release of an accumulated amino-acid from the cell into the blood takes place by diffusion. Samiy and Spencer[63] came to the same conclusion after studying the uptake of L-phenylalanine

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by small intestinal segments. On the other hand Newey and Smyth[234] [235] have postulated that the "exit" phase is energy dependent and possibly responsible for the concentration gradient.

In 1959 Jervis and Smyth[64] showed that L-methionine and L-histidine were absorbed from the rat intestine by a rate limiting process with kinetics approximating to the Michaelis-Menten Scheme. The low Michaelis constant obtained for L-methionine suggested a relatively high affinity of L-methionine for the transport mechanism. Their data also suggested that the D-enantiomorphs had a much lower affinity for the transport carrier.

The specificity of transport demands not only the correct stereo-isomerism, but also an unchanged carboxyl group[65], an α β, or very exceptionally a ω amino- group[65][66], an α hydrogen[65][67] and an uncharged side chain[65][68], if present. In addition there is good evidence that the active transport of amino-acids is pyridoxine dependent[68][70].

Finally, dinitrophenol[22][51][71], cyanide[62] and anaerobic conditions[51] inhibit intestinal amino-acid transport providing supportive evidence for the presence of an active process. Matthews and Smyth[72] also noted that low temperatures reduced the ratio of L - to D - amino-acids absorbed by loops of gut in vivo

Section 4 - Amino-acid Transport Groups

It has recently become clear that there are on the whole

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specific transport groups of amino-acids and that these groups are identical both in the intestine and in the kidney[73]. These groups have been classified as follows : -

(a) Monoamino-monocarboxylic (neutral) amino-acids:

alanine, serine, threonine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, asparagine, histidine, cysteine, methionine, and citrulline.

(b) Dibasic amino-acids:

Lysine, arginine, ornithine and cystine.

(c) Dicarboxylic amino-acids:

Glutamic acid and aspartic acid.

(d) The imino-acid and glycine group:

Proline, hydroxyproline and glycine.

The evidence for these groups has come from the work of several teams of investigators.

(i) <u>In the Intestine</u>.

Wiseman[74][75] clearly demonstrated that some neutral amino-acids compete with each other for transport, and that some are more potent inhibitors than others. He also showed that certain amino-acids have a greater affinity for the transport system than others. Finch and Hird[76] applied Michaelis-Menten kinetics to the rate of uptake of amino-acids by the intestine and on this basis predicted the degree of inhibition of the uptake of one amino-acid by another. They found a good correlation between predicted and observed values. The exceptions were lysine,

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ornithine and arginine and they suggested that these amino-acids did not compete for the same common transport system as the neutral amino-acids. Indeed the active transport of the "dibasic" amino-acids, lysine, arginine and ornithine, was in doubt until recently. Wiseman[74] had been unable to demonstrate the active transport of either L-lysine or L-ornithine although previous work had suggested that more than diffusion was involved.[56][77].

The studies of Milne, Asatoor and Loughridge[78] showed that in patients with cystinuria, the intestinal absorption of lysine and ornithine was defective. It was inferred that in cystinuria the genetic defect in the transport of cystine, lysine, ornithine and arginine by the renal tubules was shared by the small intestine. (This work is referred to in more detail in Chapter III). As a result of this stimulating work, Hagihira, Lin and Wilson reinvestigated the transport of these amino-acids in vitro and did indeed show that they were transported against a concentration gradient[79]. This had been known to be so in the case of L-cystine[80]. The maximal rate of absorption of these amino-acids was much lower than that of most of the neutral amino-acids[74], explaining the difficulty that had been experienced in demonstrating active transport. Hagihira, Lin and Wilson[79] confirmed that the three dibasic amino-acids and cystine shared the same transport system as had previously been pointed out by

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Milne and his co-workers. This is of particular interest in as much as cystine is a neutral amino-acid. In the gut the three dibasic amino-acids had been shown to be unable to inhibit the transport of L histidine[81][75], or of iodotyrosine[82], and L-lysine did not inhibit the transport of L-isoleucine[76] or L-methionine[60]. Thus there is very good evidence for a separate transport system for the dibasic amino-acids plus L-cystine in the gut. That this is not absolute is shown by the fact that L-methionine does cause some inhibition of L-lysine intestinal transport.

As already indicated, Wiseman[74][75] and Finch and Hird[76] had provided excellent evidence of a specific transport system in the intestine for most neutral amino-acids and this has been confirmed by a number of workers[71][76][81][82][83][84][85][86]. Finally it was noted that while most neutral amino-acids had little if any inhibitory effect on betaine transport in the gut, L-proline and hydroxy-L-proline were exceptions to this rule[87]. The reverse has also been shown to be the case i.e. N-methyl compounds like betaine can inhibit imino-acid transport but not that of neutral or dibasic amino-acids.

There is evidence in favour of a separate transport system for proline, hydroxyproline, and possibly glycine in the gut, and sarcosine, N, N-dimethylglycine and betaine may also belong to this group[87][88]. Some studies do suggest that the imino-acids and glycine share at least one step in the transport process with the neutral amino-acids[75][87][89]. Indeed glycine

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really belongs to both the "neutral" group and the "imino" group.

While there is no direct evidence of the active transport of dicarboxylic amino-acids by the intestine[75], the experimental demonstration of such transport may well be hindered by the extensive metabolism which these amino-acids undergo in the intestinal cell.

(ii) <u>In the Kidney</u>.

The existence of these four transport groups of amino-acids in the kidney has been confirmed by conventional clearance tests for amino-acids both in man and in experimental animals; by infusions of amino-acids and subsequent study of amino-acid excretion in the urine; as well as by "stop-flow" experiments in dogs. Since the excretion of amino-acids in the urine is normally negligible, reabsorption by the renal tubules must be essentially complete[90][91][92]. In the 1940's it was proposed that several amino-acids were reabsorbed by a single tubular mechanism with limited transport capacity[92][93]. However Beyer and his colleagues[94][95][96] and Kassim and Handler[97] showed conclusively that there were several transport groups involved. The latter authors infused dogs with various amino-acids at constant rates, and measured the rate of excretion of eight "endogenous" amino-acids. While the ratio of the excretion during the infusion to the excretion during the control period

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varied widely, in general the excretion of an amino-acid was most increased by the infusion of an amino-acid of similar acidic properties. Brown, Samiy and Pitts[98] used the "stop-flow" method of analysis in dogs and observed that infusion of lysine or ornithine diminished the reabsorption of arginine only. They also confirmed that amino-acids are reabsorbed in the proximal tubule as had been suggested by the work of previous workers[99][100][101][102][103]. Brown, Samiy and Pitts[98] were able to confirm that the movement of amino-acids across the tubular cell took place against a concentration gradient and that therefore this constituted active transport.

Robson and Rose[104] showed that infusion of lysine into normal subjects caused increased renal clearance of cystine, arginine and ornithine. However other reports[105][106] have indicated that the inhibition of the transport of lysine by cystine might not be demonstrable in renal tissue from several species including man. It would seem that in this respect the intestinal transport of cystine and lysine might differ from the renal transport of these amino-acids. Further work is needed to confirm this in view of the conflicting data.

In 1962 Schafer, Scriver and Efron[107][108] demonstrated that in hypoprolinaemia there was a secondary renal amino-aciduria involving hydroxyproline and glycine. This suggested that these amino-acids plus glycine constituted another renal transport group.

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Kopelman, Asatoor and Milne have recently confirmed these observations in another family[109].

Finally Webber[110] studied the reabsorption of dicarboxylic amino-acids by the kidneys of dogs and was able to show that acidic amino-acids inhibited the reabsorption of each other; while neutral amino-acids inhibited on the whole the reabsorption of other neutral amino-acids.

Section 5 - Conclusions

1. Amino-acids move from the lumen of the intestine into the intestinal cell by a process of active transport and probably also by diffusion.

2. The active movement of amino-acids is highly specific; inter alia the L-stereo-isomer is much more readily transported than its D-antipode.

3. Amino-acids are transported in the gut in four specific transport groups; which are in the main identical to the four specific transport groups in the kidney, where the transport of amino-acids takes place by an active process.

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CHAPTER III

Comparison of Amino-acid Transport Defects in the Intestine and in the Kidney.

As outlined in Chapter II, there is now good evidence that similar transport mechanisms for amino-acids exist in the intestine and in the kidneys. In this Chapter the significance of this physiological phenomenon will be discussed in more detail.

In the Bradshaw Lecture delivered at the Royal College of Physicians of London, Milne[73] gave an outstanding review of various aspects of amino-acid transport. He pointed out that amino-aciduria might occur as : -

(i) A pure "overflow" amino-aciduria, where there is an increase in the blood of the amino-acid concerned.

(ii) Mixed "overflow" and "renal" amino-aciduria, with partial or complete saturation of an amino-acid transport system in the kidney.

(iii) Pure "renal" amino-aciduria, involving a specific amino-acid transport system without other evidence of disordered tubular function, and with normal or low plasma levels of the relevant amino-acids.

(iv) "Renal" amino-aciduria associated with generalised tubular damage.

Section I of this Chapter will deal with the evidence for a defect in amino-acid transport in both the renal tubule and the gut in diseases characterised by genetically determined "renal" amino-aciduria. Section 2 will present the evidence for the same

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phenomenon in patients with acquired "renal" amino-aciduria.

Section 1 - Evidence for genetic defects of amino-acid transport occurring simultaneously in the intestine and kidney.

The development of this important concept is the result of the studies of Milne and his associates and was discussed in detail in the 1964 Bradshaw Lecture[73]. As pointed out on that occasion, the two genetically determined diseases in which "linked" transport defects in amino-acids occur in the intestine and the kidney, are Hartnup disease and Cystinuria.

Hartnup Disease

This disease was first described by Baron, Dent Harris and Jepson in 1956[111]. They described four of eight children of a first cousin marriage, the name of the family being Hartnup. The metabolic disease which these children manifested was called therefore Hartnup disease. Earlier briefer reports by the same workers had referred to it as "Hart's disease" after Dr. E.W. Hart under whose care the family was first investigated and treated. The most constant clinical feature was a tendency to develop pellagra. On occasion a severe but fully reversible cerebellar ataxia developed in association with the pellagra, and, in one patient, followed on an infective illness without any accompanying pellagra. The older affected siblings were mentally retarded.

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The patients had a constant gross amino-aciduria which was thought to be "renal" in type in view of the fact that Dent and Fowler[112] had shown that the level of α-NH_2-N in the plasma of all four siblings with amino-aciduria was normal in the fasting state. Evered[113] had also reported that individual plasma amino-acid levels were normal in these patients. It was predominantly the neutral amino-acids that were involved in the renal defect. There was also a constant large excretion in the urine of Indole-3-acetic acid and a less constant large excretion of indican. The faeces contained a moderately increased quantity of protoporphyrin. Subsequently Cusworth and Dent[114] confirmed that the renal clearances of the Group 1 amino-acids were very high in this disease, and that consequently there was an excessive excretion of these amino-acids in the urine, with normal or low plasma levels. Eighteen cases of Hartnup disease in ten families have been described to date[111][115][116][117][118][119][120][121][122][123]. Of interest is the observation that none of the more recent cases has shown evidence of cerebellar dysfunction, while in the original patients, this is no longer a feature.

Cystinuria.

This disease is characterised by an excess of group 2 amino-acids in the urine with normal or low plasma levels of these amino-acids. Dent and Rose[124] showed that this was due to an

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isolated defect in the reabsorption of these amino-acids from the proximal renal tubule. Their results, based on paper chromatographic methods, have been confirmed by more refined techniques[124][125][126][127][128][129][130][131]. Cystine is relatively insoluble in urine, unlike the mixed disulphide of cystine and homocystine which is also excreted in cystinuria. Cystinuria is responsible for about 1% of urinary tract calculi in adults, and for a larger percentage in children.

As Milne[73] has pointed out, there was no difficulty in recognising the presence of a renal transport defect of amino-acids in these two diseases because of the recognition of the "renal" amino-aciduria.

Defects of intestinal transport of amino-acids are more difficult to demonstrate. Milne[73] summarised the evidence obtained from five methods used by himself and his co-workers.

(i) An intestinal load of the relevant amino-acid should result in a lower plasma concentration of the amino-acid in the patients than in normal controls. This lowered plasma concentration should not be due to excessive renal amino-acid loss. At the same time intravenous loads should cause the same rise in plasma amino-acid concentration in both patients and controls. Using these methods, results have been suggestive after the ingestion of tryptophan in Hartnup disease. Milne, Crawford, Girao and

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Loughridge[132] gave L tryptophan (70mg./kg. body weight) by mouth, and blood samples were taken at two-hour intervals. In control subjects, tryptophan was at its greatest concentration in plasma two hours after the ingestion of the amino-acid, and had fallen to basal values in eight hours. In the two asymptomatic patients with Hartnup disease, the maximum concentration of tryptophan in the blood was found in the four hour specimen, and this was regarded as suggestive evidence in favour of impaired absorption of tryptophan. Asatoor, Lacey, London and Milne[133] gave 10g. of L-arginine hydrochloride in 100ml. of water by mouth to patients with cystinuria and to controls, and measured plasma arginine concentration after 1,2,3 and 5 hours. In the normal controls there was a rapid rise within the first hour to concentrations averaging 2.5 times the basal value and this was followed by a gradual fall almost to basal level 5 hours after the ingestion of the amino-acid. In the patients with cystinuria, there was only a slight increase in the plasma levels of arginine. There was a slow rise to a mean value of only 25% higher than the basal concentration after 3 hours. Despite the greater variation in the control subjects, the mean concentrations in the plasma samples which were obtained one hour after amino-acid ingestion, were significantly different at the 1% probability level. There was no significant difference in plasma arginine concentration between patients with cystinuria and controls after intravenous infusion of 10g. of L-arginine hydrochloride. These results proved a

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transport defect of arginine in the gut in patients with cystinuria.

(ii) If the relevant amino-acid is ingested, an excessive amount should appear in the faeces if intestinal absorption is defective. This has been shown to be the case in two patients with Hartnup disease[132], who excreted considerable amounts of tryptophan in the stools after a tryptophan load. No tryptophan was detected in faecal extracts from normal subjects. Milne and his co-workers were able to demonstrate defective arginine, lysine and ornithine absorption from the intestine of patients with cystinuria in this way. Using paper chromatography of faecal extracts they were able to show that arginine was present in the faeces of five out of eight cystinuric patients after arginine ingestion. There was only a trace in one out of six normal subjects[133]. After giving L-lysine or L-ornithine monohydrochloride by mouth to five patients with cystinuria and twelve controls[134], it could be shown that a considerable fraction of the ingested amino-acid was unabsorbed in the patients with cystinuria, and consequently appeared in the faeces unchanged. By contrast in the normal controls, absorption was complete except in three subjects who developed intestinal hurry and watery diarrhoea.

(iii) If an imperfectly absorbed amino-acid is ingested, the unabsorbed amino-acid can be degraded by bacteria in the gut, and

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the resultant products might then appear in excessive amounts in the faeces. Asatoor, Craske, London and Milne[135] studied seven cases of Hartnup disease and showed that incubation of their stools produced more indole and tryptamine than did incubation of stools from normal controls. Indole and tryptamine are derived from tryptophan by bacterial action. Recently de Lacy, Hooft, Timmermans and Snoeck demonstrated that the indoles in the stool of a patient with Hartnup disease, disappeared with antibiotic treatment[136].

Asatoor, Lacey, London and Milne[133] fed arginine to patients with cystinuria and found citrulline subsequently present in the faeces in seven or eight patients so tested, and putrescine in four of seven. Only a trace of citrulline was present in three of six controls, and no putrescine was found in any controls. Both citrulline and putrescine are derived from arginine by bacterial action in the gut; the latter being derived from ornithine or agmatine, which in turn are breakdown products of arginine. While there was no evidence of agmatine formation, ornithine was formed in these patients.

(iv) If an amino-acid is inadequately absorbed not only will an excess of bacterial breakdown products of that amino-acid appear in the faeces, but also some of these substances will be absorbed from the gut and excreted in the urine in excessive amounts.

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The substance excreted in the urine may be identical with that absorbed from the gut, or may be a compound derived metabolically from the absorbed breakdown product. In patients with Hartnup disease, so tested, malabsorption of tryptophan has resulted in : -

(a) An excess of indole in the gut with excretion of derived indican (indoxyl sulphate) and bis-indolyl-indoxyl in the urine;

(b) an increase in intestinal tryptamine concentration with consequent excretion of indolyl-3-acetic acid in the urine;

(c) consequent increase in the intestinal indolyl-3-acetic acid and its breakdown resulting in the urinary excretion of indolylacetylglutamine; and

(d) Indolyl-3-proprionic acid in excess in the gut and consequent urinary excretion of indolyl-3-acrylic acid and indolylacrylylglycine.

In the same disease impaired absorption of phenylalanine from the intestine has resulted in :-

(a) An excess of β -Phenylethylamine in the gut with consequent urinary excretion of phenyl-acetylglutamine; and

(b) increased intestinal benzoic acid with subsequent urinary excretion of hippuric acid.

Malabsorption of tyrosine in Hartnup disease causes increased intestinal tyramine and urinary p-hydroxylphenylacetic acid and other phenolic acids[132][73]. Of interest also is the recent report of the disappearance of the indicanuria on antibiotic

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therapy[136]. The results are also positive in the patients with cystinuria studied by the same group of investigators. A lysine load by mouth results in the appearance of cadaverine and piperidine in the faeces and urine although cadaverine is only very occasionally detectable in the urine. Ornithine ingested in excess by patients with cystinuria results in the excretion of increased amounts of putrescine and pyrrolidine in both faeces and urine; again putrescine is only very occasionally detected in the urine. An oral arginine load given to patients with cystinuria results in the excretion of excessive amounts of citrulline and ornithine in the faeces and consequently citrulline in the urine; putrescine in the faeces and very occasionally in the urine, and pyrrolidine in the stool and urine[132][73].

(v) The exposure of colonic bacteria to increased concentrations of amino-acids as a result of malabsorption may induce preferential selection of unusual bacterial strains. This has been shown to occur in some patients with cystinuria from whose stools an Escherichia coli has been cultured having an increased capacity for decarboxylating lysine and ornithine to the corresponding diamine[133]. No such organism has been isolated from the stools of patients with Hartnup disease[135].

In addition to these five indices of amino-acid malabsorption from the intestine of man, so ably studied in Hartnup

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disease and cystinuria by Milne and his colleagues, two other methods are available which may be used to study intestinal amino-acid transport in man : -

(i) The <u>in vitro</u> study of amino-acid transport in material obtained by jejunal biopsy. There are two recent reports of such a study in cystinuria and they both confirm the presence of an intestinal transport defect.

Thier, Fox, Segal and Rosenberg[137] found that the uptake of labelled L-lysine and L-cystine in normal and cystinuric subjects was most rapid during the first thirty minutes of incubation and then approached a plateau. They therefore used an incubation period of forty-five minutes for most of their studies and were able to show that intestinal biopsy material from normal volunteers concentrated lysine 13-fold and cystine 4.5-fold. Similar tissue from four cystinuric subjects failed to concentrate either of these amino-acids significantly. They were also able to show that under their experimental conditions cystine accumulation could be inhibited by lysine in the intestine and that similarly lysine accumulation could be inhibited by cystine in the gut. This was in keeping with the data of Hagihira and his co-workers in their studies of amino-acid transport in the gut of the hamster[79].

McCarthy, Borland, Lynch, Owen and Tyor[139] reported

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similar studies on duodenal biopsies in two patients with cystinuria and four controls. Their control subjects were able to concentrate C^{14}-L-arginine, C^{14}-L-lysine, C^{14}-DL-ornithine, S^{35}-L-cystine, C^{14}-DL-leucine, C^{14}-L-phenylalanine and C^{14}-DL-cystine to average ratios between mucosal tissue water and incubating fluid of 4.7 to 11.7. The average ratios for C^{14}-L-arginine, C^{14}-L-lysine, C^{14}-DL-ornithine and S^{35}-L-lysine in the patients with cystinuria were uniformly less than 1.0. The ratios for C^{14}-DL-leucine, C^{14}-L-phenylalanine and C^{14}-DL-cystine did not differ significantly between the two groups. This finding for DL-cystine was unexpected and not adequately explained.

(ii) The other approach is the study of amino-acid intestinal transport in man using the double lumen tube technique which has been so ably utilized to study carbohydrate absorption[140].

