CHARACTERIZATION OF L-LEUCINE TRANSPORT IN THE TOADFISH LIVER IN VIVO

by

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DEDICATION

This dissertation is dedicated to the memory of my father, Robert Kenneth Persell, whose love and encouragement remain with me.

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ABSTRACT

The transport of L-leucine by liver has been studied in vivo at two temperatures in a marine teleost, Opsanus tau. A kinetic model is developed which permits analysis of amino acid exchange between liver and blood in vivo and of the simultaneous incorporation of transported leucine into intrahepatic proteins. At normal plasma leucine concentration (0.1 mM), uptake occurs rapidly ($t_{1/2}$ = 0.25 min), and flux for the liver as a whole is 0.6 μmol/min. The distribution of leucine between extracellular and intracellular compartments and in protein-bound form was analyzed at times of 30 s to 5 min after injection. The results are consistent with the operation of a concentrative or uphill transport system accounting for 40% of uptake at 21° at normal plasma concentration. Calculation of intracellular pool dilution from protein synthesis data indicates that about 20-30% of liver intracellular space is occupied by the leucine pulse during the first 2 min after portal injection. Characteristics of leucine transport in liver in vivo are comparable to results obtained in vitro: the saturation of uptake with increasing leucine concentration and competitive inhibition by isoleucine and phenylalanine. Estimated Michaelis-Menten kinetic parameters were: maximal flux V_{max} = 4.1 μmol/min/7-g liver, and concentration

at one-half maximal rate K_m = 0.6 mM.

Adaptability of the poikilothermic toadfish to a wide range of body temperatures permits examination of the temperature dependency of L-leucine transport by liver in vivo. When compared with data at 21°, transport data at 10° indicate a large temperature effect on both leucine influx and incorporation into protein, with a smaller effect on leucine efflux. Results suggest a reduced contribution of active transport to total leucine uptake at 10°. The study confirms in vivo several effects of temperature which have been demonstrated in vitro. Results are discussed in relation to the natural temperature range for the toadfish, and possibilities for future studies are considered.

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INTRODUCTION

A. The role of liver in amino acid movements.

The first description of the fate of amino acids released hydrolytically from dietary proteins in the intestine was presented in the pioneering work of van Slyke and Meyer (1913a-1913d). They found that the free amino acids were absorbed into the blood stream, from which they rapidly moved into body tissues against a concentration gradient, with the liver being the site of the greatest concentration differences. Since a dietary load increased this concentration gradient to a tissue/plasma amino acid ratio of between 5 and 10, van Slyke and Meyer dismissed a simple osmotic effect. They concluded that amino acid movement into tissue cells must occur by an "adsorption" process or formation of readily-dissociable compounds within the cells. Their data indicated that amino acids are exchangeable and in equilibrium between plasma and liver. Furthermore, since the amino nitrogen concentration of liver was highly variable, depending on time of day, nutritive status, or elapsed time after a meal, van Slyke and Meyer concluded that amino acids were metabolized there.

Peters and van Slyke (1940) realized that the anatomical position of the liver--receiving blood directly from the intestine--was critical to its role in regulating

dietary sources of proteins. In a study of the partitioning of dietary amino acids, Friedberg and Greenburg (1947) observed that the total concentration of free amino acids is lower in the emerging hepatic vein than in the afferent portal vein, thereby demonstrating that the liver extracts amino acids directly from the hepatic circulation. Kamin and Handler (1951) showed that the liver is not indiscriminately permeable, but selectively accumulates certain amino acids, such as glycine and lysine, while exhibiting almost no concentration of arginine. Denton and Elvehjem (1954a; 1954b) showed that the liver released amino acids to the circulating plasma during fasting or several hours after the ingestion of a protein meal. Their studies suggested a very rapid turnover and exchange of amino acids between blood and liver.

Using dog liver perfused in situ, McMenamy et al. (1962) sought to establish the quantitative role of the liver in maintaining or altering blood amino acid levels and in total amino acid metabolism. They showed that depending upon the amino acid and the load, the liver extracts most of an added amino acid during a single passage. Of the amino acids extracted, up to 90% may be catabolized to urea, while the rest may be stored as hepatic proteins which may be degraded later to free

amino acids and released into the general circulation.

Elwyn (1970) extended these quantitative measurements of the movement of amino nitrogen in dog and found that, in terms of net balances, 57% is converted to urea in the liver, 23% enters the general circulation as free amino acids and 6% as liver-synthesized plasma proteins within 12 h. The remaining 14% form a temporary increase in intrahepatic protein, constituting the storage of amino acids noted by McMenamy et al, (1962). Elwyn points out that the actual percentage of dietary amino acids which enters one pathway or another will vary depending upon several factors, e.g. amino acid composition of the meal. These figures, nevertheless, demonstrate the "extensive buffering action of the liver" between diurnal or long-term changes in protein ingestion and the requirement of other tissues for a constant amino acid supply.

Woods and Handschumacher (1971) have shown that the liver's ability to maintain plasma levels of amino acids, at least for asparagine, is not abolished by inhibitors of protein synthesis, nor interrupted by removal of neuronal or hormonal influences. The liver can apparently monitor directly the plasma concentration and absorb or release amino acids to maintain plasma concentrations within a very narrow range, 20-30 μM for asparagine.

Recently, Elwyn et al. (1976) have demonstrated that the liver's central function in regulating amino acid supplies extends beyond variations in diet to the physiological extreme of hemorrhagic shock. They conclude that by releasing amino acids to the general circulation and to peripheral organs during shock, the liver serves to limit peripheral protein catabolism.

B. Liver protein metabolism and the amino acid pool.

Bollman et al. (1924) perfected a technique for total hepatectomy and thereby demonstrated that the liver is a primary site of amino acid catabolism, including urea synthesis and deamination (Bollman et al., 1926). Compared with proteins of skin, muscle and bone, liver proteins show a very high proportion of incorporation of infused amino acids, reflecting a high protein synthetic activity (Shemin and Rittenberg, 1944). Liver is now known to be a primary site of plasma protein synthesis. It is the major, if not the sole, source of albumin and fibrinogen, and it produces 75-90% of the α-globulins and 50% of the β-globulins (Kukral et al., 1963; Miller, 1962), as well as clotting factors and complement proteins (Asofsky, 1974). Furthermore, liver is a site of extensive protein degradation, indicated by both widely-fluctuating levels of many liver enzymes and

by the rapid turnover of several liver proteins (Tarver, 1963), for example ferritin, with an 18-h half life (Schimke, 1970).

The extensive anabolism and catabolism of proteins is only one aspect of the liver's regulation of an intracellular pool of free amino acids. The free amino acid pool occupies a central position in protein and amino acid metabolism (Miller, 1962; Tarver, 1963). Munro (1970) has extensively reviewed its general features. The free amino acid pool reflects the relative contributions of the routes into and outlets from the pool, with the transfers from one compartment to another, for example amino acid transport from plasma to liver intracellular space, being potential sites for the regulation of amino acid and protein metabolism (Munro, 1970).

Complications have arisen, however, in the interpretation of experiments in which radioactively-labeled amino acids have been used to trace amino acid movements into liver, particularly when the amino acids are involved in subsequent protein metabolism (Christensen, 1975; Munro, 1970). In an early study Loftfield and Harris (1956) had difficulty in changing the specific radioactivity of intracellular valine during infusion of labeled valine in rat liver. They postulated that the intracellular pool of valine was rapidly diluted by unlabeled valine from intrahepatic albumin proteolysis. Although this explanation

has been criticized on the basis that proteolysis is slow relative to amino acid uptake (Christensen, 1975), Gan and Jeffay (1967) have shown that protein breakdown may contribute up to 50% of the liver free amino acid pool. Mortimore, Woodside, and Henry (1972) proposed the existence of two intrahepatic pools which mix slowly: one, the precursor pool for protein synthesis, receives amino acids directly by liver uptake from plasma, and the second, a lysosomal pool, is supplied directly by intracellular proteolysis. This view has been modified by more recent work of Khairallah and Mortimore (1976), who measured the specific radioactivity in perfused rat liver of the immediate precursor to newly-synthesized proteins, aminoacyl-tRNA. They found that for valyl-tRNA the specific radioactivity was intermediate between that of the extracellular and intracellular valine pools. The authors concluded that protein synthesis may utilize both an intermediate intracellular pool and an extracellular pool. No clear role for amino acid transport in the regulation of the precursor pool for protein synthesis has yet been proposed.