One difficulty which would be encountered here is the high concentration of amino-acids occurring physiologically in the fluid recovered. This could probably be overcome by studying individual amino-acids in sufficiently high concentrations. To date no such study has been reported.

There has been one case report of a fourteen year old child with cystinuria who also developed malabsorption of fat, xylose, vitamin B_{12} and glucose[141]. This child also had vidamin D resistant rickets. The authors give some evidence for intestinal

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malabsorption of lysine but autopsy failed to reveal the cause of the generalised malabsorption. There was a recent report of a patient with cystinuria who developed classical coeliac disease[142]. There is no evidence that the malabsorption of fat and other substances in these two patients was related in any way to the cystinuria.

From the evidence presented it is clear that there is defective transport in both the intestine and the kidney of some neutral amino-acids in Hartnup disease, and of dibasic amino-acids in cystinuria. Both these diseases are recessive hereditary conditions and the assumption is that an abnormal gene has caused defective synthesis of a protein, presumable an enzyme, important in amino-acid transport at these sites.

Recently Drummond, Michael, Ulstrom and Good[138] described the "blue diaper" syndrome in which in addition to hypercalcaemia and nephrocalcinosis, the one infant well studied also had indicanuria. They presented evidence for defective intestinal absorption of tryptophan. There was no amino-aciduria on paper chromatography but no quantitative studies were done. It seems possible that the defect in amino-acid transport is confined to the gut in this syndrome, the etiology of which is quite obscure.

Section 2 - Evidence for acquired defects of amino-acids transport occurring simultaneously in the intestine and kidney.

If a toxic substance can reach a sufficiently high

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concentration in the cells of the renal tubule and of the small intestine, defects in amino-acid transport might occur at both sites. This has been shown to be the case in man after the administration of neomycin by mouth.

Neomycin is poorly absorbed and frequently causes a malabsorption state which disappears when the drug is withdrawn. Jacobson, Chodos, and Falcon[143] did forty-five studies on thirty-three subjects giving them 12 grams of neomycin sulphate in three divided doses daily. They noted reversible malabsorption of carotene, iron, vitamin B_{12}, xylose and glucose plus a fall in serum cholesterol. Jacobson, Prior and Falcon noted histological changes in the jejunum in patients taking neomycin by mouth, not unlike those seen in idiopathic steatorrhoea[144]. Jacobson and Falcon also demonstrated an increase in faecal fat in patients receiving neomycin by mouth[145]. This abnormality also disappeared after neomycin was withdrawn. Hvidt and Kjildsen[146] obtained very similar results in their studies with neomycin.

At the same time neomycin is clearly nephrotoxic if given parenterally. It has recently been shown in the experimental animal that the earliest and most severe damage is in the proximal renal tubule[73]. Further, Milne has clearly shown that patients receiving neomycin not only have amino-aciduria, but also have an excess of amino-acids in the faeces on chromatography or de-proteinised extracts. All transport groups are involved but neomycin has least effect on the transport of group 2 amino-acids

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This is the only example of acquired "linked" defect in amino-acid transport in kidney and intestine in the literature. There are a number of examples of acquired tubular damage however.

Recently Frimpton, Timpanelli, Eisenmenger, Stein and Erlich[147] observed three patients including two children aged thirteen, with an illness resembling the Fanconi syndrome after therapy with degraded tetracycline. These patients had inter alia generalised amino-aciduria and the whole syndrome resolved when the drug was withdrawn.

Many poisons have been shown to produce tubular damage both in man and in the experimental animal[148][149][150][151][152][153][154][155][156][157][158].

Clarkson and Kench[149] pointed out that evidence collected over a number of years had shown the kidneys to be particularly susceptible to poisoning by heavy metals. Indeed they were able to demonstrate that in man cadmium and uranium poisoning produced quite a profound amino-aciduria affecting particularly threonine and serine, while one third of the men tested after exposure to lead had an increase in alanine in the urine. An increase in urinary glycine was observed in half of those tested who were exposed to mercury.

Furthermore, as Milne[73] has pointed out, metabolic diseases may result in the formation of abnormal metabolites, or a very high concentration of normal metabolites and secondary proximal tubular damage. This is known to occur in galactosaemia[159] where

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galactose-1-phosphate has been incriminated, hepatolenticular degeneration where copper is thought to be responsible for the tubular damage[160]; and in multiple myeloma[161][162][163]. Further renal amino-aciduria has been described in severe cases of the nephrotic syndrome[164][165]; and in severe vitamin D[166] or vitamin C deficiency[167]. Tubular damage also occurs in the de Toni-Fanconi syndrome[168][169]; Lowe's syndrome[170] and in Lignac-Fanconi disease with cystinuria. In the latter, cystine may be the agent toxic to the tubular cells.

In none of these disease states has a jejunal transport defect hitherto been described and as far as is known this has not been studied in these patients.

Section 3 - Conclusions

There is a clear evidence that both inherited and acquired defects in amino-acid transport may be shared simultaneously by both the cells of the proximal renal tubule and of the saml1 intestine.

The recognition of this phenomenon is largely due to the work of Milne and his co-workers.

CHAPTER IV

The Development of the Hypothesis

Section 1

(i) Galactosaemia.

a. Clinical Manifestations and Biochemical Defects.

Galactosaemia, a genetically determined disorder, is characterised by an impairment to utilise galactose and to convert it to glucose or energy. The clinical manifestations are directly related to galactose administration and may be fatal if galactose ingestion continues. If galactose is removed from the diet before irreversible changes occur, most of the symptoms and signs regress and even disappear completely[171]. Over forty cases of galactosaemia have been reported[172][173][174], but there are many more patients with this disease than have been recorded in the medical literature. The symptoms of galactosaemia usually manifest shortly after birth and patients classically develop the triad of cataract formation, hepatosplenomegaly and mental retardation, if exposure to galactose continues. The fundamental biochemical fault is a blocking or deficiency in the enzyme, galactose-1-phosphate uridyl transferase which is necessary for the following reaction : -

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Galactose-1-phosphate + uridine diphosphate glucose $\rightleftharpoons$
uridine diphosphate galactose + glucose-1-phosphate.

This represents the main pathway in galactose utilisation. Further it is important to note that uridine diphosphate galactose is necessary for the synthesis of galactose derivatives, such as lactose, galactolipids and galactose-containing polysaccharides[171]. Mammalian liver also contains a pyrophosphorylase[175] which catalyses the following reaction : -

Uridine triphosphate + galactose-1-phosphate $\rightleftharpoons$
uridine diphosphate - galactose + pyrophosphate.

This enzyme therefore provides an additional pathway by which galactose-1-phosphate may be utilised and incorporated into uridine diphosphate galactose. Its activity in neonatal liver is very weak and increases with age. It probably represents the pathway by which patients with galactosaemia are able to tolerate galactose better as they grow older.

b. Amino-aciduria in Galactosaemia

Patients with galactosaemia exposed to galactose develop galactosuria, proteinuria and amino-aciduria. The amino-acid pattern has been similar in most cases, with a predominance of the neutral simple aliphatic chain type i.e. serine, glycine, alanine, threonine, glutamine and valine. In addition, small quantities of

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phenylalanine, lysine, cystine, glutamic acid, methyl histidine tyrosine and aminoisobutyric acids have been detected[176][177][178]. Kemrower[176] and Cusworth, Dent and Flynn[159] have shown that the amino-aciduria of galactosaemia is of the renal type. Further Cusworth and his colleagues[159] demonstrated that the amino-aciduria became manifest only after several days of galactosuria, and that the excessive urinary amino-acid excretion persisted for as long as seven days after the galactosuria had ceased. It has therefore been postulated that a metabolite of galactose, such as galactose-1-phosphate, may accumulate in the renal tubular cells and inhibit amino-acid transport[171]. This will be discussed in more detail in subsequent chapters.

(ii) Hereditary Fructose Intolerance.

a. Clinical Manifestations and Biochemical Defect.

This inherited disease was first described in 1956 by Chambers and Pratt[179]. This publication was followed by the report of Froesch and his co-workers in 1957[180]. Recently Froesch, Wolf, Baitsch, Prader and Lablart have reviewed our knowledge of the disease.[181] A total of eleven families, comprising twenty three patients with hereditary fructose intolerance have been found in seven European countries. There is evidence that the mode of inheritance is of the autosomal recessive type and the primary

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defect has been shown to be the lack of 1-phosphofructaldolase[181]. The patients develop hypoglycaemia and vomiting after weaning, when fructose or sucrose are added to the diet. Hepatomegaly develops and hypophosphataemia has been observed. Two adult patients had no residual organic manifestations of the disease but had developed a strong aversion to fructose-containing foods. The mechanism for the development of the symptoms is a matter for debate but it seems likely that secondary inhibition of phosphoglucomutase and 1,6-diphosphofructaldolase may play a part[181]. Hereditary fructose intolerance must be sharply distinguished from the benign disorder of essential fructosuria, in which patients have fructosuria and no other abnormal symptoms or signs.

b. Amino-aciduria in Hereditary Fructose Intolerance.

Both proteinuria and amino-aciduria develop rapidly when patients with hereditary fructose intolerance are given fructose[180][182][183][184]. It is thought that the amino-aciduria is probably due to the accumulation of fructose-1-phosphate in the tubular cells[181] and this will be discussed further in subsequent chapters. The amino-aciduria disappears promptly when fructose is omitted from the diet[183]. Transient intolerance to exogenous fructose has recently been described in the new born, presumably due to delayed maturation of 1-phosphofructaldolase[185]. There was no study of amino-acid excretion in these neonates.

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(iii) Glycosuria Associated with Amino-aciduria

Gray and Illing reported their findings on amino-acid metabolism in diabetes mellitus in 1952[186] and reviewed previous work on this subject. They were able to study thirty-three 24 hr, specimens of urine from twenty-six diabetic subjects as well as from six normal controls. Qualitative chromatographic analysis showed a gross increase in all the amino-acids identified (including L-lysine and glycine) in ten samples. Seven of the samples contained aceto-acetic acid but three did not. Gray and Illing were inclined to equate the amino-aciduria with ketosis rather than glycosuria. Of four patients with ketosis studied before and after treatment the amino-aciduria disappeared with correction of acidosis in three. Quantitative estimation of total urinary α-Nitrogen/24 hrs. tended to confirm the role of ketosis. Recent unpublished work has shown that the infusion of glucose into normal volunteers does result in a "renal" amino-aciduria, as does the infusion of galactose or fructose. In contrast the infusion of xylose does not result in an increase in urinary amino-acids[187].

While there is both glycosuria and "renal" amino-aciduria in the de Toni - Fanconi syndrome[188] as well as other proximal tubular defects in transport, there is no evidence to support a causative role for glucose as regards the amino-aciduria. Indeed the contrary is the case, because in simple renal glucosuria there

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is no associated amino-aciduria[189]. All the evidence points to the presence of multiple renal tubular transport defects in the Fanconi syndrome and the cause of the proximal tubular damage is quite unknown. No studies of intestinal amino-acid transport have been reported in this disease.

Section 2 - The Hypothesis

In view of the fact that : -

1. "Renal" amino-aciduria occurs in galactosaemia, hereditary fructose intolerance and in some states associated with glycosuria (Chapter IV - Section 1); and
2. It is known that inherited or acquired causes of "renal" amino-aciduria may be associated with an intestinal defect in amino-acid transport (Chapter III),

it was postulated that : -

1. Intestinal amino-acid transport might be inhibited in vitro if galactose, fructose or glucose were present in the medium;
2. Patients with galactosaemia and hereditary fructose intolerance might have defective intestinal amino-acid transport when exposed to the offending sugar.

Because of the relative infrequency with which it would have been possible to study such patients with amino-

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aciduria, and because it was considered unethical to expose known cases to the sugar which was harmful to them, it was not possible to test this hypothesis directly in such patients. In was known that rats fed 30% galactose for two to three months developed a syndrome resembling galactosaemia in man in many respects, including the occurrence of "renal" amino-aciduria[190]. It was therefore postulated that such animals would also have defective amino-acid transport in the small intestine. Further it was postulated that rats fed 30% fructose for two to three months would also develop both renal and intestinal defects in amino-acid transport, and that those fed 30% glucose for a similar period might be similarly affected.

The experiments recorded in this Thesis have tested this hypothesis and found it to be essentially correct. Other experiments aimed at trying to elucidate the mechanisms involved are also recorded here.

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CHAPTER V

The Inhibitory Effect of Sugars on Amino-acid Transport by Rat Intestinal Segments *In Vitro*.

Section 1 - Method

(i) Choice of Method

Amino-acid transport in the gut was measured according to the method used by Rosenberg, Blair and Segal[191] in studying amino-acid transport in rat kidney slices. This is very similar to the method used by Agar, Hird and Sidhu[62] to study the accumulation of L-histidine by small segments of rat intestine. The method is very useful because it is simple and reproducible and the segments can be randomised. Crane and Mandelstam[192] have used this technique to advantage in studying sugar absorption. It gives an index of intracellular accumulation of the amino-acid in question. Such a demonstration of intracellular accumulation does not prove conclusively that active transport has occurred, for as pointed out by Christensen[27] solutes may theoretically become bound to cellular components, accumulate in the cell, and give a false impression of active transport. This was not regarded as a valid objection to the use of this method to test the hypothesis studied in this thesis, as it has clearly been shown that active transport is involved in amino-acid transfer in the intestine although diffusion may play a role. (Chapter II). Indeed there is very little doubt that active transport is being measured as shall be shown by the influence of temperature, anaerobiasis and a metabolic

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inhibiter.

Alternative *in vitro* methods considered were the perfusion of the isolated intestine[193]; circulation techniques[21][61]; and the everted gut sac method[194]. Of these three alternative methods, only the everted sac technique was seriously considered for the others are much more difficult technically, and have no real advantage over the method used. As there was nothing really to choose between the use of intestinal segments and everted sacs, the former method was selected because it was clear that a very large number of experiments would have to be done, and that many more animals would be necessary if everted sacs were used.

The technique used involved the measurement of the extracellular fluid volume (E.C.F.) of the intestinal segments.

A great number of substances of different chemical composition, electrical charge and molecular weight have been used to estimate the E.C.F. both *in vivo* and *in vitro*, and there is no general consensus among workers in the field on the identity of the substances which accurately measure the total extracellular space of all tissues, and only this space. The first attempt to measure the E.C.F. was made visually using frozen muscle preparations, and the value obtained for the E.C.F. was 15% of the wet tissue weight[195]. It was then surmised that if the total quantity of chloride in muscle was limited to this histological space, its concentration would be equal to that in plasma, making allowance for the Donnan effect. This led to the conclusion that all chloride

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was extracellular[196]. This assumption was extended to include the whole body, and, for a time, chloride and sodium were used to measure the E.C.F.[197]. Since these attempts, many other ions including bromide, sulphate, thiosulphate and thiocyanide have been used to estimate the E.C.F. in man, the dog and the rat[198][199]. However, each of these substances yielded values for the E.C.F. which strongly suggested variable cellular penetration and metabolism. Because of these difficulties, attention was focused on saccharide molecules, and mannitol[198][200], sucrose[200][201][202][203], raffinose[198][204][205] and inulin[198][199][200][201][202][206][207][208] have been studied in man, the dog, the rabbit and the cat by both in vitro and in vivo techniques.

The great range of values obtained with these substances and the controversy over proper analytical methods bear testimony to the fact that this problem is far from solved. However, the use of labelled compounds to overcome the analytical problems has been of great help, and the consensus of opinion is that inulin is the most reliable measure of the E.C.F. Perhaps it is better to refer to this as the "inulin space" but there seems little doubt that it closely approximates the E.C.F., and it was for this reason that carboxyl-C^{14}-inulin was used in this study. In experimental work of this kind any objections to the use of inulin to measure the E.C.F. are largely overcome by the fact that a comparison is being made, the important point being that the E.C.F. or "inulin space" should not change undetected during the experimental conditions.

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(ii) The Method

Male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 120 - 150g. were fasted overnight. The rats were killed by a blow on the head and the small intestine removed immediately and put into ice cold Krebs-Ringer bicarbonate buffer (pH 7.4). The gut was then perfused twice with ice cold normal saline and then everted over a glass rod. Segments measuring 3 - 5 mm. were cut from the distal jejunum and proximal ileum. Six everted segments weighing together 200 - 300 mg. were placed in 25ml. Erlenmeyer flasks containing 5ml. of Krebs-Ringer bicarbonate buffer of the following composition : -

Sodium 143 mM., Chloride 125mM., Potassium 5.9mM., Calcium 1.27mM., Bicarbonate 25mM., Phosphate 1.18mM., Sulphate 1.18mM., Magnesium 1.18mM.

The final buffer was made up fresh each day from more concentrated solutions, which were stored at 4°C. The concentrated solutions were gassed for 30 minutes with 95% oxygen and 5% carbon dioxide before the buffer was made up. The pH of the Krebs-Ringer bicarbonate buffer was 7.4 and this was again gassed with 95% oxygen and 5% carbon dioxide for thirty minutes at 37°C in a Dubnoff shaking incubator before the segments were added. The flasks were gassed throughout the incubation period with the same mixture of oxygen and carbon dioxide. The time of incubation varied from ten to ninety minutes depending upon the experimental design. The amino-acid concentration in the incubation medium varied from

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0.05mM. to 5mM., and the stable amino-acids added were obtained from Mann Laboratories Inc. or Calbiochem Inc. All the amino-acids used were in the laevorotatory or L. form (except glycine), and the amino-acid isotopes used were uniformly labelled with C^{14} except in the case of hydroxy-L-proline which was labelled with H^3 in the 5 position. All isotopes were purchased from the New England Nuclear Corp., Boston, Mass. and were chromatographed on paper in a butanol, glacial acetic acid and water solvent to check their purity, the colour development being done with ninhydrin. In no instance was any impurity detected. The specific activity used in the incubation medium was in all instances 0.02 uC/u.mole. The sugars, when added to the medium, were at final concentrations varying from 5.8mM. to 28mM. All the sugars were obtained from the Fischer Scientific Corp. and were of analar grade. They were in the D + form and the galactose used was 99% pure. No glucose or other sugar could be detected in the galactose on paper chromatography.

After incubation the segments were removed, washed twice in normal saline, blotted and boiled for five minutes in 2ml. of distilled water. A glass marble was used to prevent evaporation. The final volume was checked and found to be 2ml. Radioactivity was assayed in a Packard Tri-carb liquid scintillation spectrophotometer using dioxane and ethanol as the solvents, diphenyl oxasole as the primary phosphor, and p,bis 1, 2 (phenyl-oxasolyl) -1-benzene as the secondary phosphor. The counting solution also contained naphthalene. 100ul of each radioactive

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solution was counted in a glass phial containing 15 ml. of counting solution and every determination was done in duplicate. The counts/min. were 30 - 300 times background.

Efficiency of counting was 60% to 70%. When degree of quenching was the same throughout any one day's experiments, the counts were not converted to absolute disintegrations per minute as the calculation was unaffected by quenching provided it was constant for all counts in any one series of experiments. Quenching was assessed by the ratio of the counts per minute obtained on the red and green channels on the spectrophotometer in all samples, including known standards which were counted daily. If the degree of quenching varied in any one experiment (which was extremely rare) then the counts were converted into absolute disintegrations per minute after the method of Bush(209).

Recovery of radioactivity from the segments was in all cases greater than 85%. Paper chromatography (butanol, glacial acetic acid and water solvent) of the medium, before and after incubation, and of the segment fluid, with subsequent scanning of the chromatogram, confirmed that more than 95% of the label had remained attached to the amino-acid used. In some instances segments were cut out of chromatograms running concurrently with amino-acid standards and counted in the spectrophotometer in 15ml. of counting solution. Again more than 95% of the label was shown to have remained attached to the amino-acid used. No radioactivity was detectable elsewhere in these chromatograms.

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Extracellular fluid volume (E.C.F.) was measured with carboxyl-C^{14}-inulin with a molecular weight of over 5000. This isotope was also purchased from the New England Nuclear Corporation and segments were incubated and treated in precisely the same way as described previously but in this instance in the presence of inulin (specific activity 1 uc/u.mole, and at a concentration of 8×10^{-5} mM./ml.).

The total water content of the segments was measured by drying to constant weight at 110°C.