Fern and Garlick (1974) have questioned whether amino acids move directly from an extracellular pool into protein without mixing with an intracellular pool. They have suggested as an alternative explanation that the aminoacyl-tRNAs themselves exist in heterogeneous pools

(Fern and Garlick, 1976). In an analysis of albumin and ferritin synthesis in rat liver, they concluded that the two proteins are synthesized from different pools of activated tRNAs, each pool associated with a separate "ribosomal entity". This picture of the regulation of hepatic amino acid pools may include a complex relationship between the activation of tRNA and amino acid transport, as indicated by a recent study in cultured mammalian cells (Moore et al., 1977). The authors found that a shift to permissive temperatures in cells with mutant leucyl-tRNA synthetase resulted in an increased transport of leucine without affecting the uptake of other amino acids.

The nature of the intracellular amino acid pool or pools, however, is still not clear (McKee et al., 1978). Some authors claim that no significant compartmentation exists and that intracellular amino acids are in a single, homogeneous precursor pool for protein synthesis (Li et al., 1973; Blackburn et al., 1975; Seglen, 1976). Christensen (1975) has summarized the problem and suggests that more than one intracellular pool would be "possibly a new important point of regulation" of protein metabolism.

C. Regulation of intracellular amino acid concentrations.

Although a clear understanding of the nature of intracellular amino acid pools is not yet possible, alterations

in total intracellular free amino acid concentrations are known to occur in response to several physiological factors. For example, changes occur in response to diet (Adibi et al., 1976; Tews and Harper, 1976), elapsed time after a meal (Elwyn, 1970), stages of early development (Guidotti et al., 1975), and phases of the mitotic cell cycle (Bhargava et al., 1976). An extensive review of several of the factors which alter amino acid concentrations may be found in Munro (1970).

1. Transport as a site of regulation of amino acid uptake and metabolism.

Special attention has focused on the transport of amino acids across biological membranes as a possible site for the regulation of amino acid and protein metabolism through the effect of transport on intracellular amino acid concentrations (Christensen, 1964; 1977b). Amino acid transport has been implicated in diseases, such as Hartnup's disease which is characterized in part by defective renal transport of neutral amino acids and accompanying neurological disorders (Christensen, 1975). A pronounced congenital protein intolerance, followed by severe growth failure, has been identified as a defect in the renal, intestinal, and liver transport of basic amino acids, leading to insufficient cellular concentrations of otherwise non-essential amino acids (Greenstein et al.,

1956). Altered transport mechanisms in general may be responsible for increases in intracellular concentrations of nutrients that have been associated with the unregulated growth characteristic of cancer malignancies (Holley, 1972). For example, increased rates of glucose transport are among the earliest events associated with transformation of chick fibroblasts by Rous sarcoma virus (Lang and Weber, 1978). In addition, transformation of rat hepatic cells increases glucose transport (Graff et al., 1978). Moreover, the authors have shown that inhibition of transport can reduce glycolysis to the same rate as in untransformed cells. Induction of hemoglobin synthesis is also accompanied by changes in transport (Mager and Bernstein, 1978).

A link between transport and general hormonal regulation of protein metabolism was suggested early by Noall et al. (1957). Reviews of this large topic include those by Munro (1970), Heinz (1972), and Christensen (1975). Recent attention has focused on the effect of glucagon on amino acid transport. Intraportal infusions of glucagon in sheep in vivo caused net uptake of alanine by liver to increase from a control value of 3.8 mmol/h to 5.9 mmol/h over a period of 2 h (Brockman and Bergman, 1975). Glucagon elevates net uptake of primarily the neutral amino acids, α-aminoisobutyric acid, alanine, and glycine in liver (Chambers et al., 1970).

The role of both glucagon and insulin (Young et al., 1975) in altering amino acid transport has led several investigators to examine the possible role of cyclic nucleotide "second messengers" in amino acid transport (Tews et al., 1975; Le Cam and Freychet, 1976). Dibutyryl cyclic AMP stimulates α-aminoisobutyric acid uptake in liver slices by a factor of 4-6 over control values, but quantitation of the effect has remained difficult since elevated intracellular cyclic AMP was not always associated with increased amino acid uptake (Tews et al., 1970). Phang et al. (1975) suggested that hormonal stimulation of amino acid uptake in rat calvaria may be due to an overall increase in protein synthesis, particularly of specific membrane proteins. Foury and Goffeau(1975), however, found that the stimulatory effect of dibutyryl cyclic AMP on leucine uptake in yeast continued even in the presence of cycloheximide, a potent inhibitor of protein synthesis.

Munro (1970) and Heinz (1972) have emphasized that the regulatory role of transport across biological membranes is still not clear, in part because transport is so rapid compared to other metabolic steps and has been difficult to measure quantitatively, especially in conjunction with physiological regulation.

2. Models of amino acid transport.

In spite of a limited understanding of the regulation of amino acid transport and its role in overall protein

metabolism, considerable information about the transport process itself has emerged. Transport of most natural amino acids across biological membranes is generally accepted as being mediated by membrane components, usually described as carrier molecules (Heinz, 1972). Transport characteristics are defined largely by kinetic parameters, including the relationships of Michaelis-Menton kinetics: concentration at half-maximal flux and maximum velocity of of flux (Hoare, 1972a). Neame and Richards (1972) have written a general introduction to the kinetic study of membrane carrier transport, and Kotyk (1973) has reviewed how kinetic formulations relate to schematic models of membrane carrier function in non-electrolyte transport. In the absence of any significant knowledge about the chemical mechanisms of amino acid transport (Heinz, 1972), kinetic analysis remains a powerful tool in analyzing data upon which hypothetical molecular mechanisms are based (Christensen, 1975).

The work of Halvor Christensen and his colleagues has been instrumental in developing models of amino acid transport (Christensen et al., 1948; Christensen and Riggs, 1952; Christensen and Handlogten, 1968). Oxender and Christensen (1963) found evidence for two predominant systems in their pioneering studies with Ehrlich ascites tumor cells. System L is independent of extracellular Na^+ concentrations and exhibits its greatest activity

with leucine, isoleucine, and phenylalanine, while System A is Na+ dependent and shows greatest affinity for alanine and the model amino acid, α-aminoisobutyric acid (AIB) (Christensen, 1975). Both systems, however, transport all neutral amino acids to some extent (Christensen, 1975, 1977a). Glycine appears to be transported by a highly-specific, Na+-dependent system that shows additional affinity only for sarcosine (Vidaver, 1974). A fourth system identified by Christensen et al. (1967), System ASC, is also Na+ dependent and has an intermediate range of specificity for 3- to 5-carbon straight chain amino acids, asparagine, and proline. In spite of overlapping affinities, System ASC remains distinct from System A (Christensen, 1977a). System L has recently been reconstituted in partially-purified membrane vesicles from Ehrlich ascites cells (Cecchini et al., 1977).

Alton Meister and his colleagues have developed the first detailed molecular model for amino acid transport based on the enzymes of the γ-glutamyl cycle, involved in synthesis and degradation of the ubiquitous tripeptide, glutathione (Meister, 1973, 1976; Meister and Tate, 1976). Meister has proposed that the actual translocation step may be mediated by the widely-occuring membrane-bound enzyme, γ-glutamyl transpeptidase, which catalyzes the

the addition of L-amino acids to glutathione to form L-γ-glutamyl-L-amino acid and L-cysteinylglycine. Abnormal excretion of 5-oxoproline has been linked to deficiency of a γ-glutamyl cycle enzyme, glutathione synthetase (Meister and Tate, 1976). Recently, it has been shown that increases in amino acid transport that accompany malignant transformation of cultured hepatocytes correlate with increased activity of γ-glutamyl transpeptidase (Cheng et al., 1978). In addition, the cycle may be a pathway for the transport of peptides (Tate and Meister, 1974). Meister's model has been criticized on the grounds that the energy requirement is too high for amino acid transport, that structural specificity is low, and that the kinetic observation of trans-stimulation (in which higher extracellular substrate concentrations increase the exit rate of intracellular tracer substrate) cannot be readily explained (Samuels, 1977). A recent proposed modification of the intermediate cycle steps may have removed some of these objections, at least for neutral amino acids (Samuels, 1977). Moreover, Karkowsky and Orlowsky (1978) have recently shown that in humans in vivo, γ-glutamyl transpeptidase does exhibit marked specificity for amino acids, with highest affinity for glutamine.

Non-electrolyte transport processes in general show considerable variability from one tissue to another (Heinz, 1972). Systems A and ASC, for example, are absent in the mature mammalian erythrocyte, although they exist in reticulocytes (Wise, 1976). Benderoff et al. (1978) have recently characterized the changes that occur in amino acid transport systems as the red cell matures. The specific glycine system is missing in the Ehrlich ascites cell (Heinz, 1972). Studies in rat brain in vivo suggest that only System L may be operating in the capillary cells (Zanchin et al., 1976). Nevertheless, similarities among transport processes are becoming more apparent, especially with respect to the structural features that determine transport specificity (Christensen, 1975). For example, System L specificity appears to be based on the presence of an adequate apolar side chain, with steric features only secondarily important (Mathews and Zand, 1977).

Differential responses to hormones could be useful in discriminating different amino acid transporting systems in liver (Christensen, 1975). Harrison and Christensen (1971) demonstrated that concentration by rat liver of norbornane amino acid, a model substrate which mimics the characterisitics of System L, was not responsive to glucagon administration in liver in

vivo over a 38 h period. Under the same conditions, however, they found that uptake of AIB, a model substrate for System A, was stimulated by glucagon.

Since its first description by Oxender and Christensen (1963), System A has been the focus of much attention because of the relationship between its dependency on Na+ and the energization of amino acid transport (Heinz, 1972; Christensen, 1975). The driving role of Na+ has been characterized as a cotransport phenomenon, similar to the gradient hypothesis of Na+-glucose cotransport originally formulated by Crane et al. (1965) for rabbit intestine. Recent work by Heinz et al. (1977) has revealed that an electrogenic potential may be sufficient to drive amino acid transport even in the absence of an adequate Na+ concentration gradient. The role of Na+ in amino acid transport has recently been reviewed (Heinz and Geck, 1978). Christensen (1977a) has expanded the models of energization to include an oxidoreductase system within the plasma membrane, fuelded by reducing equivalents of NADH and possibly involving an intramembrane cycle of deprotonation and reprotonation (Christensen and Handlogten, 1975).

3. Temperature dependency of amino acid transport.

Although there are few detailed studies on the effect

of temperature on amino acid uptake, available evidence indicates that amino acid transport is strongly temperature dependent (Jacquez et al., 1970; Hoare, 1972b; Seglen and Solheim, 1978). These studies are of particular interest because they provide information on the activation energies of amino acid transport processes. Changes in these activation energies with temperature provide criteria for detecting rate-determining steps, selecting between possible molecular mechanisms, and correlating transport with structural alterations in membrane components during metabolic adjustments to normal environmental temperature ranges (Hoare, 1972b; Precht et al., 1973; Price and Thompson, 1969; Plagemann, 1975).

In a study of temperature dependency in Ehrlich ascites cells, Jacquez et al. (1970) found that phenylalanine transport at low temperatures was mediated by System A to a greater extent than expected, suggesting that System A and System L distinctions begin to disappear as temperature decreases. They showed that K_m for phenylalanine transport was affected to a greater extent than maximum velocity, J_{max}, with both parameters falling with temperature. More recently, Im et al. (1978) found that K_m for phenylalanine influx in Ehrlich

cells did not change as the temperature was lowered from 37° to 17°, suggesting that the enthalpy change for phenylalanine binding to its carrier is minimal.

Berlin (1973) found a discontinuity in the Arrhenius plot (log rate vs. inverse of absolute temperature) for lysine transport in rabbit leukocytes. The discontinuity, or temperature break, represents a change in activation energy with temperature; for lysine uptake into leukocytes, the break occurs at $22.2 \pm 0.5°$. Berlin found that K_m decreases with temperature, and he concluded that the membrane carrier may exist as two or more conformers, each being favored in a different temperature range.

A theoretical explanation of the temperature breaks in Arrhenius plots for transport phenomena was advanced by Kumamoto et al. (1971), who associated the discontinuities with temperature-related phase changes in the lipid components of the membrane. The lipids change from a liquid-crystalline state to a crystalline state upon cooling, with the phase change occuring at a characteristic transition temperature for each lipid (Christensen, 1975). Plagemann and Erbe (1975) found different critical break temperatures for different substances transported into cultured rat Novikoff hepatoma cells. Transition temperatures ranged from 17° to 23° for nucleosides and from 29° to 32° for choline

and deoxyglucose. The authors conclude that the lipid environments of the two transport groups are different and undergo independent phase transitions. Results from studies in living cells indicate that the more highly ordered the lipid structure, whether due to low temperature or increased lipid saturation, the more transport is impeded (Wilson and Fox, 1971). In artificial lipid bilayers cholesterol has been shown to help maintain structural integrity during phase changes (Tsong, 1975). Studies on membrane changes during temperature acclimation indicate that a fatty-acid desaturase enzyme may be induced or activated at low temperatures to increase the relative concentration of unsaturated lipids and thereby offset the decreased fluidity that occurs below the transition temperature (Kasai et al., 1976; Martin et al., 1976). The transition temperatures for phenylalanine transport in Ehrlich ascites cells are higher in cells grown in mice fed highly-saturated fats than in cells grown in mice fed unsaturated fats (Im et al., 1978). The authors conclude that the membrane lipid composition can directly influence System L transport. Moreover, they showed that phenylalanine influx and efflux show different transition temperatures for each ascites cell system, suggesting that the two processes occur by different molecular pathways.

The temperature dependency of D-glucose influx into and efflux from human erythrocytes led Bolis et al.(1970) to conclude that the translocation rate of a symmetric membrane carrier differs when the carrier is loaded and empty. Hoare (1972b) extended Bolis' work to a major study of the temperature dependency of leucine transport in human erythrocytes. Hoare's results are generally consistent with the earlier work of Winter and Christensen (1964) who found a temperature coefficient (Q_{10}) of 1.7 for leucine transport into erythrocytes in the 27-37° range. At lower temperatures Hoare found a marked increase in the activation energy from 71 kJ/mol at 37° to 203 kJ/mol at 2.5°, equivalent to a Q_{10} for influx of 2.5 in the 27-37° range and a Q_{10} of nearly 12 in the 10-20° range. Hoare's data showed a discontinuity at around 25°. More recently, McGivan et al. (1977) found a sharp temperature break at 18° for leucine transport into isolated rat hepatocytes. Their measured activation energies were 32.6 kJ/mol between 18-37° (Q_{10} = 1.5) and 80.7 kJ/mol between 4° and 18° (Q_{10} = 3.4).

Using an extensive kinetic analysis, Hoare (1972b) determined that the change in activation energy is associated with the transport velocity term, V, rather than with the dissociation constant, K_m. Hoare confirmed

the conclusion of Bolis et al. (1970) that carrier movement and not carrier-substrate dissociation is rate limiting over the entire 0^{o} to 37^{o} range, at least for leucine transport in erythrocytes. Hoare, however, concluded that there must also be an asymmetric distribution of the leucine carrier between inner and outer membranes to account for the marked differences between the kinetics of entry and exit, which become greater at low temperatures. While Hoare allows that the relationship between molecular changes in transport mechanisms at low temperatures to normal physiological control over intracellular amino acid concentrations remains unclear, he suggests that changes in the asymmetric distribution of carrier may explain different relative contributions of exchange diffusion and net transport at different temperatures.