The results were expressed as follows : -

1. The Extracellular space (E.C.F.) was calculated as a percentage of total tissue water, and the equation used was as follows : -

$$E.C.F. = \frac{\frac{\text{Counts/min. } C^{14}\text{inulin in intestinal segments}}{\text{Counts/min. } C^{14}\text{ inulin/ml. of medium}}}{\text{Wet weight of Segments - Dry weight of Segments}} \times 100$$

The counts/min. C^{14} inulin in the intestinal segments equalled the counts/min. of C^{14} inulin in the supernatant after boiling the intestinal segments in 2ml. of water for five minutes. These segments were previously incubated in the presence of inulin. It was assumed that inulin is confined to the E.C.F. and that the E.C.F. is in equilibrium with the medium in respect to inulin. Therefore the counts/min. C^{14}inulin/ml. medium after incubation equalled the counts/min. C^{14} inulin/ml. in the E.C.F. This value divided into the counts/min. C^{14} inulin in the intestinal segments equalled the

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volume of the E.C.F. in mls. If this volume was divided by the total amount of water in the intestinal segments (wet weight of segments - dry weight of segments) and multiplied by 100, the E.C.F. was expressed as a percentage of total tissue water.

2. The transfer of each amino-acid was expressed as the distribution ratio of the concentration of the amino-acid in the intracellular fluid to that in the E.C.F.

The concentration of each amino-acid studied was expressed as net counts/min./ml. of intracellular fluid (I.C.F.) or of extracellular fluid (E.C.F.)

The net counts/min./ml. of I.C.F. was arrived at thus : -

Net counts/min./ml. of I.C.F. =

$$\frac{(\text{Net tissue counts/min.}) - (\text{Counts/min./ml. of medium})(\text{E.C.F. in mls.})}{(\text{Volume of total tissue water in mls.} - \text{E.C.F. in mls.})}$$

The net tissue counts/minute were obtained from the supernatant after placing the segments in a test-tube in boiling water bath for five minutes after the addition of 2ml. of water. The intestinal segments had previously been incubated in the presence of the labelled amino-acid. The assumption that the amino-acid in the intracellular water would be fully and completely distributed throughout the water bathing the segments, after boiling, proved to be correct. Indeed as previously stated at all times

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more than 85% and usually more than 90% of the radioactivity could be accounted for

The counts/min./ml. of medium were obtained by counting the medium after incubation and this fluid was assumed to be in equilibrium with the E.C.F. The volume of the E.C.F. was based on the experiments previously performed with carboxyl-C^{14}-inulin. As the E.C.F. proved to be virtually constant under the conditions of the experiments (section 2 of this chapter), the calculations were made using 28% of total tissue water as the E.C.F. The volume of the total tissue water was obtained by subtracting the dry weight from the wet weight of the intestinal segments. The volume of the E.C.F. in mls. multiplied by the counts/min./ml. of medium gave the counts/min. in the E.C.F. when this was subtracted from the net tissue counts/min., the net counts/min. in I.C.F. were obtained. This in turn was divided by the I.C.F. in mls.

The net counts/min./ml. of E.C.F. equalled the net counts/min./ml. of medium after incubation.

By dividing the net counts/min./ml. I.C.F. by the net counts/min./ml. E.C.F.; a distribution ratio was obtained. If this ratio was over unity transfer of amino-acids had occurred into the cell and the amino-acid had accumulated there against a concentration gradient. All the evidence supports the conclusion that this represents mainly active transport of the amino-acid concerned across the intestinal cell membrane into the cell (Chapter II).

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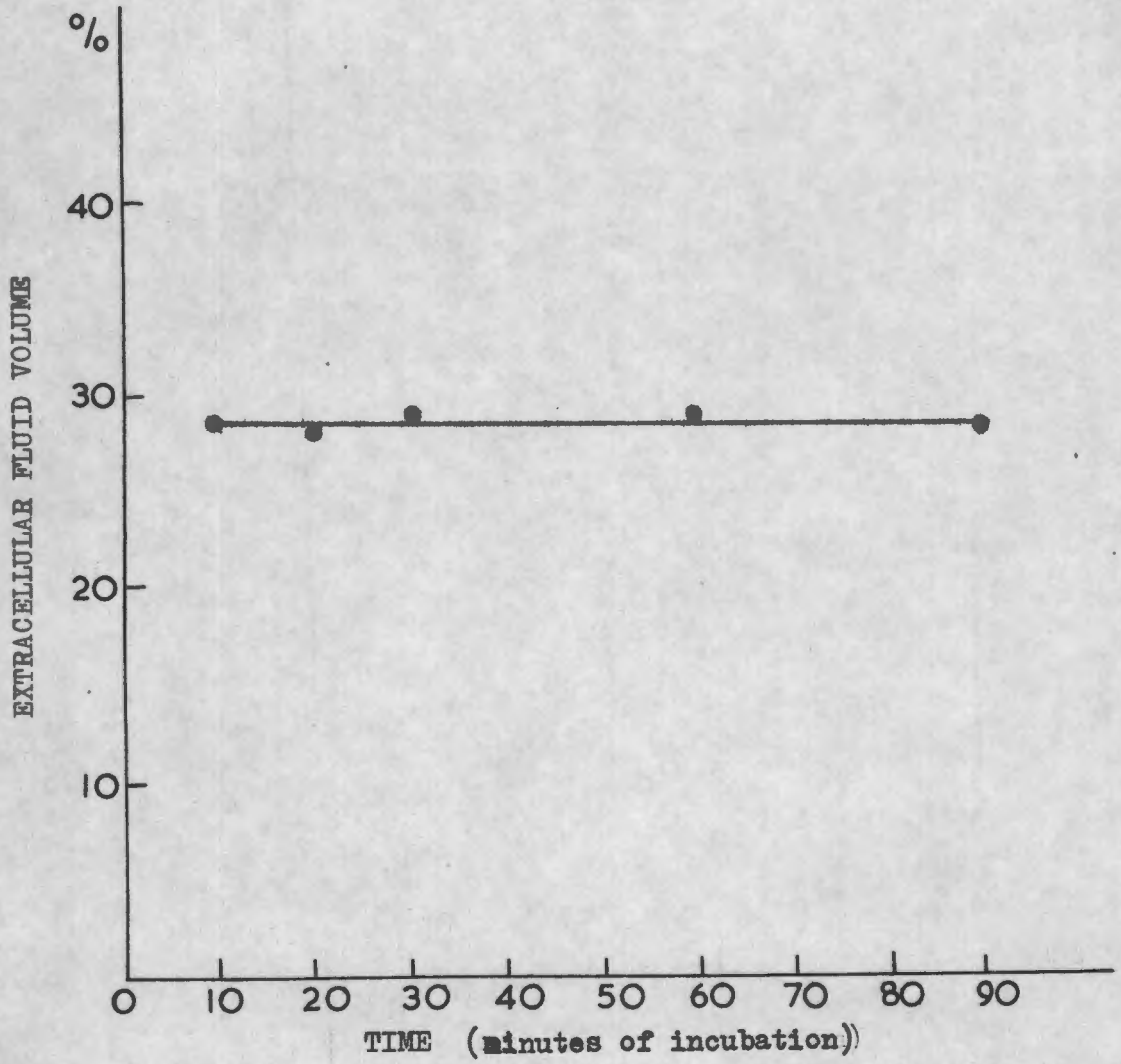

FIGURE I. The extracellular fluid volume (inulin space) expressed as a percentage of total tissue water is plotted against the time of incubation in minutes. Each point represents the mean of six determinations. It can be seen that the E.C.F. is virtually the same as measured by this method, whatever the period of incubation may be between the extremes of 10 and 90 minutes. The conditions of incubation are as described in the text.

TABLE I.

The *In Vitro* Effects of Sugars on the Extracellular Fluid Volume of Rat Intestinal Segments.

Sugar added to the Medium at a Concentration of 28mM.	Extracellular Fluid Volume of Rat Intestinal Segments expressed as a percentage of Total Tissue Water.*
None	28.7 ± 3.1 (22)
D-Galactose	28.9 ± 3.4 (15)
D-Fructose	28.1 ± 2.9 (10)
D-Glucose	26.8 ± 3.0 (10)
D-Xylose	26.4 ± 3.0 (3)

* The results are shown as the means ± Standard Deviation with the number of determinations in parentheses. Each flask contained 5 ml. of Krebs-Ringer bicarbonate buffer (pH 7.4) and six everted segments weighing 200 - 300 mg. Incubations were carried out for 60 minutes at 37°C and the flasks were gassed continuously with 95% oxygen and 5% carbon dioxide. The initial concentration of carboxyl-C^{14}-inulin in the medium was 8×10^{-5} mM./ml. with a specific activity of 1.0 uC/u.mole.

Section 2 - Results

(i) Measurements of Extracellular Fluid Volume (E.C.F.)

Figure 1 shows the E.C.F. expressed as a percentage of total tissue water in relationship to the time of incubation. It can be seen that the E.C.F. was about 28% of total tissue water regardless of the duration of incubation and that the inulin had permeated throughout the E.C.F. after as short a period as 10 minutes.

Table 1 shows the effect on the E.C.F. of incubating rat intestinal segments for sixty minutes in the presence of galactose, fructose, or glucose at concentrations of 28mM. It can be seen that there was no significant change in the E.C.F. under these conditions. It was also noteworthy that there was no change in the water content of segments incubated with or without sugars.

(ii) *In Vitro* Study of Amino-acid Transport in Rat Intestinal Segments in the Presence of Sugars.

The relationship of the distribution ratio of L-alanine in rat intestinal segments to the time of incubation, is shown in Figure 2. It can be seen that the distribution ratio was significantly above unity after only 10 minutes of incubation, and that the distribution ratio had doubled after incubation for fifteen minutes. Thereafter the distribution ratio showed no

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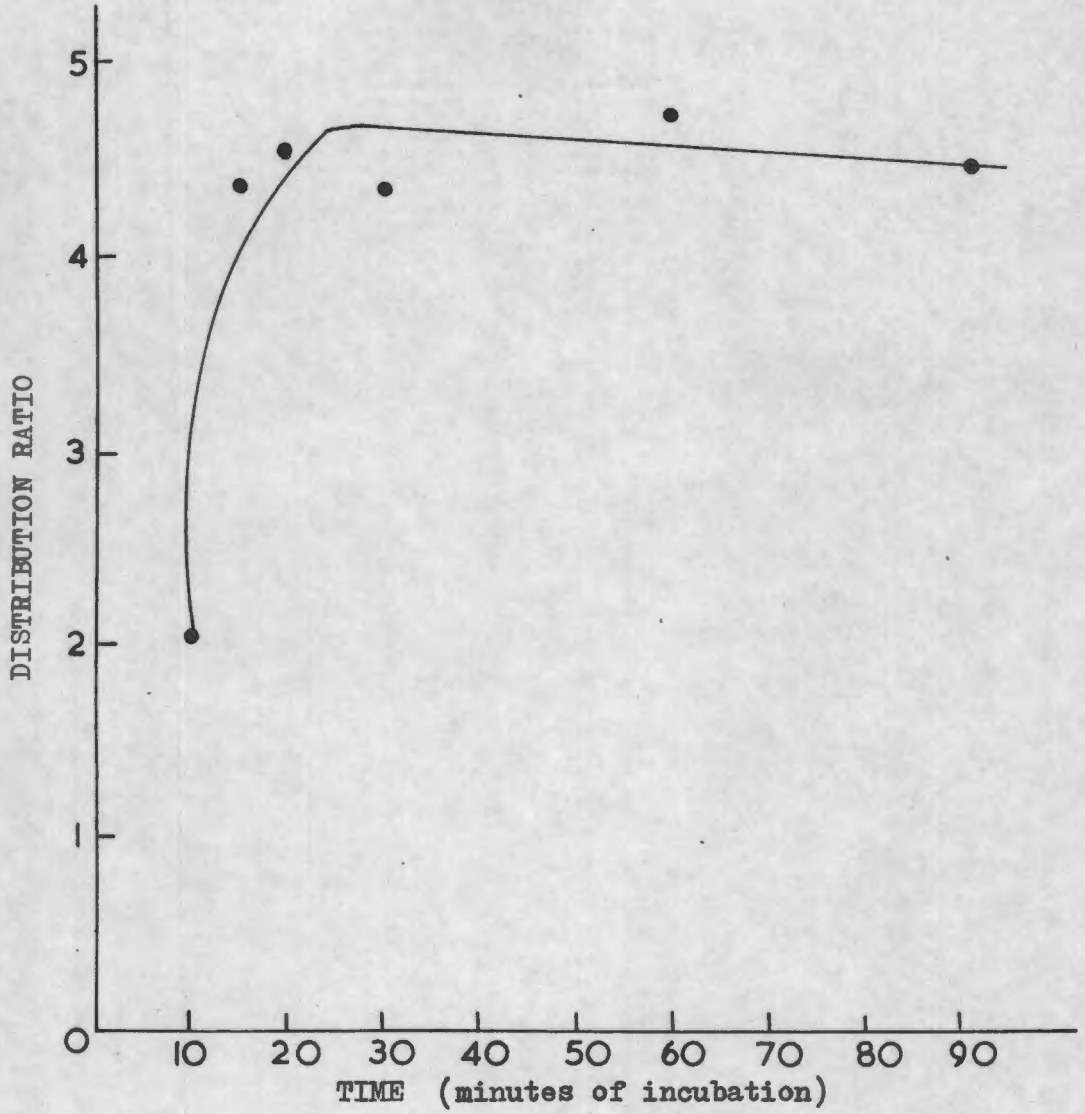

FIGURE 2. The distribution ratio for C^{14}-L-alanine in rat intestinal segments is plotted against the time of incubation in minutes. Each point represents the mean of 9 experiments except the distribution at 60 minutes, which is the mean of 31 experiments. The conditions of incubation are as described in the text. It can be seen that significant transfer of C^{14}-L-alanine has occurred after incubation for 10 minutes, but that the distribution ratio has more than doubled after incubation for 15 minutes. Thereafter no significant increase in the distribution ratio has taken place.

TABLE II

The Effect of D-Galactose on the *In Vitro* Transport of Amino-acids by Rat Intestinal Segments.

(Initial Amino-acid Concentration of 5 mMoles per Litre.)

Addition to Medium**		Amino-acid Distribution Ratios*				
		L-Alanine	L-Valine	Glycine	L-Lysine	Hydroxy-L-proline
None		4.7 ± 0.9 (31)	4.2 ± 1.1 (20)	2.2 ± 0.5 (17)	1.4 ± 0.4 (16)	2.0 ± 0.5 (19)
D-Galactose	5.6mM.	2.7 ± 0.7 (7) p<0.001***	3.7 ± 0.9 (10)	1.6 ± 0.4 (6) p<0.01		2.1 ± 0.5 (4)
	28 mM.	2.7 ± 0.7 (9) p<0.001	2.8 ± 0.3 (9) p<0.001	1.3 ± 0.4 (9) p<0.001	0.9 ± 0.1 (6)	1.5 ± 0.4 (8) p<0.025

* Ratios shown are means ± S.D. with the number of determinations in parentheses. Each flask contained 5ml. of Krebs-Ringer bicarbonate buffer (pH 7.4) and six everted segments weighing a total of 200 - 300 mg. Incubations were carried out for 60 minutes at 37°C and the flasks were gassed continuously with 95% oxygen and 5% carbon dioxide.

** Galactose was present at the final concentrations indicated.

*** Significance (p values) of the difference between the distribution ratios in slices incubated in the absence of sugar and those obtained in the presence of galactose. Where p values are not given no significant difference in amino-acid transport was observed.

TABLE III

The Effect of D-Fructose on the In Vitro Transport of Amino-acids by Rat Intestinal Segments.

(Initial Amino-acid Concentration of 5 mMoles per Litre.)

Addition to Medium[mm]		Amino-acid Distribution Ratios[m]				
		L-Alanine	L-Valine	Glycine	L-Lysine	Hydroxy-L-proline
None		4.7 ± 0.9 (31)	4.2 ± 1.1 (20)	2.2 ± 0.5 (17)	1.4 ± 0.4 (16)	2.0 ± 0.5 (19)
D-Fructose	5.6mM.	3.3 ± 0.3 (7)	-	-	-	2.7 ± 0.2 (4)
	28 mM.	2.9 ± 0.7 (11) p <0.001 [mmm]	4.1 ± 1.0 (9)	2.6 ± 0.4 (4)	1.2 ± 0.2 (7)	1.9 ± 0.7 (7)

m Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

mm Fructose was present at the final concentrations indicated.

mmm Significant (p values) of the difference between the distribution ratios in slices incubated in the absence of sugar and those obtained in the presence of fructose. Where p values are not given no significant difference in amino-acid transport was observed.

TABLE IV

The Effect of D-Glucose on the *In Vitro* Transport of Amino-acids by Rat Intestinal Segments.

(Initial Amino-acid Concentration of 5 mMoles per Litre.)

Addition to Medium[mm]		Amino-acid Distribution Ratios[*]				
		L-Alanine	L-Valine	Glycine	L-Lysine	Hydroxy-L-Proline
None		4.7 ± 0.9 (31)	4.2 ± 1.1 (20)	2.2 ± 0.5 (17)	1.4 ± 0.4 (16)	2.0 ± 0.5 (19)
D-Glucose	16.8mM	4.3 ± 0.2 (3)	-	-	-	-
	28 mM.	4.1 ± 0.5 (6)	3.8 ± 1.1 (9)	2.0 ± 0.3 (6)	1.2 ± 0.2 (6)	2.0 ± 0.2 (4)

[*] Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

[mm] Glucose was present at the final concentrations indicated.

No p values are given as no significant difference in amino-acid transport was observed.

TABLE V

The Effects of D-Xylose, D-Ribose and 3-O-Methyl Glucose on the *In Vitro* Transport of Amino-acids by Rat Intestinal Slices.

(Initial Amino-acid Concentration of 5 mMoles per Litre.)

Addition to Medium**		Amino-acid Distribution Ratios*				
		L-Alanine	L-Valine	Glycine	L-Lysine	Hydroxy-L-proline
None		4.7 ± 0.9 (31)	4.2 ± 1.1 (20)	2.2 ± 0.5 (17)	1.4 ± 0.4 (16)	2.0 ± 0.5 (19)
D-Xylose	28mM.	5.4 ± 0.7 (4)	3.7 ± 1.2 (5)	2.0 ± 0.1 (2)	1.2 ± 0.1 (3)	2.7 ± 0.2 (3)
D-Ribose	28mM.	5.0 ± 0.4 (3)	3.7 ± 1.0 (6)	1.9 ± 0.3 (5)	1.1 ± 0.1 (3)	2.7 ± 0.2 (3)
3-O-Methyl Glucose	28mM.	4.2 ± 0.4 (5)	4.4 ± 0.8 (7)	2.2 ± 0.2 (6)	-	-

* Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

** Sugars were present in the final concentrations indicated.

*** No p values are given as no significant difference in amino-acid transport was observed.

significant change even after incubation for ninety minutes. In view of these findings, the studies of the effects of sugars on amino-acid transport reported in this chapter were done in all instances with an incubation period of sixty minutes. Table II shows effects of incubating rat intestinal segments for sixty minutes in the presence of amino-acids (5mM.) without added sugar, as well as with galactose in the medium at concentrations of 5.6mM and 28mM. In can be seen that galactose significantly inhibited the transport of L-alanine, L-valine, glycine and hydroxy-L proline.

Table III shows the results obtained when rat intestinal segments were incubated in the presence of amino-acids (5mM.) and fructose (5.6mM. and 28mM.). Fructose inhibited L-alanine transport only. From Table IV it can be seen that glucose (16.8mM. and 28mM.) had no inhibitory effect on amino-acid transport, when the amino-acids were present at a concentration of 5mM.

Table V shows the results for xylose, ribose and 3-O-methyl-glucose. These sugars did not inhibit amino-acid transport. When L-alanine was present in the medium at an initial concentration of 5mM., and both glucose and galactose were added, the concentration of each being 28mM., inhibition of L-alanine transport by galactose was still observed. Distribution Ratio for L-alanine = 3.3 ± 0.9 (mean $\pm$ Standard Deviation of six experiments). When the initial amino-acid concentration was reduced one hundred fold to 0.05mM. and all the sugars tested were used at an initial concentration of

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TABLE VI

The Effect of D-Galactose on the In Vitro Transport of Amino-acids by Rat Intestinal Slices.

(Initial Amino-acid Concentration of 0.05 mMoles per Litre.)