Changes in amino acid uptake in rat brain at different temperatures have been investigated both in vivo (Embirbekov et al., 1977) and in vitro (Banay-Schwartz et al., 1977), with particular attention to elucidating the relationships between amino acid transport and physiological changes with temperature such as hypothermia. For brain tissue in vitro, all amino acids studied exhibit temperature dependency for both uptake and exit, although different transport systems are apparently affected independently (Banay-Schwartz et al,, 1977). The results confirmed

Hoare's (1972b) findings that entry and exit are differentially responsive to temperature changes, and, therefore, changes in total amino acid distribution must depend on the balance between uptake and efflux, rather than on the absolute value of unidirectional amino acid flux.

D. Amino acid transport in liver.

Net amino acid uptake by liver has been studied in a number of species both in vivo (Aikawa et al., 1973; Felig et al., 1973; Bloxan, 1972; McMenamy et al., 1962) and in vitro (Jefferson et al., 1972; Lacy, 1970; Lindsay et al., 1975; Tews, 1969; Crawhall and Purkiss, 1973). Felig (1975) has reviewed the general pattern of liver amino acid uptake, with special attention to uptake in humans.

1. Liver slices.

Tews (1969) and Crawhall and Segal (1968) both demonstrated concentration of AIB (α-aminoisobutyric acid) in rat liver slices, although Crawhall and Segal found that the model System L amino acid, cycloleucine (1-aminocyclopentane-carboxylic acid) was not concentrated. Crawhall and Davis (1971) found that lysine and valine were not concentrated by liver slices; however, an abnormally high inulin extracellular space was reported.

Nallathambi et al. (1972) also observed that within a few hours liver slices exhibited increasingly large extracellular spaces. Christensen (1975) has called attention to studies of amino acid transport in liver slices because they show dramatic alterations in ion distribution, up to a 50% increase in intracellular Na^+, for example. MacKnight et al. (1974) confirmed that liver slices exhibit altered cell volumes over a 60 min period due to K^+ loss and Na^+ gain, which may reflect damage caused by slicing or decreased metabolic rates associated with low oxygen tension.

2. Perfused liver.

Perfused liver preparations have been extensively used in investigation of amino acid transport, especially with respect to changes in net amino acid uptake or loss in response to hormone adminstration. Schimassek et al. (1973) have reviewed the literature on liver perfusion studies, with an emphasis on perfusion techniques. Transport activity in perfused liver is highly selective: up to 90% of perfused phenylalanine and tryptophan is accumulated, but only about 20% of administered threonine, valine, and leucine is accumulated (McMenamy et al., 1962).

Jefferson, Schwarer, and Tolman (1975) have concentrated their studies on the effect of growth hormone and hypophysectomy on liver amino acid uptake. They found

that growth hormone increases uptake of AIB by 84% over control levels in liver taken from intact rat and by 108% in liver from hypophysectomized rat. Kinetic analysis revealed a greater effect of growth hormone on the V_{max} of both AIB and cycloleucine uptake than on K_m. The authors concluded that the hormonal effect was a direct one on transport, based on the observation that the hormone increased net liver concentration of only a few amino acids, in particular threonine, serine, proline, glycine, alanine, and arginine. Perfused liver concentrates asparagine when the perfusing medium contains an asparagine concentration greater than 20 μM, and it appears that asparagine enters the liver both by active transport and by passive diffusion (Woods and Handschumacher, 1973).

Liver, perfused or in vivo, exhibits a low net uptake of glutamine (Felig, 1973) and the branched-chain amino acids, leucine, isoleucine, and valine (Elwyn, 1970). Uptake of the branched-chain amino acids, however, is known to increase in livers showing high rates of protein synthesis (Lindsay et al., 1975). Elwyn et al. (1968) have also maintained that the net uptake of leucine and isoleucine is accounted for by liver protein synthesis.

3. Isolated and cultured hepatocytes.

McGivan et al. (1977) have recently studied leucine transport in isolated hepatocytes. They found a low distribution ratio, $[Leu]_{in}/[Leu]_{out}$ = 1.15, but leucine flux was high (2.6 nmol/min/mg hepatocyte protein). These findings are consistent with those of Oxender et al. (1977) in their recent studies on cultured mouse fibroblasts and ascites cells and may be a general characteristic of System L transport. Using a similar rat hepatocyte system, Seglen and Solheim (1978) recently found that valine uptake is non-concentrative and energy-independent.

Active accumulation of AIB in cultured liver hepatocytes has been observed by several authors (Ayala and Canonico, 1975; Kletzien et al., 1976). AIB uptake is increased in the presence of insulin, suggesting that the cultured cells have retained regulatory properties (Kletzien et al., 1976). Consistent with their conclusion is the fact that System A transport is inducible in hepatocytes in response to starvation (Heaton and Gelehrter, 1977) and under conditions of growth media supplementation with different amino acids (Hume and Lamb, 1974). Oxender et al. (1977) have also shown that amino acid transport rates are altered under conditions of differential cell growth.

Glucagon stimulates neutral amino acid uptake by System A in isolated hepatocytes (Le Cam and Freychet, 1976, 1977), confirming the earlier observations of Mallette et al., (1969) in perfused liver. AIB uptake in this system exhibits a saturable and non-saturable component. The saturable component, accounting for 75% of AIB uptake, is Na+ dependent and cross inhibited by alanine, the major criteria for System A transport.

4. Liver in vivo.

Pardridge and Jefferson (1975) have measured uptake of amino acids by rat liver in vivo utilizing an indicator dilution technique originally developed for studies on rat brain transport (Oldendorf, 1970). They found that in vivo, net uptake of model amino acids (AIB and cyclo-leucine) was low after a single circulatory passage compared to the uptake of naturally-occuring amino acids. AIB, for example, gave a liver uptake index, relative to a diffusable ^{3}HOH standard, of only 22.0 compared to an uptake index of 78.2 for leucine. Their results confirm earlier work by Bravo and Yudilevich (1971) who found that for times less than 1 min, uptake of AIB by liver in vivo was only slightly higher than that of the extracellular space marker ^{22}Na. Pardridge and Jefferson conclude that AIB uptake by liver is a

concentrative process, but it is slow relative to liver uptake of natural amino acids. The acidic L-amino acids showed the greatest net uptake after a single circulatory passage in vivo; basic amino acids, except for histidine, exhibited the lowest net uptake. Stereospecificity was demonstrated in vivo by comparison of the uptake index of L-alanine (45.7) and D-alanine (23.5). Recently Pardridge (1977) has extended his studies to include estimates of unidirectional influx based on fractional liver clearance in rat at several time points between 0 and 90 s. Transport capacity of liver in vivo is several times greater than that of brain or muscle. Pardridge confirms the emerging view (e.g. McGivan et al., 1977) that branched-chain amino acids undergo rapid unidirectional flux across the liver plasma membrane, although net accumulation is low. Fractional extraction of leucine, for example, during an 18-s circulatory passage is 56% of the dose. Studies of net uptake only may, therefore, lead to erroneous conclusions about transport events. Short-term starvation increased liver transport of acidic and neutral amino acids in vivo, an observation consistent with the demonstration that starvation induces System A transport in hepatocytes (Heaton and Gelehrter, 1977). Pardridge (1977) concluded that amino acid transport in liver in vivo is characterized

by low affinity but high capacity, in contrast to the high-affinity, low-capacity system of brain.

Work by Haschemeyer and Persell (1973), establishing the basis and direction for this thesis, introduced the use of poikilotherms for studying rates of amino acid transport in vivo. They showed that the liver of the toadfish, Opsanus tau, extracts as much as 75% of a mixture of amino acids after a single circulatory passage through the liver. Cooperstein and Lazarow (1977) have confirmed that toadfish liver in vivo concentrates all amino acids five min after injection into the gill arch, with lysine, glutamine, and alanine being concentrated most. The advantage of the poikilotherm is that lower temperatures can be used to achieve a reduction in reaction rates. This can facilitate experimental measurement of very fast processes such as amino acid transport.

In summary, it has been known since the pioneering work of van Slyke and Meyer at the beginning of the century that liver is a major site for regulation of amino acid movements. Liver accumulates amino acids against a concentration gradient and can release them to the general circulation in response to the metabolic needs of peripheral organs. Within the liver a complex relationship may exist between the movement of amino acids into intracellular pools and subsequent incorporation

of amino acids into protein. A probable site of regulation of amino acid movements by liver is at the point where they cross the liver plasma membrane, i.e. at the amino acid transport system. Although amino acid transport has been studied extensively in several isolated systems, few quantitative measurements of the transport of naturally-occuring amino acids have been made under in vivo conditions. Pardridge and Jefferson (1975) have identified the main problems in obtaining quantitative measurements in vivo as a) the very rapid equilibration between blood and liver of transported substrates and b) the effects of other metabolic reactions including protein synthesis on amino acid pools.

E. Thesis rationale.

The rationale for this thesis is two-fold:

1. to characterize quantitatively the kinetic parameters of L-leucine transport utilizing a method that takes account of both the fraction of leucine in the intracellular free amino acid pool and the fraction incorporated into polypeptide chains after transport. These measurements can establish a basis for monitoring changes in amino acid transport that occur in the whole animal as a direct result of altered physiological parameters such as circulating hormone levels and diet.

2. to extend the characterization of L-leucine

transport to low temperatures as a means of establishing the temperature dependency of amino acid transport in vivo as well as identifying further the controlling steps in amino acid transport.

MATERIALS AND METHODS

A. Fish.

Adult toadfish, Opsanus tau, body weight 220 ± 20 g, about 80% male, were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, in May and June and utilized during the following two months. Collection procedures are based on the mating and brooding habits of toadfish and rely on the fact that toadfish choose empty collection cans as nesting sites (Gray and Winn, 1961). The cans are collected during the period when spent males are guarding the eggs, thereby resulting in the predominance of males. Spent females, however, may be caught when baited traps are used (Farmanfarmaian et al., 1972).

The fish were maintained in running sea-water aquaria at ambient temperature, 22 ± 2°. Live killifish, Fundulus heteroclitus, or fresh mussel meat were fed to the fish to appetite until 48 h before an experiment. For temperature-dependency studies, when acclimation and experimental temperatures differed , 1 h was allowed for adjustment to the new temperature before the experiment.

Animals that showed any liver abnormality or signs of being unhealthy were discarded. Experiments were

performed on healthy animals which had empty stomachs and upper intestines, but which showed white or grey fecal matter in the lower intestine, indicating a normal feeding pattern preceding the 48 h starvation period.

B. Experimental procedure.

1. Surgery.

Experimental fish were transfered from a 48-h starvation tank into an oxygenated sea-water tank containing a standard fish anesthetic solution. The solution was prepared by dissolving 0.5 g MS-222 (ethyl m-aminobenzoate methanesulfonate, Eastman Chemical Co.) in 1 liter of sea water maintained at experimental temperature. When the opercular movements became reduced, and the animal could not right itself, it was removed and placed on a sponge-covered V-shaped rack in a supine position, ventral side up. The rack was in a plastic pan containing a minimal-level, or maintenance, anesthetic solution of 0.1 g MS-222/l sea water. A Dynaflow pump chamber pumped the solution, after it was saturated with oxygen, through a Y-shaped tube placed in the mouth of the fish and over the gill arches. Flow was approximately 1 l/min. Occasional opercular movements were observed

under these conditions, and the heart beat remained normal throughout the experiments at 20-25 beats/min at T = 22°.

Toadfish exposed to this procedure for more than an hour can be returned to fresh sea water, where they will resume normal swimming behavior in less than 5 min without any observable side effects. These observations on the non-traumatic effect of MS-222 anesthesia on toadfish were reported earlier by Farmanfarmaian et al. (1972) who demonstrated that intestinal absorption of glucose was not altered in fish maintained under minimal anesthesia for as long as 1 h when compared with the results from fish which were returned directly to free-swimming conditions in fresh sea water.

When the fish showed no movement on the rack, other than an occasional opercular contraction, an incision was made about 5 cm anterior to the anus, approximately 1 cm to the right of the ventral median line of the fish. The incision was extended rostrally for 2-3 cm. At the anterior end of the incision, a second, connected incision was made laterally to the left for another 2-3 cm, creating a triangular flap of skin and body wall and a corresponding triangular area of exposed splanchnic viscera. At this point, the lower edge of the liver, the swim bladder, and a considerable portion of middle intestine

are visible. A gentle rearrangement of the intestines readily exposed the gall bladder and bile duct, with the hepatic portal vein immediately dorsal to the duct. The gall bladder was lifted gently and placed on the left side of the viscera, where it was usually held in place with a small square of sterile guaze. This simple procedure requires about 2 min and exposes a minimum of 1 cm of hepatic portal vein without disturbing the position of or the blood flow to the liver.

Under anesthesia the toadfish may reflexively expand its air bladder. When an expanded air bladder threatened to decrease portal venous flow to the liver, the bladder was punctured with a small (3-5 mm) incision and allowed to deflate. This procedure re-established normal blood flow to the liver.

Unless otherwise indicated, exactly 0.10 ml of a prepared injection solution was introduced into the hepatic portal vein rapidly (injection time < 5 s) through a 28-guage hypodermic needle and glass syringe. At the desired time after injection, the liver was quickly excised and placed in a Sorvall Omnimixer cannister with 10.0 ml cold homogenizing medium, Medium A (0.25 M Tris, pH 7.4, 0.025 M KCl, 0.01 M $MgCl_2$). The liver was weighed and then rapidly homogenized for about 2 min at half speed. Approximately 20 s elapsed

from the time of liver excision to the beginning of homogenization. Homogenates were kept on ice until all fish in a given experiment, usually 8, had been finished. Previous in vitro studies on toadfish liver homogenates indicated that protein synthesis ceases under these conditions (Haschemeyer, 1969b).

Immediately after liver excision, blood was collected in a heparinized syringe from the cut end of the hepatic veins leading to the sinus venosus and the heart. External pressure was applied to the heart region to facilitate drainage. After centrifugation the plasma was treated with cold10% tricholoacetic acid (TCA).

2. Injection solutions.

The standard injection solution used to measure amino acid transport kinetics, as well as polypeptide chain assembly time, contained 2 μCi of L-[U-^{14}C]-leucine, 4 μCi of D-[1-^{3}H(N)]-mannitol or 4 μCi of L-[3-^{3}H(N)]-phenylalanine (New England Nuclear), unlabeled L-amino acids (Sigma Chemical Company) as indicated, and a balanced-salt solution, based on the methods of Hoare (1972a), to yield a final osmolarity of about 0.3 and pH 7.4. The final concentrations of the balanced-salt solution were 0.13 M NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, and 25 mM NaH_2PO_4. Standard kinetic experiments were

carried out at concentration of 0.1 mM L-leucine and 0.3 mM D-mannitol, which served as an extracellular space marker. To prevent bleeding, the needle was kept in the vein throughout the incubation period, ranging from 3 s to 8 min.

The syringe was weighed after filling and again after the injection bolus was delivered to assure that a 0,1 ml volume of solution was administered into the hepatic portal vein. Deviations from the standard injection volume were normalized to a 0,10 ml volume.