Addition to Medium[mm]	Distribution Ratios[*]						
	L-Alanine	L-Valine	Glycine	L-Lysine	L-Arginine	L-Phenyl-alanine	Hydroxy-L proline
None	6.7±0.4 (5)	16.5±3.4 (4)	4.4±0.8 (3)	12.1±1.6 (8)	7.4±2.1 (3)	9.5±2.3 (6)	3.1±1.3(4)
D-Galactose 28mM.	4.2±0.4 (3) $p<0.01$[mmm]	8.8±1.8 (3) $p<0.001$	1.8±0.8 (3) $p<0.001$	10.9±2.5 (10)	9.1±2.3 (3)	11.0±1.1 (3)	-

* Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

mm Galactose was present at the final concentration indicated.

mmm Significance (p values) of the difference between the distribution ratios in slices incubated in the absence of sugar and those obtained in the presence of galactose. Where p values are not given no significant difference in amino-acid transport was observed.

TABLE VII

The Effect of D-Fructose on the In Vitro Transport of Amino-acids by Rat Intestinal Slices.

(Initial Amino-acid Concentration of 0.05 mMoles per Litre.)

Addition to Medium**	Distribution Ratios*						
	L-Alanine	L-Valine	Glycine	L-Lysine	L-Arginine	L-Phenyl-alanine	Hydroxy-L proline
None	6.7±0.4 (5)	16.5±3.4 (4)	4.4±0.8 (3)	12.1±1.6 (8)	7.4±2.1 (3)	9.5±2.3 (6)	3.1±1.3 (4)
D-Fructose 28mM.	4.6±0.8 (3) $p<0.025$***	19.8±4.3 (4)	1.8±0.4 (3) $p<0.001$	11.3±2.5 (3)	8.6±2.2 (3)	10.8±4.0 (3)	4.0±1.0 (4)

* Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

** Fructose was present at the final concentration indicated.

*** Significance (p values) of the difference between the distribution ratios in slices incubated in the absence of sugar and those obtained in the presence of fructose. Where p values are not given no significant difference in amino-acid transport was observed.

TABLE VIII

The Effect of D-Glucose, D-Xylose, D-Ribose and 3-O-Methyl Glucose on the In Vitro Transport of Amino-acids by Rat Intestinal Slices.

(Initial Amino-acid Concentration of 0.05 mMoles per Litre.)

Addition to Medium**	Distribution Ratios*						
	L-Alanine	L-Valine	Glycine	L-Lysine	L-Arginine	L-Phenyl-alanine	Hydroxy-L proline
None	6.7±0.4 (5)	16.5±3.4 (4)	4.4±0.8 (3)	12.1±1.6 (8)	7.4±2.1 (3)	9.5±2.3 (6)	3.1±1.3 (4)
D-Glucose 28mM.	4.3±0.8 (3) p<0.025***	10.5±4.7 (5) p<0.05	1.9±0.9 (3) p<0.01	11.1±2.4 (5)	10.0±2.4 (3)	13.1±4.3 (2)	3.9±0.7 (5)
D-Xylose 28mM.	8.1±0.9 (2)	-	-	-	-	-	-
D-Ribose 28mM.	6.2±0.3 (2)	-	-	-	-	-	-
3-O-Methyl Glucose 28mM.	6.9±1.1 (3)	-	-	-	-	-	-

* Ratios shown are means ± S.D. with the number of determinations in parentheses. The segments were incubated as described in Table II.

** Sugars were present at the final concentrations indicated.

*** Significance (p values) of the difference between the distribution ratios in slices incubated in the absence of sugar and those obtained in the presence of glucose. Where p values are not given, no significant difference in amino-acid transport was observed.

28mM., galactose again caused significant inhibition in the transport of L-alanine, L-valine and glycine (Table VI), while fructose now inhibited glycine in addition to L-alanine (Table VII). It was of interest that at this lower concentration of amino-acid, the presence of glucose did serve to inhibit the transport of L-alanine, L-valine and glycine. Xylose, ribose and 3-0-methyl-glucose did not inhibit L-alanine transport under these experimental conditions (Table VIII). When rat intestinal segments were incubated with L-alanine (5mM) for sixty minutes as described, but in an atmosphere of nitrogen, the mean distribution ratio was 0.7 $\pm$ 0.1 (6)*. When segments were incubated at 22°C the mean distribution ratio was 2.1 $\pm$ 0.1 (6)* and at 37°C but with 2 - 4 dinitrophenol (10^{-4}M) in the medium, the mean distribution ratio was 1.2 $\pm$ 0.2 (6)*. These data on amino-acid transport in the absence of oxygen, at a lower temperature and in the presence of 2 - 4 dinitrophenol, confirm that under the experimental conditions used, the transport of amino-acids is almost certainly an active process.

Section 3 - Discussion and Conclusions.

Galactose and fructose definitely inhibited amino-acid transport in rat intestinal segments at both concentrations of amino-acid

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*Expressed as means $\pm$ Standard Deviations with the number of experiments in parentheses.

used (0.05mM. and 5mM.) while glucose caused diminished amino-acid transport when the amino-acids were at an initial concentration of 0.05mM. The amino-acids which showed inhibition of intestinal transport by hexoses in vitro did not conform to the four intestinal groups as described in Chapter II. Of the neutral amino-acid group, galactose inhibited L-alanine and L-valine transport but not that of L-phenylalanine; fructose inhibited L-alanine but not L-valine or L-phenylalanine; and glucose inhibited L-alanine and L-valine but not L-phenylalanine.

Of the imino-acid - glycine group, galactose inhibited both glycine and hydroxy-L-proline but fructose and glucose inhibited glycine only.

It is of course possible that under different experimental conditions a closer correlation with transport groups might emerge, but as indicated this was not the case in the results described here. The closest correlation with the transport groups is the fact that the amino-acids predominantly affected, both here and in subsequent studies, which will be dealt with in later chapters, were those of the monoamino - monocarboxylic group plus glycine which shares this transport group as well as that of the imino-acids.

It is also noteworthy that 3-O-methyl-glucose, which is subject to active transport in the intestine, and does inhibit the transport of other sugars[216], did not inhibit amino-acid transfer. These results suggest that the inhibition of amino-acid transport by hexoses is not of a competitive nature. This is supported by the fact

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fructose was able to inhibit L-alanine transport although this sugar moves across the intestinal wall by a process of diffusion[210][211]. It is known that fructose may be partially converted to glucose before absorption (210). However the inhibition of intestinal amino-acid transport induced by fructose could not have been due to partial conversion to glucose alone for under certain experimental conditions glucose did not inhibit the transport of L-alanine, while fructose did. Fructose may also be converted to lactic acid by the intestine and partially absorbed as such[212][213][194].

Further evidence will be given later to support the contention that the observed inhibition of amino-acid transport was noncompetitive. (Chapter IX). If the sugars had exerted competitive inhibition, it would have had to have taken place at a site other than the one where members of groups of amino-acids competitively inhibit mutual transport in the gut, and also discrete from the site where sugars exert mutual inhibition of intestinal transfer.

It was interesting to note the failure of glucose to inhibit amino-acid transport when the initial amino-acid concentration was 5mM. Indeed it can be seen that the distribution ratios for all the amino-acids were greater when the initial concentration of amino-acids in the medium was 0.05mM. Presumably this was due to the greater saturation of the transport process at the higher concentration of amino-acid. Probably the correct inference is that glucose is a less potent inhibitor of amino-acid transport than either galactose or fructose.

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The failure of sugars to inhibit the intestinal transport of the dibasic amino-acids, lysine and arginine, is of great interest. In this respect the results did conform to known amino-acid transport groups, and it is noteworthy that this is in keeping with Milne's observations that, after the oral administration of neomycin to man, the absorption of the dibasic amino-acids is not interfered with as much as that of the members of the other transport groups(109).

The absence of any change in the E.C.F. or in total tissue water, under the experimental conditions used, exclude any effect of water shift on the results obtained.

Segal, Thier, Fox and Rosenberg(214) recently reported results of the influence of fructose on L-cycloleucine and L-lysine transport by rat intestinal slices. The transport of L-cycloleucine but not of L-lysine was inhibited by fructose. No other amino-acids or sugars were tested in the gut as these authors were predominantly interested in the renal transport of amino-acids.

In 1964 Newey and Smyth(215) reported that galactose inhibited, and that glucose enhanced, the transfer of glycine and L-methionine in everted rat gut sacs. Their results also indicated that simultaneous incubation with glucose could overcome the inhibition produced by galactose. In contrast to these authors the results reported here show neither enhanced transfer of amino-acids in the gut in the presence of glucose, nor

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correction by glucose of the inhibition caused by galactose.

The reason for the different results Newey and Smyth obtained with glucose is not easy to determine. It should however be pointed out that far more experiments on a larger number of amino-acids are reported here than was published by those workers.

The results detailed in this chapter are more in keeping with those reported in rat kidney cortex slices.(214)

CHAPTER VI

The Transport of Amino-acid by the Intestine of Rats fed 30% Galactose, Fructose or Glucose for Two to Three Months.

Section 1 - Methods.

Young Sprague-Dawley rats fed 30% galactose diets for 2 - 3 months develop a renal amino-aciduria[190]. In addition these animals develop other features reminiscent of galactosaemia in man such as cataract formation[201]. They also develop polyuria[217][218] galactosuria[199][200], hypercalciuria and hypercalcaemia[218], hypernatruria, hyperkaluria and hyperphosphaturia[219]. Hypoglycaemia, hypophosphataemia and depletion of liver glycogen have also been reported in rats fed 40% galactose diets[220]. More recently studies in carbohydrate and protein metabolism in the lenses of these animals have been reported[221][222][223][224][225].

Accordingly male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 50g. were fed 30% galactose diets for sixty to ninety days. The transport of amino-acids was studied in the intestine of these animals in precisely the same way as detailed in Chapter V, with the exception that sugars were at no time added to the incubation medium. Similarly amino-acid transport was measured in intestinal segments of rats fed 30% fructose or 30% glucose for 2 - 3 months, the diets commencing when the rats weighed 50g.

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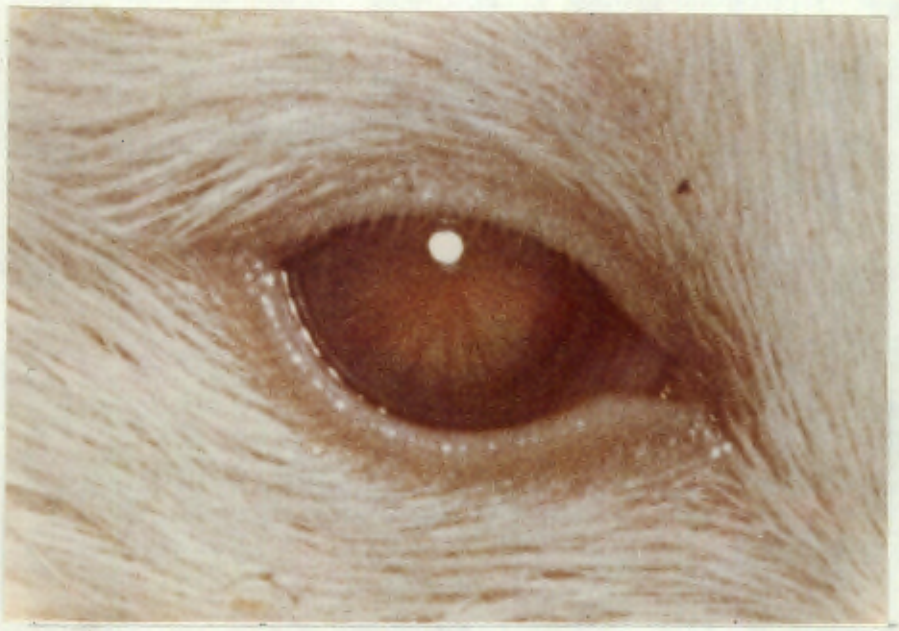

FIGURE 3. This illustrates the development of a cataract in a rat which was fed 30% galactose for 2 - 3 months.

TABLE IX

Amino-acid Transport by the Intestinal Slices of Control Versus Galactose-fed Rats.

Intestinal Slices	Distribution Ratios*										
	L-Alanine	L-Valine		L-Phenyl-alanine	Glycine		L-Lysine		L-Argi-nine	Hydroxy-L proline	
	5mM**	5mM.	0.05mM.	0.05mM.	5mM.	0.05mM.	5mM.	0.05mM.	0.05mM.	5mM.	0.05mM.
Control	4.3±0.9 (12)	4.4±1.0 (9)	16.5±3.4 (4)	8.1±2.8 (6)	2.4±0.6 (9)	4.4±0.8 (3)	1.5±0.4 (10)	12.1±1.6 (8)	7.4±2.1 (3)	1.9±0.5 (11)	3.1±1.3 (4)
Galactose-fed	2.8±1.0 (6)	2.3±0.8 (6)		9.4±1.6 (6)	1.5±0.7 (6)		1.3±0.3 (6)		8.0±0.2 (3)	1.3±0.5 (6)	
	p<0.005***	p<0.005			p<0.01						

* Ratios shown are means ± S.D. with the number of determinations in parentheses. The incubation conditions were the same as described in Table II.

** Initial concentration of amino-acid in medium.

*** Significance (p values) of the difference between the distribution ratios of control and galactose-fed rats. Where p values are not given no significant difference in amino-acid transport was observed.

TABLE X

Amino-acid Transport by the Intestinal Slices of Control Versus Fructose-fed Rats.

Intestinal Slices.	Distribution Ratios[*]										
	L-Alanine	L-Valine		L-Phenyl-alanine	Glycine		L-Lysine		L-Argi-nine	Hydroxy-L proline	
	5mM[mm]	5mM.	0.05mM.	0.05mM.	5mM.	0.05mM.	5mM.	0.05mM.	0.05mM.	5mM.	0.05mM.
Control	4.3 ± 0.9 (12)	4.4 ± 1.0 (9)	16.5 ± 3.4 (4)	8.1 ± 2.8 (6)	2.4 ± 0.6 (9)	4.4 ± 0.8 (3)	1.5 ± 0.4 (10)	12.1 ± 1.6 (8)	7.4 ± 2.1 (3)	1.9 ± 0.5 (11)	3.1 ± 1.3 (4)
Fructose-fed	2.9 ± 0.9 (10) $p<0.005$[mmm]	4.2 ± 1.1 (6)	17.3 ± 6.4 (3)	11.3 ± 1.3 (3)	2.7 ± 0.8 (6)	4.3 ± 0.7 (3)	1.7 ± 0.3 (6)	15.8 ± 1.6 (3)	13.2 ± 2.0 (3)	1.7 ± 0.7 (10)	3.0 ± 0.4 (2)

[*] Ratios shown are means $\pm$ S.D. with the number of determinations in parentheses. The incubation conditions were the same as described in Table II.

[mm] Initial concentration of amino-acid in medium.

[mmm] Significance (p values) of the difference between the distribution ratios of control and fructose-fed rats. Where p values are not given no significant difference in amino-acid transport was observed.

Section 2 - Results.

Figure 3 shows the development of a cataract which occurred in all of the rats fed galactose. The results of the studies of amino-acid transport in the small intestine of these galactose-fed rats are shown in Table IX. It can be seen that there was significantly decreased intestinal transport of L-alanine, L-valine and glycine in these animals as compared with their controls, which were litter mates receiving ordinary rat food without added galactose.

Table X shows the results of a similar experiment in male Sprague-Dawley rats (Charles River Breeding Laboratories) fed 30% fructose for sixty to ninety days. Again the controls were litter mates receiving food without added fructose. The fructose-fed animals showed an inhibition of L-alanine transport only.

The transport of L-alanine and glycine was similarly measured in intestinal segments from three rats fed glucose for two to three months and in three litter mate controls. The transport of these amino-acids was normal in the glucose-fed rats. (Mean distribution ratio of L-alanine 6.6, and of glycine 4.2.)

Section 3 - Conclusions

Galactose-fed and fructose-fed rats clearly developed a defect in the intestinal transport of certain of the amino-acids tested. There was a close correlation between the results reported

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here and the *in vitro* effect of galactose and fructose on isolated intestinal segments from normal rats (Chapter V). The one exception was the *in vitro* inhibition of hydroxy-L-proline by added galactose (28mM) when the initial amino-acid concentration was 5mM.

The glucose-fed animals tested did not have a demonstrable defect in amino-acid transport in the gut. This is in keeping with the less regular inhibition of amino-acid transport in the intestine by glucose *in vitro*.

It is possible that if more glucose-fed rats had been tested, an occasional animal might have shown defective intestinal transport of some amino-acids. As will be seen in Chapter VII the data on the renal excretion of amino-acids in these animals supports this suggestion.

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CHAPTER VII

The Development of Amino-aciduria in Fructose-fed and Glucose-fed Rats.

Section 1 - Methods.

Fructose-fed and Glucose-fed rats were put in metabolism cages. They were given no food but were allowed water ad libitum. A wire trap prevented contamination of the urine with the faeces and all the urine passed in a 24 hour period was collected. There was no significant difference between the urine volumes from the fructose-fed rats, and their controls. The glucose-fed animals had slightly smaller urine volumes than their controls. The urine was frozen immediately after collection. A ten minute volume of urine from each rat was analysed on a Technicon amino-acid analyser for the quantitative estimation of the urinary amino-acids. The resin used was Chromobeads Type B (Technicon) and the column 127 x 0.62cms.

Procedure:

0.2 N NaOH was pumped through the column for thirty minutes followed by a sodium citrate buffer of pH 2.875 for a further ninety minutes before the sample was put on with 2ml. of citric acid buffer (pH 2.2). The sample was put on the column with great care to achieve accurate quantitative results, the same carefully washed pipette being used each day. The sides of the column were washed down with the pH 2.2

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buffer to ensure that all the sample entered the resin. Norleucine (0.1 u mole/ml.) was used each day as an internal standard and was put on the column in the same careful manner. The sample * and internal standard were driven into the column with air at 100psi. Colour development was with ninhydrin under nitrogen, the amino-acids passing through an oil bath at 95°C before being cooled, and entering the flow cuvettes of the colorimeters. The amino-acids were read at 440 um and 570 um wave lengths. The results were calculated after the method of Moore and Stein[18].

The ninhydrin was made up as follows : -

45g. of ninhydrin and 3.375g. of hydrindantin were dissolved in 4.5L of methylcellusolve in a dark bottle and mixed for 15 minutes. 900ml. of a sodium acetate buffer (sodium acetate 1640g., glacial acetic acid 500ml., water to 5L) and 3600ml. of water were then added. Nitrogen was bubbled through the mixture throughout and for at least 30 minutes after the addition of the water.

The buffers were made up as follows : -

<u>pH 2.875 buffer</u>.

14.71gm. of sodium citrate (0.05m.; 0.150 N with respect to Na^+); 25.0ml. of 2.000 N (Standardised) NaOH; and 5.0ml. of thiodiglycol (Pierce Chemical Co.) were added to 900ml. of water. The pH was adjusted to 2.875 with 6 N HCl and 10ml. of Brij 35 solution (Atlas Chemical Indus. Inc.) were then added. The volume was made up to 1 L and the pH again adjusted to 2.875 with 6 N HCl if necessary.

* Ten or thirty minute deproteinised urine volume.

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pH 3.785 Buffer

This buffer was made up in precisely the same manner as the pH 2.875 buffer but the final pH adjustment was to 3.785.

pH 4.7 buffer

471.308gms. of sodium citrate were dissolved in 5.4L of water. The pH was adjusted to 4.7 with 6 N HCl. 60ml. of Brij 35 were then added and the final volume made up to 6L.

All water used was glass distilled and then deionised.

All buffers were stored at 4°C and the pH checked before use and adjusted if necessary.

The nine chambered autograd was used to obtain a graded buffer change through the column and the buffers were pumped through the column at 30ml./hour, and at a pressure of 200psi. The distribution of the buffers in the autograd was as follows : -

Chamber 1	:	75 ml. of pH 2.875 buffer
Chamber 2	:	75 ml. of pH 2.875 buffer
Chamber 3	:	75 ml. of pH 2.875 buffer
Chamber 4	:	75 ml. of pH 2.875 buffer
Chamber 5	:	40 ml.of pH 2.700 buffer and 35 ml. of pH 3.785 buffer
Chamber 6	:	6 ml. of pH 2.700 buffer, 9 ml. of pH 3.785 buffer, 60 ml. of pH 4.700 buffer
Chamber 7	:	75 ml. of pH 4.700 buffer
Chamber 8	:	75 ml. of pH 4.700 buffer
Chamber 9	:	75 ml. of pH 4.700 buffer.

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TABLE XI

Urinary Amino-acids in Fructose-fed Rats.