3. Analytical methods.

Liver homogenates were fractionated in the following manner: a 100 µl aliquot of the total liver homogenate (T fraction) was placed on a filter paper disc (#3 Watman 2,3 cm, Thomas) and allowed to dry. The total protein and charge aminoacyl-tRNA on the discs was precipitated by washing the filters in cold 10% TCA, approximately 10 ml wash fluid/filter, according to the procedure of Mans and Novelli (1961). After a second 10% TCA wash, the filters were washed twice in 3% $HClO_4$, twice in 95% ethanol, and twice in anhydrous ether. Duplicate filters were also washed in 10% TCA heated to 90° to eliminate aminoacyl-tRNA (Haschemeyer, 1969a) so that an analysis could be made of the total protein fraction and total protein plus aminoacyl-tRNA. A 0.5 ml aliquot

of liver homogenate was precipitated with cold 10% TCA. Supernatant fluid was analyzed to determine total recovery of liver free radioactivity as a $^{14}C/^{3}H$ ratio.

From the supernatants of precipitated plasma and precipitated liver homogenates, 100 µl aliquots were taken and counted directly in 10 ml Aquasol (New England Nuclear) scintillation fluid with a Packard Tri-carb Liquid Scintillation Spectrometer or a Beckman Liquid Scintillation Counter. Activity in these fractions represents total free radioactivity in the plasma (Pl FR) emerging from the liver and total free radioactivity within the liver (L FR).

Filters were counted on the same machines in 5 ml toluene scintillation fluid containing 200 mg/l POPOP and 4 g/l POP. Efficiencies for the isotope on filter were determined by first analyzing whole homogenate aliquots in a universal solubilizer, Protosol (New England Nuclear), and determining the efficiency in Protosol by adding a known quantity of standard isotope preparation (New England Nuclear). The efficiency of the homogenate aliquot on filter paper could then be directly calculated. Free radioactivity efficiencies and channel overlaps for the two ^{14}C and ^{3}H isotopes were also determined by using an internal standard. Counter settings

on the Packard Tri-Carb Liquid Scintillation Spectrometer were: ^{14}C windows from 50 to 1000, gain at 15%; ^{3}H windows from 30-250, gain at 50%.

D. Measurement of protein synthesis.

To determine the fraction of transported amino acid that enters into protein synthesis, as well as to monitor the normal metabolic activity of the liver, measurements were made on the rate of polypeptide chain translation, or elongation rate. Polypeptide chain elongation times were determined according to the method developed by Haschemeyer (1969a) and Mathews, Oronsky, and Haschemeyer (1973). Sodium deoxycholate (DOC) (Fisher) is added to a 5,0 ml aliquot of total homogenate to give a final concentration of 1.0% in order to free ribosomes and completed proteins from membrane structures. This mixture was centrifuged for 4 h at 100,000 x g in a Spinco model L preprative ultracentrifuge at 4^{o} to sediment glycogen, ribosomes, and microsomal fractions. The supernatant was drawn off and aliquots were used for determining the total soluble protein (S fraction), representing completed polypeptide chains, by a filter counting method similar to that used for the total protein fraction (T fraction), representing complete and incomplete polypeptide chains. The ratio of S/T is used to calculate

polypeptide chain assembly time, as first described by Haschemeyer (1969a).

E. Chemical analysis of free amino acid concentrations.

Aliquots of liver homogenate and plasma were precipitated with 55% sulfosalicylic acid (Fisher) to give a final concentration of 5%. An internal amino acid standard (1.0 mM norleucine) was added to each sample, and each sample was then dried for analysis on a Beckman automatic amino acid analyzer.

F. Analysis of amino acid movements.

1. Theoretical background.

At any time t after portal injection of a radioactive L-amino acid (A), the recovery in the excised liver will be the sum of the activity present in the intracellular (i) and extracellular (e) spaces:

$$A_{liver} = A_i^{total} + A_e \qquad (1)$$

The intracellular contribution may be further subdivided as:

$$A_i^{total} = A_i^{free} + A_i^{protein} + A_i^{aa\text{-}tRNA} \qquad (2)$$

The quantities $A_i^{protein}$, $A_i^{aa\text{-}tRNA}$ and the sum $(A_i^{free} + A_e)$, which represents total free radioactivity in the liver, may be determined directly by the use of hot and cold acid extraction procedures described earlier. The sum of these quantities thus yields A_{liver}. The value of A_e must be determined by use of an extracellular space marker and then subtracted from the total liver free radioactivity to obtain the desired parameter, A_i^{free}, which is the fraction of the injected isotope appearing in the hepatic intracellular spce as free amino acid.

The recovery of the marker substance B in the liver will represent that amount present in the extracellular space B_e plus any of the marker that has reached intracellular spaces by diffusion or other non-specific uptake processes, e.g. pinocytosis:

$$B_{liver} = B_e + B_i^{diffusion} \qquad (3)$$

In the present study markers were used which showed negligible accumulation in liver during the short time course of the experiments. The term, $B_i^{diffusion}$, may then be neglected without significantly affecting the final determination of the active transport of A. Upon neglecting the diffusion component, equation 3 becomes

$$B_{liver} = B_e \qquad (4)$$

Comparing equations 1 and 4, it is apparent that under circumstances of active accumulation of amino acid A by liver, the total recovery, A_{liver}, will exceed B_{liver} by an amount proportional to the extent of specific or active transport (above that associated with any non-specific processes.)

In order to eliminate the unknown, A_e, from equation 1, a calculation is made based on the measured value, $(A/B)_{plasma}$, obtained as the ratio of $^{14}C/^{3}H$ in the supernatant fluid from plasma draining from the liver. The term, $(A/B)_{plasma}$, is considered approximately equal to the term (A_e/B_e) in the extracellular space of the liver since it is known that there is no significant kinetic barrier between blood flowing through the liver and the extracellular space of Disse or the liver sinusoids (Goresky, 1970). The plasma ratio of amino acid activity to marker activity, as a measure of liver extracellular space ratio, leads to a direct calculation of A_e from experimental data:

$$A_e = (B_{liver})(A_e/B_e) \qquad (5)$$

Substituting $(A/B)_{plasma}$ for A_e/B_e gives

$$A_e = (B_{liver})(A/B)_{plasma} \qquad (6)$$

Once A_e has been calculated, it may be subtracted from A_{liver} to yield a value for A_i^{free} by means of equations 1 and 2. In summary,

$$A_i^{free} =$$

$$\left[A_{liver} - (A_i^{protein} + A_i^{aa\text{-}tRNA})\right] - (B_{liver})(A/B)_{plasma} \quad (7)$$

to give the fraction of injected dose that appears as free, intracellular amino acid. Control experiments indicated that blood cells trapped in the liver (toadfish hematocrit = 16%) do not make a significant contribution to the radioactivity recovered in the various fractions over the time course of these experiments.

2. Amino acid compartments.

Movement of the amino acid A was evaluated in terms of reversible monomolecular reactions:

$$A_{body} \underset{K_3}{\overset{K_5}{\rightleftharpoons}} A_e \underset{K_4}{\overset{K_1}{\rightleftharpoons}} A_i^{free} \xrightarrow{K_2} A_i^{protein} \quad (8)$$

The reaction equations represent the fraction of A in various compartments under consideration. From left to right these compartments are a) the fraction of A in the body excluding the liver, b) the fraction of A in the liver extracellular space, c) the fraction of A in

the liver intracellular free pool, and d) the fraction of A that has been incorporated into liver protein. The fraction of A that is bound to tRNA is small compared to total incorporation into protein (Haschemeyer and Persell, 1973) and is, therefore, included in the total protein fraction. No consideration was given to a hypothetical intracellular non-free, non-protein-bound pool.

Movements of the space marker, B, follow that of the amino acid for exchange between the extrahepatic body space and liver extracellular space:

$$B_{body} \underset{K_3}{\overset{K_5}{\rightleftharpoons}} B_e \qquad (9)$$

where B_e represents the fraction of B recovered in liver and attributed to extracellular and diffusion-accessible space. B_{body} is that fraction of the injected dose of B that is lost to the rest of the body.

The monomolecular equations reflect amino acid and space marker compartmentalization after a bolus injection. Measurement of mannitol (B) disappearance from liver yielded an average time for the passage of the bolus through the liver of 0.25 min, which compares well with the average time of 18 s for bolus transit through rat liver in vivo (Pardridge and Jefferson, 1975).

During the 0.25 min period for passage of the bolus through the liver, all of A and B are within the liver, and

K_3 and K_5 from equation 9 equal zero. After this time period, however, K_3 and K_5 have positive values which must be determined by an analysis of equation 9. Since this analysis is normalized to a dose of 1.0 μCi, and compartment and kinetic values are given as fraction of dose, initial values of A_e and B_e are unity. All other compartments are given initial values of zero. No term for protein turnover is included because the time course of these experiments is short relative to that required for one complete round of polypeptide chain synthesis, measured earlier by Haschemeyer and Persell (1973) to be 4.0 ± 0.4 min for toadfish acclimated to and measured at 22° and 11.5 ± 1.5 min for toadfish acclimated to and measured at 11°. Catabolic oxidation of leucine by the liver is also neglected, since liver activity of specific leucine or general branched-chain amino acid deamination enzymes is very low relative to other tissues. A discussion of liver leucine metabolism is included in Appendix I.

3. Computer analysis and equations.

The movement between compartments for amino acid A, described by the reaction scheme of equation 8, and the movement between the body and liver extracellular space of the marker B, described by equation 9, can be

written as a set of simultaneous first-order differential equations:

for the amino acid A,

$$dA_{body}/dt = K_3A_e - K_5A_{body} \quad (10)$$

$$dA_e/dt = K_5A_{body} + K_4A_i^{free} - (K_1 + K_3)A_e \quad (11)$$

$$dA_i^{free}/dt = K_1A_e - (K_2 + K_4)A_i^{free} \quad (12)$$

$$dA_i^{protein}/dt = K_2A_i^{protein} \quad (13)$$

for the marker B,

$$dB_{body}/dt = K_3B_e - K_5B_{body} \quad (14)$$

For a time course sufficiently long to make the recycling of labeled amino acid due to proteolysis significant, equation 8 and equations 12 and 13 would include an additional term, $K_{11}A_i^{protein}$, to reflect the contribution to the free pool from protein turnover. For these rapid time studies, the term is not included.

Direct solution of equations 10 - 14 can be accomplished by the simple Euler method of numerical integration in which a single evaluation of the derivative of all the

variables is made. These derivatives are multiplied by a small step in time to determine the increment to add to the previously-existing value of each variable. The new value of each variable is then calculated, and the process is iterated to some pre-determined end point. This approach to solving a set of differential equations was suggested by the work of Moore and Ramon (1974) who used the same procedure to solve equations describing ion fluxes across excitable membranes. Preliminary work for this thesis in solving the equations by computer program was carried out in the FOCAL language for graphical output using a PDP8/m computer with Tektronix 4010-1 cathode-ray graphics terminal (Persell, 1974). Subsequent work was written in FORTRAN-IV for use with an IBM 370 computer at the City University of New York. Programs written in FORTRAN-IV are included in Appendix II.

RESULTS

A. Time course of mannitol recovery in liver.

Averaged data for the recovery of D-mannitol in toadfish liver at 20-22° as a function of time after hepatic portal vein injection of a 0.1 ml bolus are presented in Figure 1. An analysis of Fig. 1 reveals an exponential loss of the mannitol marker from liver until equilibrium is reached at a level of 10% of the injected dose. If the equilibrium value of B_e is subtracted from the data, the resulting curve represents only the portion declining exponentially and may be solved from the equation

$$d(B_e - B_e^{equil.})/dt = B_e^{initial} \times \exp^{-K_3 t} \qquad (15)$$

Since the values of all compartments are normalized to the fraction of dose, the initial value of B_e at $t = 0$ is set at unity. A solution of equation 15 for K_3 may be obtained by plotting $\ln(B_e - B_e^{equil.})$ vs. t, with the slope equal to K_3, as shown in Figure 2. The best fit to equation 15 was found for the following values of the constants: $K_3 = K_5 = 0.0$ for the period the bolus remains within the liver, $0 \leq t \leq 0.25$ min; $K_3 = 0.9$ min^{-1} and $K_5 = 0.1$ min^{-1} for subsequent values of t up to 6 min where recovery roughly equilibrates.

Previous studies by Haschemeyer and Persell (1973)

Figure 1. Recovery of D-[^{3}H]-mannitol in toadfish liver as a function of time after hepatic portal vein injection. B_e, presented as standard error bars, is fraction of injected dose retained by liver. Injection time = 5-10 s; t = 0 represents end of injection; T = 20-22°.