Animal	Urine Volume (ml./24 hrs.)	Urinary Amino-acids (u.moles/24 hrs.)		
		Glutamic acid	L-alanine	Glycine
1. Fructose-fed	12.5	1.13	1.91	19.84
2. Fructose-fed	7.2	2.57	28.60	26.00
3. Control	8.5	3.47	1.64	5.10
4. Control	15.3	1.86	3.05	10.11

The Fructose-fed rats had received a diet of 30% fructose for 2 - 3 months before the urine was collected. The controls were litter mates which received a normal diet. The amino-acids were estimated quantitatively by column chromatography. No other amino-acids were present in measurable amounts.

TABLE XII

Urinary Amino-acids in Glucose-fed Rats.

	Animal	Urinary Volume (ml./24 hours)	Urinary Amino-acids (u moles/24 hours)		
			Glutamic acid	L-alanine	Glycine
1.	Glucose-fed	10	1.77	1.22	4.45
2.	Glucose-fed	12	0.75	1.16	24.69
3.	Glucose-fed	14	1.98	0.89	3.70
4.	Glucose-fed	10.5	2.09	0.95	3.79
5.	Control	15	1.66	0.96	9.25
6.	Control	22	1.51	1.12	5.02
7.	Control	15	3.80	1.77	14.14
8.	Control	18.5	2.89	2.01	8.70

The glucose-fed rats had received a diet of 30% glucose for 2 - 3 months before the urine was collected. The controls were litter mates which received a normal diet. The amino-acids were estimated quantitatively by column chromatography. No other amino-acids were present in measurable amounts.

The temperature of the column was kept at 37°C for the first ninety minutes, and thereafter was increased to 60°C for the remaining 19½ hours of the chromatogram.

Section 2 - The results in the fructose-fed rats are shown in Table XI. There was a significant increase in the excretion of glycine in both of the fructose-fed animals. One of the fructose-fed rats also showed a considerable increase in the excretion of L-alanine No plasma levels were estimated but it seems very likely that this is a renal amino-aciduria similar to that which occurs when rats are fed galactose[190]. The amino-acids not shown in Table XI were not present in amounts sufficient to estimate accurately.

Table XII shows the results for the glucose-fed animals. Only one of the four glucose-fed rats tested showed an excessively high glycine excretion. No other amino-acids were increased in amount. When the results were expressed per mg. creatinine/24 hours, this single example of glycinuria was confirmed.

This rat excreted 2.88 u moles of glycine/mg. of creatinine. The other three glucose-fed animals excreted a mean of 0.50 u moles of glycine/mg. of creatinine, and the four controls a mean of 0.80 u moles/mg. creatinine.

Section 3 - Discussion and Conclusions

It has been clearly demonstrated that fructose-fed rats develop an amino-aciduria involving L-alanine and glycine only. This corresponds fairly closely to the effect of fructose on the

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transport of amino-acid in normal rat intestine in vitro (Chapter V), and to the transport defect in the intestine of fructose-fed rats (Chapter VI) where only L-alanine and not glycine showed decreased transport. It is believed that this is a demonstration of the development of a defect in both the intestinal and renal tubular transport of amino-acids in fructose-fed rats. Galactose-fed rats also develop "renal" amino-aciduria as mentioned previously[190] and this involves inter alia L-alanine and L-valine. There is no increase in lysine, phenyl-alanine or glycine. Arginine and hydroxyproline have not been estimated in the urine of these animals. The close correlation between these reported results, the experiments in galactose-fed animals reported in Chapter VI, and the in vitro studies with galactose in Chapter V is again obvious.

One glucose-fed rat developed glycinuria. None of the four tested had an increase in any other urinary amino-acids. It seems that the development of an amino-aciduria is less frequent in glucose-fed than in fructose-fed or galactose-fed animals.

Unfortunately intestinal amino-acid transport was not studied in the one glucose-fed rat with glycinuria. None of the glucose-fed rats had glycosuria.

Recently there has been a report on the effect of sugars on amino-acid transport in rat kidney cortex slices in vitro. In 1962 Segal, Thier, Fox and Rosenberg[226] showed that galactose, fructose and glucose inhibited the accumulation

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of L-aminoisobutyric acid, glycine, L-cycloleucine and L-valine by rat kidney cortex slices, and not that of L-histidine, L-lysine or L-phenylalanine. The sugars were present at a concentration of 16.8 mM. The initial amino-acid concentration in the incubation medium varied from 0.03mM. to 0.28mM. They found that glucose was not inhibitory at 5.6mM. but exerted an effect at 11.2mM. Galactose and fructose caused inhibition even at a concentration of 5.6mM. Raffinose, sucrose, xylose, ribose, 3-O-methyl-glucose, 2-deoxy-glucose and 3-deoxy-glucose at concentrations of 16.8mM. did not effect any inhibition of amino-acid transport. They have reported further on this very recently(227) and in this paper confirmed their previous work with the exception that they now reported that at a concentration of 5.5mM. only fructose depressed amino-acid accumulation. They also found no inhibition with D-fucose, D-Mannose or D-mannoheptulose.

In addition unpublished observations have shown that the intravenous administration of galactose, fructose or glucose to man, results in a "renal" amino-aciduria during the infusion of sugar(187).

It would seem therefore that both the <u>in vitro</u> and the <u>in vivo</u> effects of sugars on amino-acid transport in the gut are shared by the kidney.

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CHAPTER VIII

The Effect of Sugars on the Incorporation of an Amino-acid into Brain and Liver Cellular Protein in the Rat.

Section 1 - Introduction

The brain and liver may be affected in galactosaemia. If the clinical manifestations of this disease extend beyond four to eight weeks, one can usually detect evidence of mental retardation. If exposure to galactose continues, gross mental deficiency often results. In addition jaundice commonly occurs in galactosaemia. The liver is initially fatty and shows focal cellular necrosis but readily proceeds to cirrhosis if milk ingestion persists[171].

Similarly in hereditary fructose intolerance, jaundice and disturbed liver function tests commonly occur on exposure to the offending sugar[181]. The incidence of mental retardation in this syndrome remains to be established. Of course the associated episodes of hypoglycaemia may play a role in this regard.

In view of the involvement of the brain and the liver in galactosaemia, and the liver and possibly the brain in hereditary fructose intolerance, it was decided to study the effects of sugars on the incorporation of amino-acid into brain and liver protein. This is of especial interest in view of the demonstration that galactose, fructose and glucose inhibited amino-acid transport in the gut and kidney. It seemed feasible that interference with

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amino-acid transport across brain and liver cell membranes might play a role in the pathogenisis of the lesions in those organs in galactosaemia and hereditary fructose intolerance.

Section 2 - Choice of Method.

After confirming that slices of rat brain and liver did not accumulate amino-acids sufficiently well to allow moderate inhibition of transport to be demonstrated, it was decided to measure the incorporation of an amino-acid (C^{14}-L-alanine) into brain and liver protein. This can be regarded as an indirect measure of transport as well as an index of the cells' ability to incorporate amino-acids into protein.

The Method:

Incorporation of L-Alanine into Brain and Liver Protein.

10 uC of C^{14} uniformly labelled L-alanine was injected intravenously into the tail vein of a rat which had been fed either 30% galactose or fructose for 2 - 3 months. Controls received the same dose of the isotope. In other experiments normal rats were given four intraperitoneal injections of galactose, glucose or fructose (300mg./injection) over three hours and the C^{14}-L-alanine was given intravenously at the mid-point. In each case a weighed amount of isotope was given and a weighed standard used for counting purposes. The rat was killed 2½ hours after the injection of the isotope, the brain removed, the cerebral cortices separated, weighed

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and homogenised in 2 ml. of 0.9% saline. Three ml. of 10% trichloracetic acid were added and the suspension again homogenised and transferred to a centrifuge tube. Two further portions of 10% TCA of 1 ml. each were added, and the contents of the homogenising tube transferred quantitatively to the centrifuge tube. After centrifuging the supernatant was discarded and the tube left overnight in the refrigerator. The precipitate was then extracted once with a 2:1 ethanol/ether mixture for five minutes at 60°C, and once with 10% TCA for fifteen minutes at 90°C. Thereafter it was washed with ethanol, then with the ethanol/ether mixture, and then with ether, and then dried. One ml. of 1.0 N NaOH was then added, and after the precipitate had gone into solution, 0.5 ml. aliquots were counted in a Packard Tricarb. liquid scintillation spectrophotometer in a 2% Cabosil solution of toluene and dioxane and with the same phosphors as were used in the previous experiments.

Incorporation of C^{14}-L-alanine into liver protein was measured in the same way, the liver being perfused with ice cold 0.9% NaCl via the portal vein immediately after the brain had been removed, and a portion of the perfused liver then homogenised in 0.9% NaCl.

The results were expressed as the percentage of the injected dose incorporated into trichloracetic acid precipitable protein, per gram of tissue. All counts/min. were converted to absolute disintegrations/min. as described in Chapter V.

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TABLE XIII

The Incorporation of C^{14}-L-alanine into Brain and Liver Protein

Rats*	Brain (% of dose in protein/g. of tissue)	Perfused Liver (% of dose in protein/ g. of tissue.)
Controls	0.0290 ± 0.0037 (3)	0.0885 ± 0.0085 (3)
Galactose-fed	0.0390 ± 0.0086 (3)	0.1176 ± 0.0040 (3)
Rats given Intraperitoneal Galactose	0.0397 ± 0.0020 (2)	0.1681 ± 0.0091 (2)
Rats given Intraperitoneal Fructose	0.0258 ± 0.0015 (2)	0.1733 (1)
Rats given Intraperitoneal Glucose	0.0132 (1)	0.1351 (1)

* Each animal was given 10 uC of C^{14}-L-alanine in 0.5ml. of 0.9% NaCl intravenously and was killed 2½ hours later. Weighed amounts of brain and liver were homogenised in saline and the protein precipitated with 10% trichloracetic acid. The incorporation of the C^{14}-L-alanine into tissue protein was recorded as the percentage of the administered dose recovered from trichloracetic acid precipitable protein, per gram of tissue. The results given are the means ± S.D. with the number of experiments in parentheses.
Those given sugars received them in 300mg./injections hourly over three hours, intraperitoneally, and the C^{14}-L-alanine was given intravenously at the mid-point.

Section 3 - Results

The results are shown in Table XIII. In both the brain and the liver of the galactose-fed rats there was no decrease in the incorporation of C^{14}-L-alanine into trichloracetic acid precipitable protein. In fact, if anything, there was a slight increase in the incorporation of amino-acid into protein. The same is also seen to be true for the rats in which galactose and fructose were given acutely as intraperitoneal injections.

Section 4 - Conclusions .

No inhibition of L-alanine incorporation into brain or liver protein is detectable under the conditions of the experiments cited above. The above findings would be in keeping with the observation that in none of the other syndromes of linked intestinal and renal amino-acid transport defects, has any other cell membrane been shown to fail to transport amino-acids normally.

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CHAPTER IX

The Mechanism of Inhibition of Amino-acid Transport by Sugars in the Small Intestine of the Rat.

Section 1 - Kinetic Studies.

(i) Introduction :

As discussed in Chapters V and VI the indications are that the inhibition of the intestinal transport of certain amino-acids by galactose, fructose and glucose is noncompetitive in nature. As previously stated the evidence for this is as follows :

(a) The amino-acids inhibited do not conform strictly to known transport groups.

(b) 3-O-methyl-glucose, which competes for transport in the intestine with glucose and galactose, does not inhibit amino-acid transfer in the gut.

(c) Fructose is not actively transported in the gut yet it inhibits the transport of certain amino-acids.

It was accordingly decided to study the kinetics of the inhibition of amino-acid transport in the gut by sugars. Galactose was chosen as the sugar for study and L-alanine as the test amino-acid.

The usual concept of active transport involves the combination of the substance transported with some other substance, which may be a carrier or possibly an enzyme involved in the formation of the carrier, as discussed in Chapter II. If this process is the rate limiting factor then the relation between the rate of absorption and the concentration might be expected to conform with the scheme of

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kinetics outlined by the Michaelis and Menten in 1913[228]. Investigations of this sort have been made on the intestinal absorption of glucose by Fisher and Parsons[229] and of phosphate by McHardy and Parsons[230], while Jervis and Smyth[231] have studied amino-acids. All these investigators were able to show that absorption of these substances in the intestines of experimental animals was a rate limiting process with kinetics approximating to the Michaelis-Menten scheme. Jervis and Smyth[231] were able to show that this was true for both the D- and L- forms of methionine and histidine in rat intestine.

Outline of the Michaelis-Menten Hypothesis.

The Michaelis-Menten hypothesis was developed as a result of a study of enzyme kinetics. The most important feature of this theory is the assumption of an intermediate enzyme-substrate complex. A further assumption is that the rate of conversion of the substrate to the products of the reaction is determined by the rate of conversion of the enzyme-substrate complex to reaction products and the enzyme.

These events may be written thus : -

$$\text{Enzyme (E)} + \text{Substrate (S)} \rightleftharpoons \text{Enzyme-substrate complex (ES)} \rightarrow \text{Enzyme (E)} + \text{products (P)}.$$

In terms of transport phenomena E = carrier or mobile site which may have enzymic properties; S = transported solute; ES = the combination of carrier with solute; P = solute on other side

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of the cell membrane which by definition (Chapter II) equals S in a transport process.

Thus the rate of appearance of P depends upon the concentration of ES. If the rate of formation of P in enzyme kinetics had depended directly on the concentration of S, $[S]$, then at constant $[E]$ a linear relationship could be expected between the velocity and $[S]$. As this is not the case, Michaelis and Menten proposed their hypothesis which is derived thus : -

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad (1)$$

Where k_1, k_2 and k_3 are the respective velocity constants of the three assumed processes. It is also assumed that the concentration of substrate is greater than that of enzyme in this system.

For the rate of formation of ES we may write : -

$$\frac{d[ES]}{dt} = k_1 ([E] - [ES])[S] \qquad (2)$$

Where $[E] - [ES]$ is the concentration of uncombined enzyme. The rate of formation of ES is $\frac{d[ES]}{dt}$ and is shown in (2) to be proportional to the concentration of the uncombined enzyme and substrate.

The rate of disappearance of ES is then : -

$$-\frac{d[ES]}{dt} = k_2[ES] + k_3[ES] \qquad (3)$$

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When a steady state is reached and the rate of formation and disappearance of ES are equal then $\frac{d[ES]}{dt} = -\frac{d[ES]}{dt}$ and this is expressed in equation (4).

$$k_1([E] - [ES])[S] = k_2[ES] + k_3[ES] \quad (4)$$

These terms may be rearranged to give : -

$$\frac{[S]([E] - [ES])}{[ES]} = \frac{k_2 + k_3}{k_1} = Km \quad (5)$$

Further the relationship between substrate concentration, enzyme concentration and the velocity of the reaction, can be developed as follows : -

By arrangement of equation (5) to solve for $[ES]$, the steady state concentration of the enzyme - substrate complex is

$$[ES] = \frac{[E][S]}{Km + [S]} \quad (6)$$

To derive the Michaelis - Menten constant (Km) the following equations are developed : -

$$V = k_3[ES] \quad (7)$$

Where V is the observed initial velocity. When the substrate concentration is made so high in relation to the enzyme concentration that all the enzyme is present as ES, then the velocity of the reaction is maximal (V max) and may be written : -

$$V\ max = k_3[E] \quad (8)$$

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By substitution for ES in equation (7) its value in equation (6), and by dividing equation (7) by equation (8), one obtains ;

$$V = \frac{V\,max\,[S]}{Km + [S]} \quad or \quad Km = [S]\left(\frac{V\,max}{V} - 1\right) \qquad (9)$$

This is the Michaelis - Menten equation.

This equation may be arranged as suggested by Lineweaver and Burk[232] in the following way : -

$$\frac{[S]}{V} = \frac{[S]}{V\,max} + \frac{Km}{V\,max} \qquad (10)$$

A plot of $\frac{[S]}{V}$ vs $[S]$ gives a straight line. The intercept of the line on the $\frac{[S]}{V}$ axis is $\frac{Km}{V\,max}$ and the slope is $1/V$ max.

Similarly equation (9) can be arranged to give : -

$$\frac{1}{V} = \frac{[S] + Km}{V\,max\,[S]} = \frac{Km}{V\,max} \times \frac{1}{[S]} + \frac{1}{V\,max} \qquad (11)$$

Thus a plot of $1/V$ vs. $1/S$ gives a straight line in which the ordinate intercept equals $1/V$ max and the slope is Km/V max.

It was conformity with this equation (11) which allowed the conclusion that active transport phenomena conform to the Michaelis - Menten hypothesis[229][211][230][231]. In terms of transport phenomena, which as stated previously have been shown to conform to this hypothesis proposed for enzyme-substrate reactions by Michaelis and Menten, in equation (11) V = Velocity of transfer; S = concentration of transport substance.

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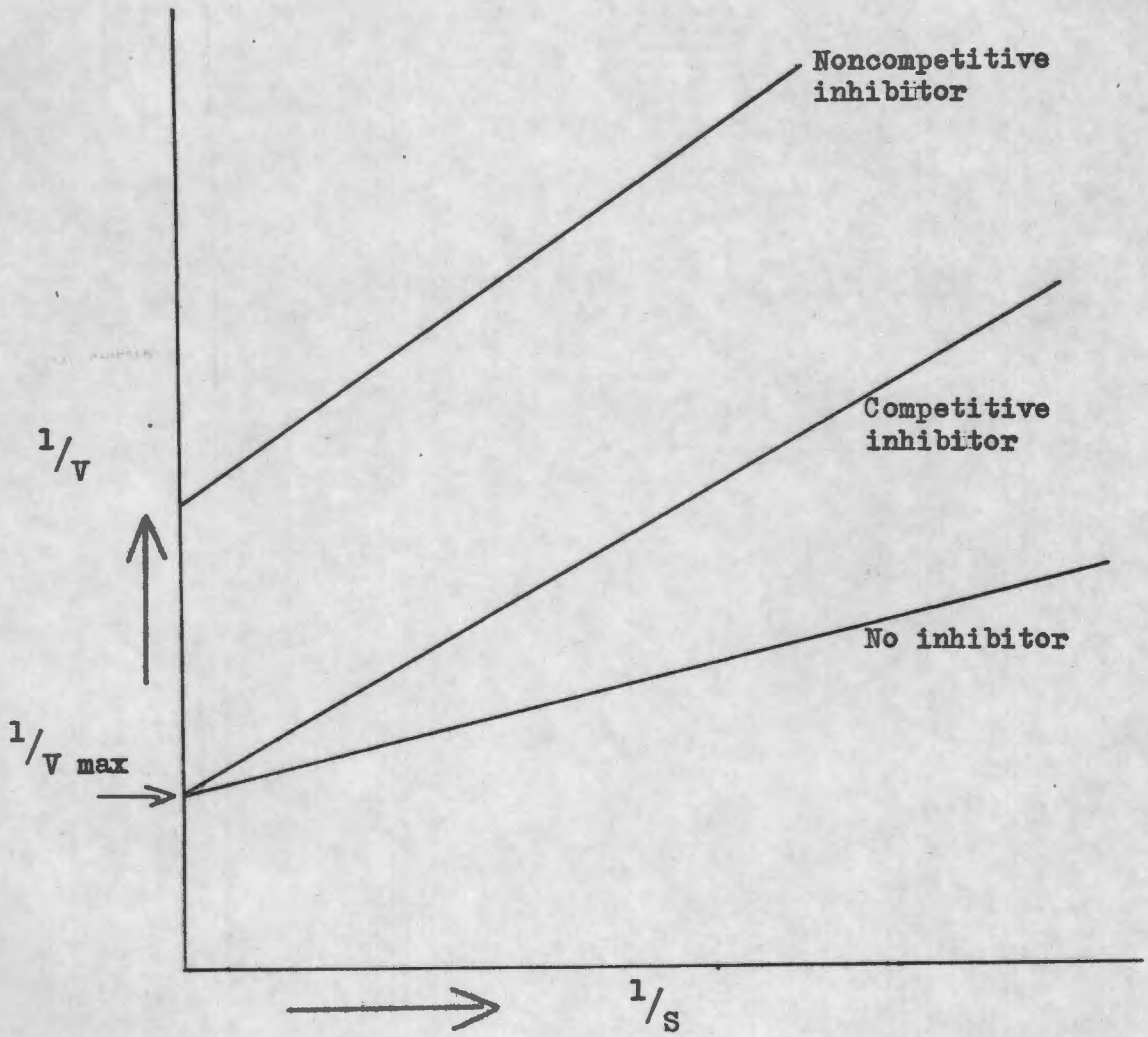

FIGURE 4. Idealised graphic representation of the effects of competitive and noncompetitive inhibition of enzyme action. The reciprocal of the substrate concentration (1/S) is plotted against the reciprocal of the velocity of the reaction (1/V).

Note that when competitive inhibition occurs the Vmax can be attained. This is not the case in the presence of a noncompetitive inhibitor.

For the action of a <u>competitive</u> inhibitor an equation may be derived incorporating the inhibitor concentration [I] and the dissociation constant of the carrier - inhibitor complex Ki, in a manner similar to that used in equation (9) thus : -

$$V = \frac{V\,max\,[S]Ki}{Km\,Ki + Km[I] + Ki[S]} \qquad (12)$$

By rearrangement of equation (12) : -

$$\frac{1}{V} = \frac{Km}{V\,max}\left(1 + \frac{[I]}{Ki}\right)\frac{1}{[S]} + \frac{1}{V\,max} \qquad (13)$$

When a plot of $\frac{1}{V}$ against $\frac{1}{[S]}$ is made in the case of competitive inhibition, the ordinate intercept, $\frac{1}{V\,max}$, is the same as in the uninhibited reaction, but the slope, which is now $\left(\frac{Km}{V\,max}\right)\left(1 + \frac{[I]}{Ki}\right)$ is increased by the factor $1 + \frac{[I]}{Ki}$.

This is shown graphically in figure 4.

It can be seen that by using a sufficiently high substrate concentration in the case of enzyme kinetics, or transported solute concentration in the case of transport kinetics, the effect of a competitive inhibitor can be overcome and V max can be reached.

In the case of non-competitive inhibition there is no relationship between the amount of inhibition and the concentration of substrate or transported substance. Inhibition here depends only on the concentration of the inhibitor. Therefore no matter how high the concentration of the substrate or transported substance may be, the V max is never reached. This is also shown in Figure 4.

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(ii) Method

Amino-acid transport in rat intestinal slices was studied precisely as described in Chapter V, but in this experiment the incubation period was in all instances limited to 30 minutes. This period was chosen because it is preferable to study kinetic phenomena during periods of marked influx and it was known (Figure2) that equilibrium was reached shortly before 30 minutes had elapsed. The initial concentration of L-alanine in the incubation medium was varied from 0.05mM to 5mM and galactose was present at a concentration of 28mM. The intracellular accumulation of L-alanine was calculated in mM/L of intracellular water/30 mins. as follows : - All counts/min were converted to absolute disintegrations/min as outlined in Chapter V. The disintegrations/min in the total tissue fluid were obtained and the number of disintegrations/min in the E.C.F. were calculated from the calculated volume of E.C.F. and the measured disintegrations/min. in the medium after incubation.

By subtracting the disintegrations/min. in the E.C.F. from the total disintegrations/min. obtained from the tissue, the number of disintegrations min. in the intracellular water was obtained. From the known specific activity of the C^{14}-L-alanine this could be converted into m Moles and by dividing this answer by the calculated intracellular fluid volume and multiplying by 1000, the amount of L-alanine in the intracellular water in mM/L was calculated, and the reciprocal of this value was plotted on the Y axis. The concentration

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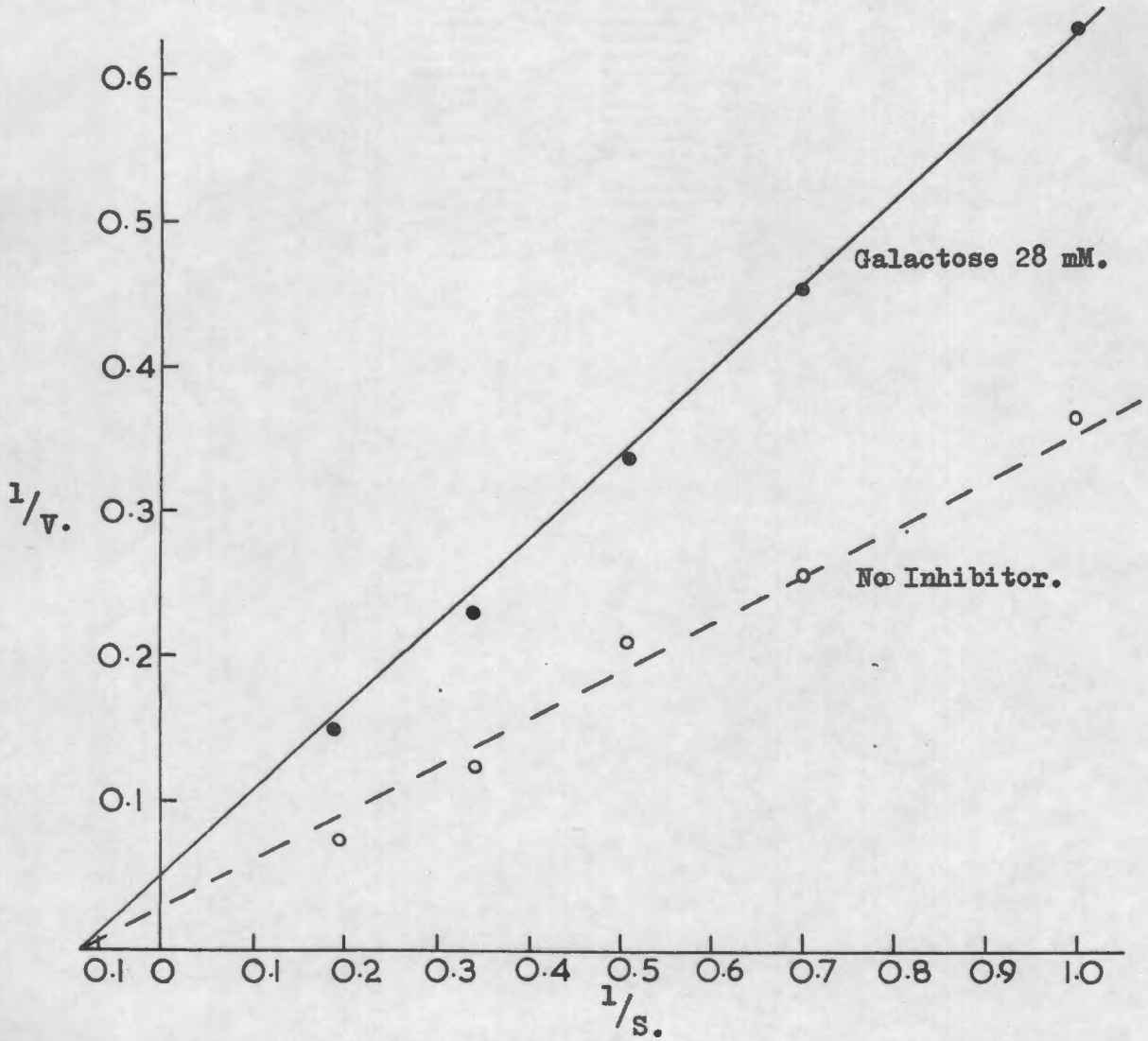

FIGURE 5. The reciprocal of the initial concentration of L-alanine (mM./L.) (1/S) in the incubation medium, is plotted against the reciprocal of the intracellular accumulation (mM./L.) (1/V) of this amino-acid after incubation for 30 minutes, as described in the text. The open circles represent uninhibited transport; the closed circles transport in the presence of galactose (28mM.). The regression lines were calculated by the least squares method from the results of a total of 77 experiments.

of L-alanine in the initial medium was also expressed as mM/L, and the reciprocal of this value was plotted on the X axis. The medium concentration of L-alanine used here may either be the average concentration or the initial concentration. Fisher and Parsons[229] have considered the implications of using the initial concentration and the average rate in the Michaelis - Menten equation, and have concluded that a similar relationship will exist between these as exists between the average concentration and the average rate, although the cohstants will have different values.

(iii) Results

The intracellular accumulation of L-alanine (mM/L/30minutes) was plotted against the initial amino-acid concentration in the medium (mM/L) according to the double reciprocal method of Lineweaver and Burk[232]. The results are shown in Figure 5. When the data for L-alanine transport without added inhibitor were plotted, the results tended to fall on a straight line, suggesting that the absorption of L-alanine in the gut conforms to the pattern of saturation kinetics described by Michaelis and Menten[228]. The regression line was calculated by the least squares method from the results of 43 experiments. When galactose was added to the incubation medium there was definite inhibition of L-alanine transport. A double reciprocal plot was drawn for L-alanine absorption in the presence of galactose. These values evidently also tended to fall on a straight line (calculated as above from 44 experiments) which, when extrapolated tended to intersect the Y axis above the point where it was

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crossed by the regression line for uninhibited L-alanine absorption. When the results of each day's experiments were plotted independently almost identical regression lines were obtained both for uninhibited L-alanine transport and for the transport of L-alanine inhibited by galactose.

In essence this implies that even when L-alanine is present at infinite concentration the inhibiting action of galactose cannot be overcome and the V max of uninhibited L-alanine transport cannot be reached. This is consistent with noncompetitive inhibition as previously discussed in this chapter.

Section 2 - The Effect of L-Alanine on Galactose Transport in the Small Intestine.

If the inhibition of L-alanine transport in the intestine by galactose were competitive in nature i.e. competing for a common transport site or sites, then it might be expected that the reverse might also occur, and that L-alanine would exert some inhibition on the transport of galactose by the gut. This was accordingly tested experimentally.

(i) Method

Intestinal segments were incubated as previously described in Chapter V but without any C^{14}-L-alanine in the medium. In all instances galactose was present in the medium at a concentration of 5.6 mM., and in half of the experiments L-alanine was added to the

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incubation medium at a concentration of 28mM. After incubation for one hour the segments were removed, washed twice in saline and blotted. They were then homogenised in 2ml. of zinc sulphate and 2ml. of sodium hydroxide, and then centrifuged. Reducing substances were assayed in the supernatant and in the medium using the method of Somogyi and Nelson[233]. Segments incubated in parallel experiments but without added galactose were used as a blank for tissue reducing substance, and this medium as a blank for reducing substances which may have entered it from the tissues. In all instances the blank values were very low. The results were expressed as distribution ratios of the concentration of galactose in intracellular fluid in mM/ml. to that in the E.C.F. in mM/ml. E.C.F. and total tissue water were determined in Chapter V.

(ii) Results

The distribution ratio for galactose transport in the absence of L-alanine was 2.9 ± 0.1 while when L-alanine was present in the medium at a concentration of 28mM, it was 2.9 ± 0.6. (These figures are the means of three observations ± standard deviations). It is clear that L-alanine did not inhibit galactose transport by the small intestine of the rat.

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Section 3 - Conclusions

The kinetic studies, and the failure of L-alanine to inhibit galactose transport by the intestine of the rat, support the previously cited evidence in favour of the conclusion that galactose inhibition of L-alanine transport is noncompetitive in type. One can reasonably assume that the same noncompetitive mechanism holds good for the inhibition of the transport of other amino-acids by galactose and for the inhibition of certain amino-acids by fructose and glucose.

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CHAPTER X

The Role of Tissue Adenosine Triphosphate in the Inhibition of Amino-acid Transport by Sugars in the Small Intestine of the Rat

Section 1 - The Role of Adenosine Triphosphate in Membrane Transport.

It is clear that adenosine triphosphate (ATP) plays a vital role in many transport reactions. In the words of Scholefield[237] "Chemical energy, usually as ATP, is required for the establishment and maintenance of concentration gradients although the biochemical reactions involved in this well documented association are virtually a mystery." It has been known for a long time that energy is required for the driving of the sodium pump, and one of the earliest theories of sugar transport[238] was based on the observation that there was a simple relationship between the transport of certain sugars and the phosphorylation of these sugars by ATP. This led to the erroneous conclusion that all sugars transported in the intestine were phosphorylated in the intestinal cell, and that this was the major physiological event in transport of these hexoses. It is now known that this is not the case, and that indeed 3-O-methyl glucose and other sugars which are not phosphorylated are actively transported by the intestinal cells[216][239]. The inhibition of transport processes by the exclusion of oxygen has supported the concept that energy and therefore ATP, are necessary for active transport.

Ascites tumour cells are able to support high rates of

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glycolysis despite the exclusion of oxygen from their atmosphere[240]. As a result their QATP under anaerobic conditions is no different to what it is under aerobic conditions. Indeed incubation of ascites tumour cells in the presence of glucose has shown that they concentrate amino-acids equally well whether oxygen is present in the environment or not[241][242][243]. This further supports the view that inhibition of transport in other cells by anaerobiasis is due to the lowering of cellular ATP levels.

Uncoupling agents such as 2 - 4 dinitrophenol (DNP) prevent the formation of ATP.

Quastel and his co-workers[244][245][246] have shown that DNP inhibits the transport of sugars and amino-acids by the intestine, while Krebs and his colleagues[247] showed similar inhibition of amino-acid incorporation into brain cortex slices by DNP. This work has been confirmed by others[248]. Christensen and Riggs[249] were able to demonstrate that DNP inhibits amino-acid uptake by Ehrlich ascites carcinoma cells. Heinz and Mariani demonstrated the same phenomena[250]. Caldwell, Hodgkin, Keynes and Shaw[251] studied the giant axon and were able to show that the injection of phosphagen, arginine phosphate, into the interior of the fibre reversed the inhibition of sodium and potassium transport across the membrane caused by poisoning with DNP. These experiments further confirm the important role of ATP in membrane transport.

As will be discussed in Chapter XII there is good evidence that the transport of certain substances in the gut, and elsewhere,

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is dependent upon the presence of certain ions, especially sodium. It has also been shown that there is a sodium-activated adenosine triphosphatase on or in cell membranes[252], underlining the importance of the breakdown of ATP by such an enzyme in transport processes.

The precise role played by ATP in the transport process is not clear and depends upon the concept of what occurs when a substance is transferred across a cell membrane.

Heins and Walsh[253] proposed in 1958 that an amino-acid entering an Ehrlich ascites carcinoma cell combined with an "active" carrier on the surface of the cell membrane, and was transported by it to the vicinity of the interface with the interior of the cell. There it was thought to be released from the carrier, which was now inactive. The inactive carrier was assumed to return to the medium-membrane interface, where it was reactivated by ATP, which in turn was dephosphorylated in the process to adenosine diphosphate (ADP). This concept allowed for a finite amount of active carrier which would give rise to kinetic requirements as described by Michaelis and Menten[228]. This concept also demanded a continued dependence on ATP as well as providing an explanation for competitive inhibition for a limited number of sites on the transport carrier. However this model required that for every molecule of amino-acid transported, one molecule of ATP must be broken down. This is almost certainly not the case in many instances[254]. Hokin and Hokin[255] have recently proposed a very similar model to that of Heins and Walsh, as applied to electrolyte transport. They suggested that the

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transport of a substance results from conformational changes in a protein, and in view of the lipid nature of the cell membrane barrier they have suggested that this protein is a lipoprotein. They also reported a rapid turnover of phosphatidic acid during transport and postulated that the lipoprotein contained phosphatidic acid. The rephosphorylation of the phosphatidic acid, which is thought to become dephosphorylated during transport, would have to occur at the expense of ATP.

Other workers have postulated a similar role in active transport for the phosphoryl - serine residues of proteins[256][257].

Wilbrandt[258] has proposed that ATP maintains the carrier in an active form at the outer surface of the cell while it is not needed on the inner surface, where the non-phosphorylated form dissociates from the substrate. There are many other complex models of active transport but suffice it to say that whatever complicated series of events takes place in amino-acid transport across all membranes, ATP is probably involved in an indirect manner, possibly as in one of the models discussed above.

One could argue that either the transport of the hexoses or their phosphorylation in the intestinal cell might result in a drop in the mucosal levels of adenosine triphosphate (ATP) with consequent inhibition of amino-acid transport.

It was therefore thought to be of importance to establish the levels of ATP in the intestine during inhibition of amino-acid transport by sugars.

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Section 2 - Method

Rat intestinal segments were incubated as described in Chapter V. Stable amino-acids were present in the medium at a concentration of 5mM. No isotopes were used. The sugars, when added to the medium, were at a concentration of 28mM. In some instances no sugar was added to the medium. The incubation period was one hour, after which the ATP content of the segments was measured. Segments from galactose-fed rats and their controls were assayed for ATP without prior incubation. For the assay, the segments were immediately transferred to 10ml. of distilled water in tubes in a boiling water bath. Care was taken to prevent undue evaporation using glass marbles on the tops of the tubes. After boiling for ten minutes the tubes were removed, cooled in ice and the segments homogenised, the tubes being kept in ice. An aliquot of 1 ml. of homogenate was taken and put into a planchette to be dried in an oven at 110°C to constant weight. From the known water content of the segments, the wet weight of the tissue could be calculated. The remainder of the homogenate was centrifuged in the cold, and the ATP assays were performed on the supernatant by the luciferin - luciferase method[259]. Fifty or one hundred microlitres of the cold supernatant were added to a mixture of 0.2ml. of firefly enzyme, 0.1ml. of 0.1 M $Na_2 HAs O_4$ (pH 7.4), 0.1ml. of 0.1 M $Mg SO_4$ and 1.0ml. of 0.05M glycine (pH 7.4).

The light measurement was then immediately recorded on a

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TABLE XIV

Adenosine Triphosphate Content of Rat Intestinal Slices after *In Vitro* Incubation for 60 Minutes with and without Sugars.

Addition to Medium**	Adenosine Triphosphate Concentration in Intestinal Slices. (u moles/g. tissue)	
	L-Alanine*	L-Valine*
None	0.158 ± 0.027 (7)	0.139 ± 0.017 (3)
D-Galactose	0.098 ± 0.017 (3)	0.110 ± 0.014 (3)
D-Fructose	0.161 ± 0.027 (4)	0.140 ± 0.014 (3)
D-Glucose	0.287 ± 0.022 (7)	0.220 ± 0.0 (3)
D-Galactose + D-Glucose	0.308 ± 0.107 (3)	

* The amino-acids were present at a concentration of 5 m Moles/L The incubation conditions were the same as described in Table II except that no isotope was added to the medium.

** Each sugar was at a concentration of 28 mM.

fluorimeter modified for the purpose. Fresh ATP standards were made up each day and the pH of the standard was adjusted to 7.4. There was a linear relationship between the quantity of ATP present and the intensity of light produced. At times it was necessary to dilute the supernatant with an equal volume of water to ensure that the readings fell within the scale used. All analyses were done in duplicate. The ATP content of the intestinal segments was expressed as u moles/g. of tissue.

Section 3 - Results

The ATP content of rat intestinal segments after in vitro incubation for 60 minutes with and without sugar is shown in Table XIV. It will be noted that in the presence of galactose the level of ATP in the intestinal slices was reduced. The ATP content of the segments was not diminished when they were incubated with fructose, even though fructose at this concentration inhibited L-alanine transport. It is therefore clear that a low cellular ATP level is not responsible for the inhibition of intestinal L-alanine transport by fructose.

Glucose caused a rise in the ATP level of the segments as might have been expected. It is clear that the inhibition of amino-acid transport by glucose is not related to a lowering of cellular ATP. Incubation of segments in the presence of L-alanine and with glucose and galactose, each in the incubation medium at a concentration

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TABLE XV

The Effect on Intestinal Amino-acid Transport of Injecting Adenosine Triphosphate Subcutaneously into Galactose-fed Rats.

	Distribution Ratios*				
	L-Alanine	L-Valine	L-Lysine	Glycine	Hydroxy-L-Proline
	5mM.**	5mM.	5mM.	5mM.	5mM.
Galactose-fed Rats plus ATP	2.9 ± 0.6 (3)	2.7 ± 0.6 (3)	1.4 ± 0 (2)	1.9 ± (1)	1.1 (1)
Galactose-fed Rats without ATP	2.8 ± 1.0 (6)	2.3 ± 0.8 (6)	1.3 ± 0.3 (6)	1.5 ± 0.7 (6)	1.3 ± 0.5 (6)

* Ratios shown are means ± S.D. with the number of determinations in parentheses. Rats were given 100mg. of ATP hourly for three hours by subcutaneous injection and amino-acid transport in the small intestine was measured thirty minutes after the last injection as outlined in Table II.

** Initial concentration of amino-acid in the medium.

TABLE XVI

The Effect of Injecting Adenosine Triphosphate Subcutaneously on the In Vitro Intestinal Transport of L-Alanine in the presence of Galactose (28mM.).

Distribution Ratios* of L-Alanine (5mM**).	
No Added Sugar	Galactose 28mM.***
4.2 ± 0.2 (2)	2.2 ± 0.3 (2)

* Ratios shown are means ± S.D. with the number of determinations in parentheses. Each rat was given 100mg. of ATP hourly for three hours by subcutaneous injection. Amino-acid transport in the gut both without sugar in the incubation medium and after the addition of galactose (28mM.) was determined as outlined in Table II.

** Initial concentration of L-alanine in the medium.

*** Initial concentration of galactose in the medium.

of 28mM., resulted in the highest ATP levels recorded, yet these experimental conditions also resulted in inhibition of amino-acid transport.

ATP measurements were made on the segments of the intestine of galactose-fed and control rats. The mean ATP level of galactose-fed animals was 0.492 uM/g (mean of six experiments) and was lower than controls 0.637 uM/g (mean of six experiments) ($p<0.05$). However individual instances were observed in galactose-fed rats where amino-acid transport was markedly inhibited, but ATP levels were normal.

In order to test further the role of ATP in the phenomenon of the inhibition of intestinal amino-acid transport by sugars, animals were injected with ATP before measuring amino-acid transfer. Shull(260) has observed that injections of ATP may increase cellular levels of this substance, and therefore systemic administration of ATP might correct for any lowering of cellular ATP. Galactose-fed rats were given ATP by subcutaneous injections in doses of 100mg/hour for three hours. Thirty minutes after the last injection of ATP these rats still showed the same degrees of inhibition in the intestinal transport of amino-acids (Table XV). In another experiment, intestinal segments from normal rats, pretreated in the same way with ATP still showed inhibition of L-alanine transport when galactose (28mM) was present in vitro (Table XVI). This dose of ATP was the largest that the rats could tolerate. Injections of 150mg of ATP hourly for three hours were fatal, the rat dying in a hypothermic state.

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The failure of injections of ATP to prevent the in vitro inhibition of L-alanine transport by galactose, and more particularly the persistence of inhibition of amino-acid transport in the intestines of galactose-fed rats despite such injections, both make a quantitative relationship between a low cellular ATP and inhibition of amino-acid transport unlikely.

Although incubation of intestinal segments with ATP has not been shown to raise the tissue levels of this substance, in three experiments ATP was added to the medium in a concentration of 10^{-3}M. Under these conditions ATP did not influence the inhibition of L-alanine transport by galactose (mean distribution ratio 3.1).

Section 4 - Conclusions.

The studies of ATP levels in intestinal segments after in vitro incubation with galactose, fructose and glucose, and the results of ATP levels in the intestine of galactose-fed rats, suggest that absolute levels of ATP in the gut are not quantitatively related to the inhibition of amino-acid transport by sugars under the experimental conditions studied. There is evidence that other agents are able to inhibit transport phenomena in many systems without affecting the level of ATP. Cardiac glycosides are able to inhibit the transport of ions without changing the cellular content of ATP(261)(262)(263).

Cardiac glycosides are also able to inhibit glucose trans-

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port in the isolated intestine[264][265], and amino-acid transport in brain slices without any change in cellular ATP levels.[267] It seems likely that ouabain acts by inhibiting ATPase activity[268] and this will be dealt with in more detail in Chapter XII, but suffice it to point out here that processes which inhibit transport do not necessarily do so by lowering cellular ATP. The fact that all the inhibiting sugars are phosphorylated in the intestinal cell[269][270][271][272] led to the idea that the inhibition they exerted might be related to the lowering of cellular ATP. The results recorded in this chapter show that this is clearly not the case.

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CHAPTER XI

The Role of Cellular Galactose-1-Phosphate in the Inhibition of Intestinal Amino-acid Transport by Galactose.

Section 1 - Introduction

It may well be of significance that galactose, glucose and fructose are all phosphorylated in the intestinal cell, while the sugars which fail to inhibit amino-acid transport are not[269][270][271][272].

As already indicated, it has been suggested that the "renal" amino-aciduria of galactosaemia is due to the effects of the accumulation of galactose-1-phosphate in the renal tubular cell[171]. Similarly the amino-aciduria of hereditary fructose intolerance has been ascribed to the presence of fructose-1-phosphate in the kidney[181].

It was accordingly decided to measure the accumulation of galactose-1-phosphate in the intestinal segments incubated in the presence of galactose. An attempt was made to see whether the presence of this phosphorylated intermediate of galactose was related to the inhibition of amino-acid transport.

Further it was decided to estimate the level of galactose-1-phosphate in the gut and other organs of galactose-fed rats.

Section 2 - Method

Galactose-1-phosphate was measured according to the method of

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Schwarz[273]. Intestinal segments were incubated with galactose (28mM) and L-alanine (5mM) as described in Chapter V but without isotopic L-alanine. After incubation the segments were homogenised in 0.9% saline. The period of incubation varied from 10 to 90 minutes. To the homogenate, three vols. of cold 20% trichloracetic acid were added, and this was shaken well and kept cool. After centrifuging, the supernatant was transferred to a tube containing three drops of 2% barium acetate, one drop of phenolphthalein and 5ml. of ethanol, previously cooled in ice. A slight excess of 40% KOH was added; the ice cold mixture acidified with a minimum amount of acid, and then rendered basic to a faint pink with 0.1 N KOH. This was then treated with 4 volumes of ethanol, reneutralised if necessary, and kept in the refrigerator for at least two hours or overnight. The precipitate was then spun down. Ten mls. of ice cold neutralized 80% ethanol were then added to the precipitate which was then resuspended. After centrifuging, 1.5ml. of 0.1 N HCl were added, the tube immersed in boiling water and shaken to disperse the precipitate. The covered tube was then put in boiling water for fifteen minutes. After cooling and centrifuging, the supernatant was put into a tube with 0.2g. of Amberlite Monobed resin MB-1. It was then centrifuged and transferred to a 5ml. volumetric flask with a Pasteur pipette. The resin was extracted three more times with 1 ml. of water each time for 5 minutes at a time. Aliquots were evaporated to dryness, taken up in 5-6 portions of 50% methanol and this transferred quantitatively to Whatman's No. 1 paper for

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chromatography. Known amounts of galactose standard, 5-50 uG, were also chromatographed. The chromatograms were run in an ethyl acetate-pyridine-water solvent (70:25:20). They were dried at room temperature and dipped in freshly prepared benzidene reagent. (0.5g. of decolorised benzidene in 5ml. of glacial acetic acid, and 20ml. of acetone to which 4ml. of 100% trichloracetic acid and acetone to 100ml. were added). The paper was heated for 2-4 minutes at 100°C until it turned pale yellow. The galactose-1-phosphate was seen as yellow spots (green-yellow fluorescence in UV light) and a quantitative estimation was made by comparison with the known standards. Numerous known standards of galactose-1-phosphate were treated in the same way and the recovery of the method was consistantly about 50%. In order to express the results in absolute terms the amount of galactose-1-phosphate estimated by this semi-quantitative method was therefore multiplied by two.

Intestinal segments and other tissues from galactose-fed rats were treated in exactly the same way but without prior incubation.

Section 3 - Results

After incubation of rat intestinal segments in the presence of galactose (28mM) for 10 minutes, galactose-1-phosphate was barely detectable (4 ug/g tissue). In order for this small amount to be quantitatively accurate, twelve flasks each with six segments were incubated simultaneously, the 72 segments were pooled and homogenised and the galactose-1-phosphate estimated in the whole amount. After

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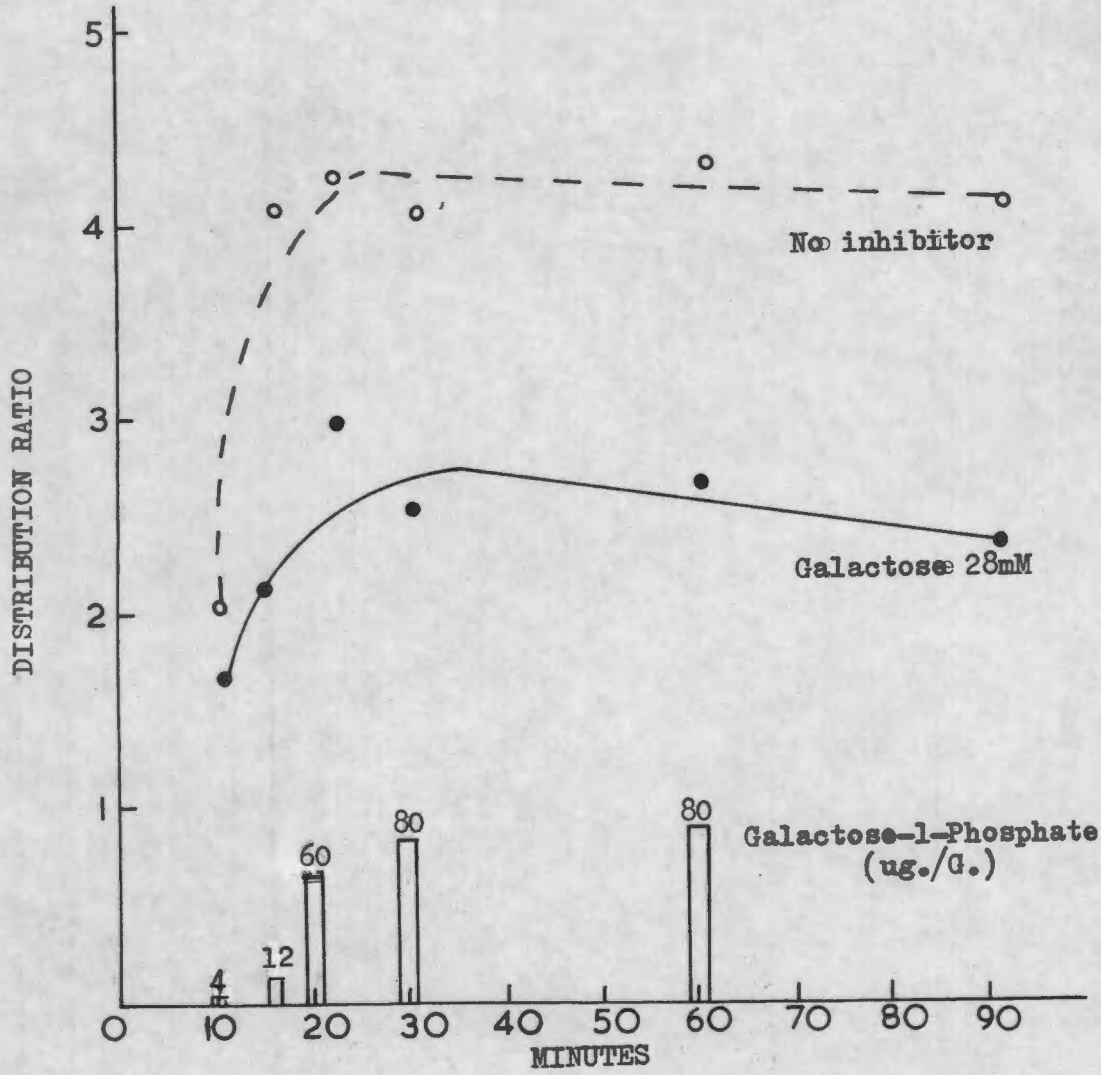

FIGURE 6. The distribution ratios for C^{14}-L-alanine transport in rat intestinal slices, incubated as described in the text are related to time of incubation in minutes. The ratios for uninhibited C^{14}-L-alanine transport are shown as open circles, while those for C^{14}-L-alanine transport in the presence of galactose (28mM) are shown as closed circles. Each point represents the mean of 9 experiments with the exception of the ratio for uninhibited transport after 60 minutes, which is the mean of 31 experiments. The amount of galactose-1-phosphate in the intestinal segments, relative to the time of incubation, is also shown and is expressed as uG/G. of tissue.

TABLE XVII

The Effect of Phlorhizin on the Inhibition Exerted by D-Galactose on C^{14}-L-Alanine Transport in Rat Intestinal Segments In Vitro.

Addition to Medium	Distribution Ratios of C^{14}-L-Alanine* .
Nothing	4.7 ± 0.9 (31)
Phlorhizin**	3.9 ± 0.4 (3)
Phlorhizin** + D-Galactose (28mM)***	4.3 ± 0.5 (6)

* The Distribution ratios are shown as means $\pm$ S.D. with the number of determinations in parentheses. Rat intestinal segments were incubated for 60 minutes in the manner outlined in Table II.

** Phlorhizin was present at a final concentration of 10^{-5}M.

*** D-Galactose was present at the final concentration indicated.

fifteen minutes incubation, under the same experimental conditions, Galactose-1-phosphate was more easily measurable (10-14 ug/g tissue) and only 24 segments were pooled for this estimation. After 20 minutes incubation galactose-1-phosphate was readily detectable (60-80 ug/g) in the intestinal segments and only six segments were needed for the estimation.

These results are shown in Figure 6. Figure 6 also shows the inhibition of intestinal L-alanine transport by galactose as a factor of time. It can be seen that there was no inhibition of L-alanine transport by galactose after 10 minutes incubation but that inhibition was apparent from 15 minutes onwards.

When phlorhizin was present in the incubation medium at a concentration of 10^{-5}M and intestinal segments were incubated for 60 minutes in the presence of C^{14} L-alanine and galactose (28mM) as described in Chapter V, galactose produced no inhibitions of L-alanine transport (Table XVII). Galactose-1-phosphate could not be detected in segments incubated in the presence of both galactose (28mM) and phlorhizin (10^{-5}M). At this concentration, phlorhizin (10^{-5}M) is known to inhibit galactose transport but does not inhibit cellular metabolism in the intestinal cell(211).

Galactose-1phosphate was readily detected in the intestines of the galactose-fed rats, as well as in the kidneys, liver and brain of these animals. No galactose-1-phosphate was detectable in any of the organs of the control rats.

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Section 4 - Discussion and Conclusions

The studies on the relationship of the inhibition of L-alanine transport in rat intestinal segments with time, show that there is a delay of more than 10 minutes before inhibition of L-alanine transport is detectable. It is probable that the mean distribution ratio of 1.7 for L-alanine after 10 minutes incubation in the presence of galactose (28mM) does represent active transport and not diffusion. This is likely to be so because after one hours' incubation with 2-4 D.N.P., the distribution ratio achieved by L-alanine was only 1.1, and under anaerobic conditions only 0.5 (Chapter V).

The failure to inhibit L-alanine transport at the 10 minute period and the observed inhibition after 15 minutes' incubation, raises the possibility that a metabolic derivative might have accumulated in sufficient concentration in the intestinal cell after fifteen minutes. Such an intermediate could then play a role in the inhibition of L-alanine transport.

The experiments reported here have indeed demonstrated the appearance of significant amounts of galactose-1-phosphate in intestinal segments incubated in the presence of galactose, and a correlation between the appearance of galactose-1-phosphate and the inhibition of L-alanine transport. The detection of very small amounts of galactose-1-phosphate after 10 minute incubation at a time when L-alanine transport is not inhibited, means that this correlation is not absolute. It is possible that the amount of

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galactose-1-phosphate in the intestinal cell after such a short period of incubation is insufficient to inhibit transport.

Such findings do not necessarily imply that there is a direct and causal relationship between the accumulation of galactose-1-phosphate in the intestinal cell and the inhibition of amino-acid transport, but it is quite possible that this is the case. The finding that phlorhizin, which interferes with galactose transport into the intestinal cell, prevented the formation of galactose-1-phosphate, and at the same time prevented inhibition of amino-acid transport by galactose, lends support to the theory that the accumulation of the phosphorylated intermediate may play an etiological role in the inhibition of amino-acid transport in the intestine by galactose. Nakazawa had found in 1922[274] that phlorhizin did not affect the absorption of amino-acids, fats, water or salts from the intestine but did inhibit glucose transport. Other workers have confirmed this[275], and the data reported in Table XVI tend to support these observations. It is also known that phlorhizin prevents the intestinal transport of galactose but not of xylose, ribose or sorbose[211][276][277][278].

It is of interest that phosphoglucomutase has been shown to be inhibited when galactose-1-phosphate accumulates in the cell[279] and it is possible that other enzymes are similarly affected. There is no evidence as yet that ATPase is inhibited by galactose-1-phosphate, but this remains a possibility as a mechanism in the inhibition of amino-acid transport. Attempts to measure ATPase by this investi-

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gator have been fraught with technical hazards. The large quantity of mucus in the gut seems to interfere with the estimation of this enzyme, and to date no meaningful results have been obtained after months of work.

Galactose-1-phosphate was also detectable in the intestine, kidneys, brain and liver of galactose-fed animals in large amounts. Despite this no inhibition of L-alanine incorporation into brain or liver protein could be demonstrated in these animals. If indeed galactose-1-phosphate is responsible for the intestinal and renal defects in amino-acid transport, one could infer that the specialised cells of the small intestine and renal tubule are more sensitive to the effect of galactose-1-phosphate just as they appear to be more sensitive to the effects of exogenous poisons.

It is clear that the study of the effects of the phosphorylated intermediates of hexoses on cellular enzymes should be a fruitful field for future studies.

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CHAPTER XII

The Dependence of Amino-acid Transport in the Intestine on Environmental Ions.

Section 1 - Introduction.

In recent years there has been considerable interest in the role of ions in transport phenomena.

In 1958 Riklis and Quastel[211] reported that the active transport of glucose by isolated surviving guinea-pig intestine was markedly affected by the concentration of cations in the medium bathing in the gut. In particular, sodium and potassium were shown to have marked effects. Csaky and Thale[280] were able to demonstrate the same phenomenon in another series of experiments. Bihler and Crane[281] explored the effects of many anions and cations, and found that the role of sodium in glucose transport in the intestine could not be duplicated. In the absence of sodium there was inhibition of intestinal glucose transport. Indeed no other ion could be shown to be essential in this regard. Studies of sugar transport into strips of hamster intestine have led Crane, Bihler and Hawkins[282][283] to postulate that glucose enters the mucosal cell as a sodium-glucose-carrier complex which then dissociates inside the cell, the sodium being removed immediately from the cell interior by a sodium pump. They suggested that the inhibiting effects of cardiac glycosides, anoxia, and dinitrocresol are due to a failure of the sodium pump, such that although glucose can still enter and leave the mucosal cell freely (coupled with sodium and carrier) no

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uphill gradient can be established.

While sodium ions have been shown to be necessary for the intestinal absorption of glucose, other ions also have some effect on this process. Increase in potassium ion concentration brings about an acceleration of the rate at which maximum velocity of active transfer of glucose is obtained. Potassium ions may be effectively replaced by rubidium but not by calcium ions. Magnesium ions play a role in intestinal glucose transport especially for securing the stimulating action of increased potassium ion concentration. Calcium (2.6m. eq/L.) and ammonium ions decrease intestinal glucose transfer[280].

Recently the intestinal absorption of substances other than glucose including bile salts[284], pyrimidines[285] and inorganic phosphate ions[286] have been shown to be sodium dependent.

There are few published studies concerning the role of extracellular sodium on the transfer of amino-acids across cell membranes. Christensen, Riggs, Fischer and Palatine[287] replaced 72 and 117m. eq/L of sodium with similar amounts of choline and noted 24% and 57% reduction in glycine accumulation by Ehrlich ascites tumour cells. Heins[288], studying glycine transport in similar tumour cells, reduced the sodium concentration in the extracellular fluid to below 90m. eq/L and kept the potassium concentration constant. He noted decreased glycine influx into the tumour cells under these conditions. Amino-acid transport into cell free suspensions of calf thymus nuclei is dependent upon the presence of sodium in the medium[289]

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While lithium is partially effective as a sodium substitute in this system, neither potassium, calcium or rubidium can replace sodium in any concentration.

Little data is available concerning the role of sodium in the intestinal transfer of the four amino-acid transport groups.

Rosenberg, Coleman and Rosenberg[290] recently reported that in the rat the intestinal transport of glycine, L-methionine and 1-amino cyclopentine-1-carboxylic acid was dependent upon sodium, while that of L-lysine was not. In addition they noted that, while the transport of the three amino-acids was inhibited by ouabain, that of L-lysine was not. Cohen and Huang[291] showed that tryptophan transport was sodium dependent in hamster intestinal sacs, while Harrison and Harrison[286] showed a similar phenomenon in the case of L-tyrosine.

Recently Fox, Thier, Rosenberg and Segal[292] have shown that the active transport of glycine and -amino 1-C^{14} isobutyric acid was abolished in rat kidney cortex slices in a sodium-free medium, while that of L-histidine was considerable reduced. In contrast the transfer of L-lysine was not totally sodium dependent. The transport of the latter dibasic amino-acid by rat kidney cortex slices was thought to be mediated by two mechanisms - one sodium and ouabain sensitive, and the other insensitive to either ouabain or sodium. This conclusion was based upon the observation that L-lysine transport was inhibited by ouabain in Krebs-Ringer bicarbonate buffer but not further inhibited by ouabain in sodium-free buffer.

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Since the active transport of a large number of substances, mostly nonelectrolyte, is dependent upon the presence of sodium, the association is probably a fundamental one[293]. A promising approach is suggested by the magnesium dependent (sodium and potassium activated) membrane adenosine triphosphatase (ATPase) which was first demonstrated by Skou in crab nerve[294]. This enzyme has been found in a large number of tissues[295], and in experiments on unfragmented red cell ghosts has been shown to be part of a coupled transport system for sodium and potassium, activated inside the cells by sodium and outside by potassium[296].

Ouabain, together with other cardiac glycosides, inhibits cationic fluxes at the cell membrane without depressing respiration or glycolysis[297][298][299]. It must therefore act by means other than those bringing about energetic exchanges. It is currently thought that ouabain combines with a variety of transport carriers, that are only able to act as transfer agents for sodium, potassium and other substances, when they are phosphorylated or stabilised by ATP. It is thought that membrane ATPase is inactivated by cardiac glycosides, this ATPase being magnesium dependent and both sodium and potassium activated.

In view of the lack of precise information on the role of sodium in the active intestinal transport of the four amino-acid transport groups, it was decided to study this phenomenon in the hope that the pattern of inhibition of transfer in the absence of sodium might be identical or similar to that induced by sugars.

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It was thought that, if this should prove to be the case, a common factor might be involved, and that this factor might well be inhibition of membrane ATPase, the enzyme activated by sodium and sensitive to ouabain.

Section 2 - Methods

Changes in the ionic constituents of the media were made by substituting in an isomolar fashion for the contents of Krebs-Ringer bicarbonate buffer. Potassium was used in place of sodium in one series of experiments, and Tris replaced sodium in another. Lithium replaced sodium in a third experiment. Tris was useful as a substitute for sodium because it served as a buffer as well as a source of cations. Osmotic studies in erythrocytes have shown that Tris does not affect the integrity of the membrane though some intracellular penetration by the un-ionised molecule does occur(300). At physiological pH, Tris chloride solution was 75 - 80% ionised and served adequately to maintain the osmolarity of the extracellular fluid. Substitution for sodium was complete or partial depending upon the experimental design.

The method of measuring the uptake of amino-acids by rat intestinal segments was in every other respect identical to that described in Chapter V. All amino-acids were at an initial concentration of 0.05mM and the period of incubation was 60 minutes in all these experiments.

In addition intestinal segments were incubated as in

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TABLE XVIII

Changes in the Extracellular Fluid Volume of Rat Intestinal Segments incubated in Media of varying Ionic Constitution.

Incubation Medium	Extracellular Fluid Volume (Percentage of Total Tissue Water)*
Krebs-Ringer Bicarbonate (Na^+ 143**; K 5.9)	28.7 ± 3.1 (22)
Replacement of Sodium in Krebs-Ringer Bicarbonate Buffer with Potassium.	
Na^+0; K^+ 148.9	29.0 ± 3.5 (5)
Na^+30; K^+ 118.9	28.1 ± 2.9 (5)
Na^+60; K^+ 88.9	26.9 ± 3.3 (5)
Replacement of Sodium in Krebs-Ringer Bicarbonate Buffer with Tris	
Na^+ 0; Tris 143	28.6 ± 2.0 (5)
Na^+60; Tris 83	27.8 ± 3.3 (5)
Replacement of Sodium in Krebs-Ringer Bicarbonate Buffer with Lithium.	
Na^+ 0; Lithium 143	28.4 ± 1.2 (5)

Rat Intestinal Segments were incubated as described in Table I.

* The E.C.F. is given as the mean ± S.D. with the number of determinations in parentheses.

** The concentration of each ion is given in mM/L.

TABLE XIX

The Effect on Intestinal Amino-acid Transport of Replacing Sodium with Potassium in the Incubation Medium.

Amino-acid (0.05mM)**	Distribution Ratios*			
	Krebs-Ringer Bicarbonate Buffer	Krebs-Ringer Buffer	Krebs-Ringer Buffer	Krebs-Ringer Buffer
	Na^+ 143***; K^+ 5.9	Na^+ 60; K^+ 88.9	Na^+ 30; K^+ 118.9	Na^+ 0; K^+ 148.9
L-Alanine	6.7 ± 0.4 (5)	1.0 ± 0 (3)	0.5 ± 0 (3)	0.6 ± 0.1 (3)
L-Valine	16.5 ± 3.4 (4)	1.3 ± 0.1 (3)	1.2 ± 0.2 (3)	0.6 ± 0.1 (3)
L-Phenylalanine	9.5 ± 2.3 (6)	1.5 ± 0.3 (3)	1.1 ± 0.1 (3)	0.8 ± 0.1 (3)
Glycine	4.4 ± 0.8 (3)	0.7 ± 0 (3)	0.9 ± 0.1 (3)	0.3 ± 0 (3)
L-Lysine	12.1 ± 1.6 (8)	2.6 ± 0.3 (3)	0.8 ± 0 (3)	0.7 ± 0.1 (3)
L-Arginine	7.4 ± 2.1 (3)	1.1 ± 0.2 (3)	1.0 ± 0.1 (3)	0.8 ± 0.1 (3)
Hydroxy-L-proline	3.1 ± 1.3 (4)	0.4 ± 0 (3)	0.3 ± 0 (3)	0.3 ± 0 (3)

The Intestinal Segments were incubated as described in Table II.

* The ratios are expressed as means ± S.D. with the number of determinations in parentheses.

** Initial concentration of amino-acid in the incubation medium.

*** The concentration of each ion is given in mM./L.

TABLE XX

The Effect on Intestinal Amino-acid Transport of Replacing Sodium with Tris in the Incubation Medium.

Amino-acid (0.05mM)	Distribution Ratios*		
	Krebs-Ringer Bicarbonate Buffer Na^+ 143 mM; Tris 0 mM.	Krebs-Ringer Buffer Na^+ 60 mM; Tris 83 mM.	Krebs-Ringer Buffer Na^+ 0 mM; Tris 143 mM.
L-Alanine	6.7 ± 0.4 (5)	2.7 ± 0.3 (3)	1.0 ± 0.0 (3)
L-Valine	16.5 ± 3.4 (4)	8.5 ± 1.6 (3)	0.5 ± 0.0 (3)
L-Phenyl-alanine	9.5 ± 2.3 (6)	5.7 ± 1.7 (3)	1.3 ± 0.1 (3)
Glycine	4.4 ± 0.8 (3)	2.0 ± 0.2 (3)	0.9 ± 0.1 (3)
L-Lysine	12.1 ± 1.6 (8)	8.9 ± 1.7 (3)	4.5 ± 0.2 (3)
L-Argi-nine	7.4 ± 2.1 (3)	10.3 ± 1.7 (3)	1.0 ± 0.2 (3)
Hydroxy-L-proline	3.1 ± 1.3 (4)	2.7 ± 1.6 (3)	0.5 ± 0.0 (3)

The intestinal segments were incubated as described in Table II.

* The ratios are expressed as means ± S.D. with the number of determinations in parentheses.

** Initial concentration of amino-acid in the incubation medium.

*** The concentration of each ion is given in mM./L.

TABLE XXI

The Effect of Ouabain 0.8mM. on Intestinal Amino-acid Transport.

Amino-acid (0.05mM.**)	Distribution Ratios* Krebs-Ringer Bicarbonate Buffer	Krebs-Ringer Bicarbonate Buffer plus Ouabain 0.8mM.
L-Alanine	6.7 ± 0.4 (5)	2.7 ± 1.2 (3)
L-Valine	16.5 ± 3.4 (4)	5.9 ± 0.4 (3)
L-Phenylalanine	9.5 ± 2.3 (6)	4.5 ± 0.8 (3)
Glycine	4.4 ± 0.8 (3)	1.8 ± 0.3 (3)
L-Lysine	12.1 ± 1.6 (8)	7.9 ± 1.7 (3)
L-Arginine	7.4 ± 2.1 (3)	5.8 ± 2.1 (3)
Hydroxy-L-proline	3.1 ± 1.3 (4)	2.5 ± 0.2 (3)

Intestinal Segments were incubated as described in Table II.

* Ratios are expressed as means ± S.D. with the number of determinations in parentheses.

** Initial concentration of amino-acid in the incubation medium.

Chapter V in Krebs-Ringer bicarbonate buffer with the various amino-acid isotopes added to the medium but in these experiments ouabain was also in the medium at a concentration of 0.8mM.

As before the pH of the medium before and after incubation was 7.4 in all instances.

The extracellular fluid space was again measured with carboxy-C^{14}-inulin (as described in Chapter V) in the presence of the incubating media of varying composition.

Section 3 - Results

The E.C.F. values obtained with Carboxy-C^{14}-inulin in the presence of the various incubation media is shown in Table XVIII. It can be seen that there was no significant variation in the E.C.F. under these conditions.

The distribution ratios for the various amino-acids, when intestinal segments were incubated in media of varying ionic concentrations, are shown in Tables XIX and XX. Table XXI shows the distribution ratios obtained when intestinal segments are incubated in Krebs-Ringer bicarbonate buffer containing ouabain (0.8mM).

It can be seen that when potassium completely replaced sodium in Krebs-Ringer bicarbonate buffer, all the amino-acids tested showed grossly diminished intestinal transport; in fact the distribution ratio in all cases was less than unity (Table XIX). This inhibition of intracellular accumulation of amino-acids was evident even when sodium was only partially replaced by potassium (Table XIX). In this series of experiments an inhibitory action

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by potassium could not be excluded [292]. When lithium replaced sodium completely in the medium no amino-acid achieved a distribution ratio greater than unity.

When Tris completely replaced sodium in the incubation medium all the amino-acids tested showed marked inhibition in intestinal transport, with only L-lysine and L-phenylalanine achieving distribution ratios over one (Table XX). Segments incubated in the "all-Tris" medium were immediately reincubated in Krebs-Ringer bicarbonate buffer in another series of experiments, and were able to accumulate L-alanine to a distribution ratio of 80% of those incubated in Krebs-Ringer bicarbonate buffer without prior incubation in a Tris medium. For this reason it did not seem as though the "all-Tris" buffer was toxic to the intestinal cells in any way.

From these results it is clear that amino-acid transport in the gut is sodium dependent, and that there is no correlation between this dependency and the inhibition of amino-acid intestinal transport by sugars.

When one considers the data obtained when sodium is partially replaced by Tris, there is some relationship, albeit tenuous, between the results of inhibition of amino-acid transport by sugars and the effects of the partial replacement of sodium in the medium. Under these conditions the dibasic amino-acids, which are not affected by sugars, are least dependent on sodium, while the converse is the case for L-alanine, L-valine and glycine. However, the results with L-phenylalanine and hydroxy-L-proline show

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that even with only partial replacement of sodium in the medium, the correlation with the effects of sugars cannot be maintained. L-phenylalanine was transported scarcely better than valine under these conditions, yet the former was unaffected by sugars and the latter inhibited. The intestinal transport of hydroxy-L-proline was inhibited by galactose (28mM) but this imino-acid was well transported when sodium was present at a concentration of 60mM/L. Very similar results were obtained with ouabain.

The validity of attempting this correlation between the inhibition of intestinal amino-acid transport by sugars and transport in the partial absence of sodium, or after the addition of ouabain to the medium, is very dubious, especially in view of the relatively small number of experiments involved. In drawing attention to this possible partial correlation I only want to point out that this was the best correlation that could be obtained from the data. Indeed no definite relationship seems to be present between the inhibition of amino-acid transport in the intestine by sugars and their sodium dependency for transport.

Section 4 - Conclusions

The lack of correlation between dependence on sodium for intestinal transport, inhibition by ouabain, and the effects of sugars on intestinal transfer of amino-acids, does not support the concept of interference with membrane ATPase or any other common

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factor, as being the fundamental biochemical mechanism for the inhibition of amino-acid transport in the intestine by sugars.

It is also of interest that phlorhizin has been observed to inhibit ATPase and that this substance does not interfere with amino-acid transport.

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CHAPTER XIII

The Implications of the Studies Reported in this Thesis.

The work recorded here has confirmed the original hypothesis that certain sugars would inhibit the intestinal transport of some amino-acids. The most significant single finding is the demonstration that galactose and fructose feeding result in defects in amino-acid transport in both the renal tubules and the intestinal cells in rats.

As discussed earlier in this thesis there is very good evidence to support the concept that patients with Hartnup disease and Cystinuria have both intestinal and renal defects in amino-acid tran port. The development of this concept has been due largely to the work of Professor Milne and his colleagues who have also shown that an acquired tubular defect in amino-acid transport as occurs in neomycin poisoning, may be associated with decreased intestinal absorption of amino-acids. It is submitted that the experiments reported here have added to and further strengthened this concept. It seems perfectly reasonable to propose that other inherited and acquired defects in the tubular transport of amino-acids are very likely to be associated with similar defects in the intestinal cell. This hypothesis needs to be tested further and as far as I know no such studies have been carried out as yet.

For example both scurvy[167] and severe rickets[166] are

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associated with a "renal" amino-aciduria which disappears when the appropriate vitamin is given. I would suggest that these patients might also have a defect in intestinal amino-acid transport which would similarly prove to be reversed by treatment with the appropriate vitamin.

The importance of defective intestinal amino-acid transport in man remains to be carefully elucidated. Collis, Levi and Milne(301) have shown that there is a significantly reduced mean stature in patients with Hartnup disease and cystinuria. However protein malnutrition is not overtly present in these patients. Patients with galactosaemia and hereditary fructose intolerance in whom it is proposed there may be defective intestinal transport of amino-acids when renal amino-aciduria is present, do not show evidence of protein malnutrition either, but in these diseases the postulated defect in intestinal amino-acid transport would only occur in relationship to exposure to the offending sugar. Milne has proposed that in Hartnup disease and cystinuria the intestinal transport defect is a partial one and there is evidence to support this contention.

Milne suggested that diseases such as Hartnup disease and cystinuria, in which there is a defect in intestinal amino-acid absorption, would cause nutritional deficiencies in communities in which there were chronic periods of protein insufficiency followed by the ingestion of a large quantity of protein over a short period of time. An example of such a community would be a primitive hunting people or a society in which children are deprived of

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protein for most of their young lives.

As far as the interference with amino-acid transport in the intestine by sugars in man is concerned, I have already speculated that patients with galactosaemia and hereditary fructose intolerance probably have such a defect when exposed to the offending sugar. In addition it should be noted that in recent years there has been renewed interest in the interrelationship of various nutrients in the diet of man and of animals(301)(302)(303)(304).

It has been postulated that carbohydrate may influence protein metabolism in two ways(301)(303).

1. By the prevention of the misuse of dietary protein in the meeting of calorie requirements. This action is shared by other energy yielding nutrients like fat and alcohol.
2. By the regulation of protein metabolism through the specific effect of blood glucose on the endocrine glands. This effect is not shared by the fat. It has been shown that the isocaloric replacement of dietary carbohydrate by fat has led to a decrease in nitrogen balance and Munro has suggested that insulin secretion and activity is of paramount importance in this regard(303).

Of great interest is the observation that pigs, fed restricted amounts of a diet of low protein value, develop pathological changes in their bones when extra carbohydrate is given. When the carbohydrate is replaced isocalorically with protein, the bones improve(306)(312). There is an associated fall in urea nitrogen excretion during the period of carbohydrate intake.

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Endocrinal changes have been invoked to explain this phenomenon but it has recently been shown that these animals may have impaired absorption of nitrogen[307].

In 1959 Truswell[308] showed that the addition of carbohydrate to the diet increased the excretion of faecal nitrogen The removal of carbohydrate had the opposite effect.

It seems possible that inhibition of intestinal amino-acid transport by carbohydrate ingestion might have played a role in these phenomena.

It is of interest that in 1926 Cori[266] first suggested that amino-acids and sugars might be mutually inhibitory during intestinal absorption. He noted mutual inhibition in the absorption of mixtures of glucose and glycine given to rats by stomach tube. These observations were not taken further at the time. The work recorded in this thesis indeed shows that amino-acid intestinal absorption is inhibited by certain sugars, although the reverse does not appear to be the case.

Platt, Heard and Stewart[302] have stated that "an insufficiency of dietary protein is a factor in the production of all forms of protein - calorie deficiency, but it is the addition of carbohydrate to the diet of the protein deficient animal that is the determining factor in the production of the acute syndrome." It should also be noted that Coles and Macdonald have claimed that the serum protein level is lowered in man by a high carbohydrate intake[313].

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Kwashiorkor has been shown to be due to a protein deficiency in the weaning child[309]. It is thought that this is the result of an inadequate intake of dietary protein. It is conceivable that the high carbohydrate diet eaten by these children further limits the amino-acids available to them by interfering with their absorption in the intestine. While this would probably be of no importance in the presence of a high protein diet, this additional interference with amino-acid absorption could play a significant role, in children whose diet is very poor in protein.

Amino-aciduria has been shown to occur in kwashiorkor. There is conflicting data as to whether this is due to raised plasma amino-acid levels or renal tubular dysfunction[310][305]. If the amino-aciduria is on the basis of an acquired enzyme deficiency, this defect in transport could be shared by the intestine, providing a second possible mechanism for impaired amino-acid absorption. It is also possible that the defect in amino-acid transport in kwashiorkor may be due to pyridoxine deficiency, which vitamin plays an important role in the transfer of amino-acids across cell membranes[70].

The importance of recognising the effect of sugars on amino-acid transport has other implications too. Many biological phenomena are studied <u>in vitro</u> in media containing sugars, and it is clear that these sugars can themselves influence some metabolic events.

A striking example of the misinterpretation of experimental

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results as a consequence of ignoring the role of sugars in *in vivo* experiments is offered by the claim that maleic acid produces an amino-aciduria[157]. It has now been shown that the defect in the transport of amino-acids in the renal tubule produced in those experiments, was due in part to lactose in the diet[311].

It is to be hoped that further work in the field of linked intestinal and renal amino-acid transport defects may help to throw light on the little understood and very complex processes involved in the transport of substances across cell membranes.

SUMMARY.

The mode of absorption of amino-acids in the intestine, and the development of the concept of "linked" renal and intestinal defects in amino-acid transport, have been reviewed.

In view of the fact that "renal" amino-aciduria was known to occur in galactosaemia, and in hereditary fructose intolerance, as well as in some states associated with glycosuria, an hypothesis was developed that these sugars might inhibit the intestinal transport of some amino-acids. The work testing this hypothesis, and finding it to be essentially a correct one, has been recorded in this thesis.

In the presence of galactose inhibition of the transport of neutral amino-acids (alanine, valine and glycine) and of hydroxyproline was noted. Studies on slices from galactose-fed rats were similar except that hydroxyproline transport was not impaired. In the presence of fructose, alanine and glycine transport was inhibited, but in the slices from fructose-fed animals only alanine transport was depressed. Glucose inhibited the intestine transport of alanine, valine and glycine *in vitro* under certain circumstances, but no defect in the intestinal transport of amino-acids could be demonstrated in glucose-fed animals. Fructose-fed, like galactose-fed rats develop an amino-aciduria. One glucose-fed rat had glycinuria.

It is clear that both galactose-fed and fructose-fed rats develop defects of amino-acid transport in both the intestine and the renal tubules.

Kinetic studies revealed that the inhibition of alanine transport by galactose was noncompetitive.

A defect in the transport of dibasic amino-acids or phenylalanine could not be demonstrated.

ATP levels were measured in intestinal slices incubated in the presence of sugars, and in the intestinal slices of rats fed galactose or fructose. No direct quantitative relationship was found between changes in intestinal ATP levels and alterations in amino-acid transport.

Galactose-1-phosphate accumulated in the intestinal slices incubated in the presence of galactose, and was also present in the intestine of galactose-fed rats. The possible role of the accumulation of this metabolite in the inhibition of amino-acid transport by galactose remains to be determined.

There was no relationship between the inhibition of amino-acid transport by sugars, and the dependence on sodium of amino-acids for transfer across the intestinal cells.

The implications of these findings have been discussed. It has been suggested that in galactosaemia and in hereditary fructose intolerance a defect in intestinal transport of certain amino-acids might occur. The possible role of carbohydrates in protein depletion in man has also been reviewed.

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