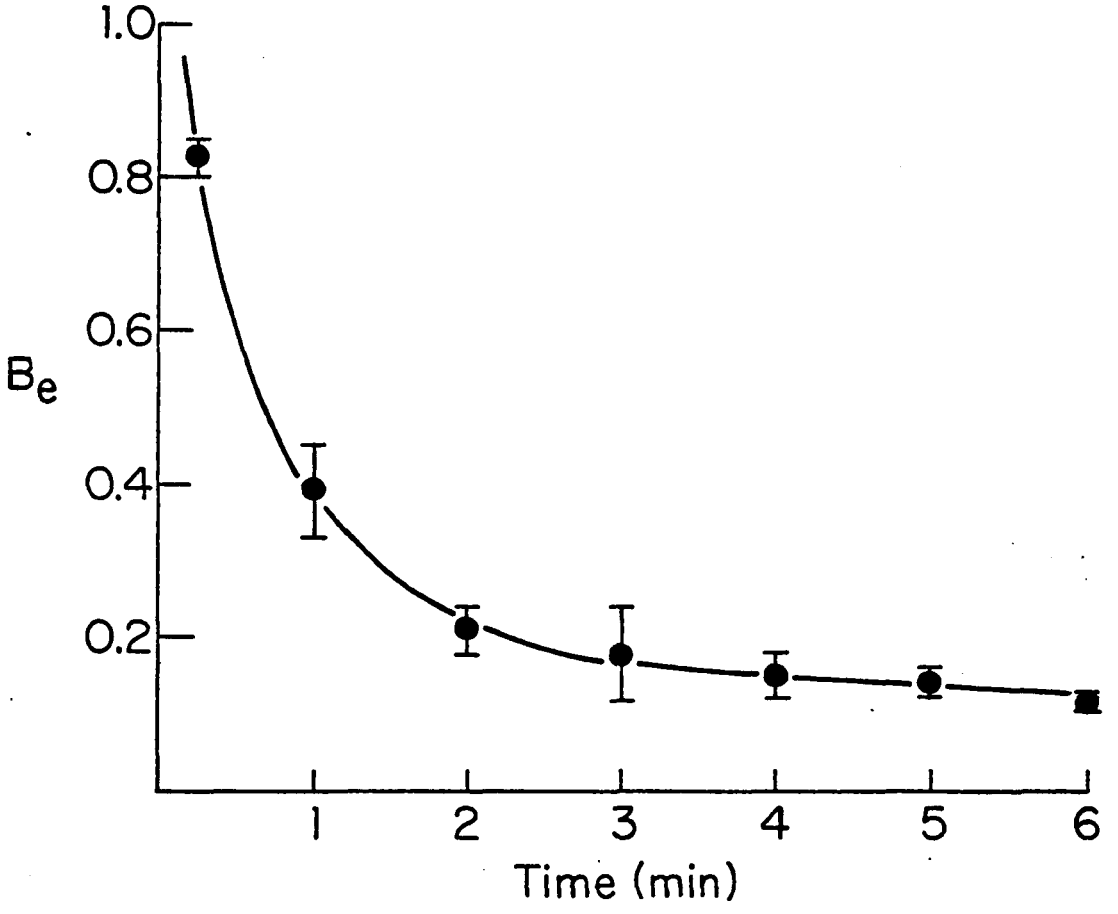
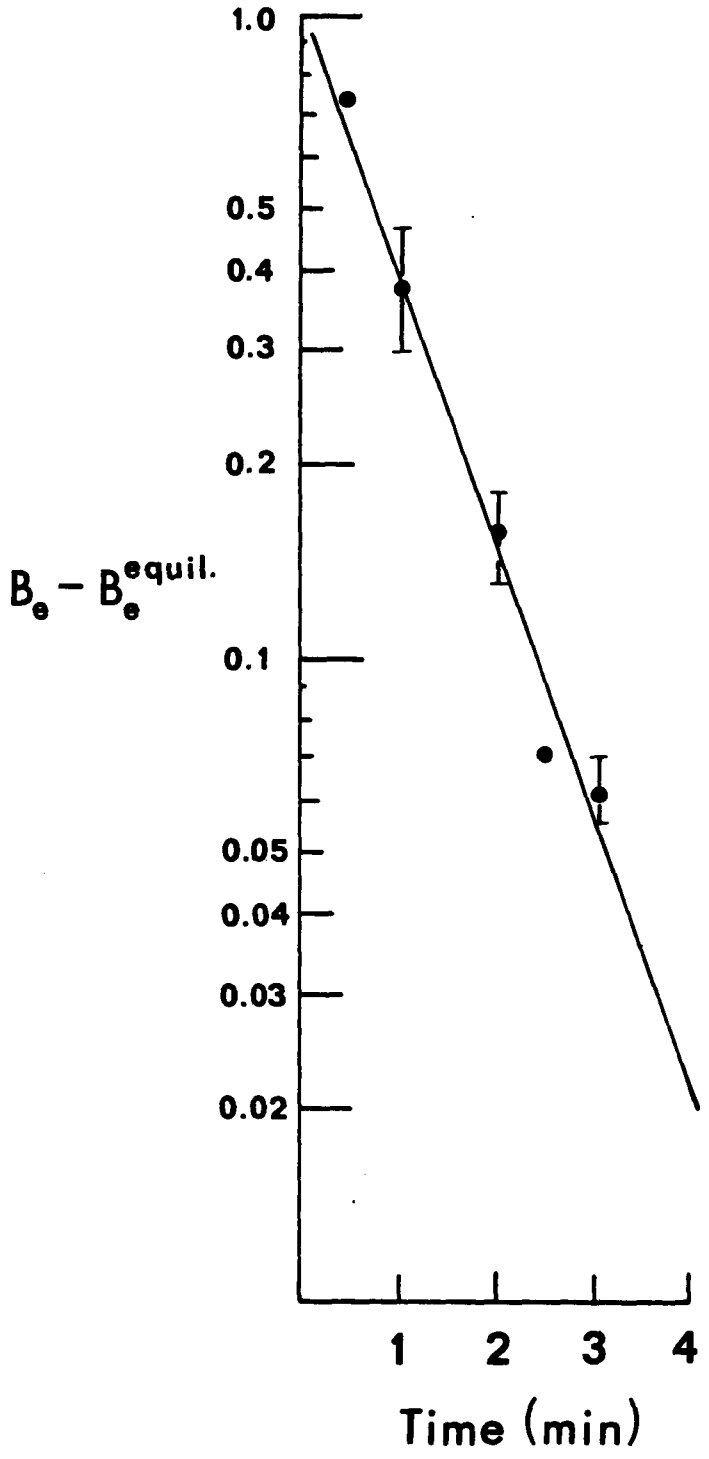

1.0
0.8
0.6
0.4
0.2
B_e
1
2
3
4
5
6
Time (min)

Figure 2. Recovery of mannitol extracellular space marker presented in Fig. 1 plotted according to equation 15, with slope equal to K_3 in equation 9. From this graph $K_3 = 0,9\ min^{-1}$.


1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
0.03
0.02
$B_e - B_e^{equil.}$
1
2
3
4
Time (min)

suggested that D-mannitol would serve as a suitable marker for extracellular space, which is rapidly occupied by small molecules (Goresky, 1970, 1973a), and for intracellular space accessible by diffusion. Although D-mannitol occupies the liver intracellular space completely within 1-3 h after injection, its half-time for uptake ($t_{\frac{1}{2}}$ = 20 min) is slow compared with the time course of the present experiments. These results confirm the earlier work of Cooperstein and Lazarow (1969), who found that mannitol was taken up by toadfish liver after incubation times of 1 h following injection into the gill arch. They concluded that mannitol could be used as an extracellular space marker for toadfish liver only for short-time experiments. In addition, the authors reported that inulin was less reliable as a marker for extracellular space because of the probability of contamination from inulin breakdown products such as fructose. Extrapolation to zero-time reveals the initial D-mannitol space or extracellular space of 0.31 ml/g wet liver compared to a water content of 0.78 ml/g wet liver (Haschemeyer and Persell, 1973). Table 1 shows the values of extracellular space obtained for inulin, D-leucine, and cycloleucine, as well as for D-mannitol. Results for D-leucine demonstrate the stereospecificity of leucine transport in toadfish liver in vivo. Based on a liver

TABLE 1. Determination of tissue spaces accessible to various substances in toadfish liver.

Substance	Time after injection	Space ml/g wet weight
Water	to dryness	0.78 ± 0.02 (8)
D-mannitol, D-leucine, cycloleucine	1 - 3 h	0.72 ± 0.03 (4)
D-mannitol	zero (extrapolated)	0.31
Inulin	zero (extrapolated)	0.31
Inulin	10 - 30 min	0.30 ± 0.08 (5)

Values include standard error and number of observations. Determination of zero-time space was based on a plot of log (1 - marker space/H_2O space) as a function of time (Haschemeyer and Persell, 1973).

density of 1.05 g/ml (Claret and Mazet, 1972), the water content is equivalent to a water space of 74% in toadfish liver, compared with reported values ranging from 71% in rat liver slices (Tews and Harper, 1969) to 77% in rat liver in vivo (Neame, 1962). The extracellular space is 29% in toadfish liver. In their earlier study, Cooperstein and Lazarow (1969) found a liver extracellular space of only 22% in toadfish, and an extracellular space of 24% has been measured for rat liver in vivo (Shanker and Hogben, 1961).

The behavior of mannitol in toadfish liver contrasts strinkingly with findings in mammalian liver. Pardridge and Jefferson (1975) report that mannitol uptake is 80% that of freely-diffusible ^{3}HOH during a single 18-s circulation period. Experiments in rat liver similar to those reported here for toadfish also show a very rapid uptake of D-mannitol into intracellular space (Mathews and Haschemeyer, unpublished observations). Comparable results have been reported for experiments in dog liver in vivo (Bravo and Yudilevich, 1971).

B. Time course of leucine uptake.

The levels of free intracellular radioactive L-leucine (A_i^{free}), determined by means of equations 1 and 2, reach a maximum level at about 1 min after injection and subsequently decline as shown in Figure 3.

Superimposed on the data are theoretical curves, based on the solutions by numerical integration of equations 10-14, which reflect the effect of different sets of kinetic parameters on the theoretical recovery of A_i^{free}. Kinetic parameters were chosen to meet three requirements, discussed in detail in Part 3 of this section (page 64): that K_1 (influx) be close to 2.7 min^{-1}, that K_2 reflect measured incorporation of ^{14}C-leucine into protein (Fig. 4), and that a new parameter, R, which is defined by equation 20 on page 65, reflect the fractional contributions of concentrative, active transport to total ^{14}C-leucine uptake. (If ^{14}C-leucine uptake were accomplished exclusively by concentrative, active transport mechanisms, R would be equal to 1.0, and the efflux kinetic parameter, K_4, would equal 0.0 from equation 20. At the other extreme, R = 0.0, ^{14}C-leucine uptake is exclusively by non-concentrative or passive exchange mechanisms, and the ratio of influx to efflux ,K_1/K_4, would be equal to the volume ratio of liver intracellular and extracellular spaces.)

In contrast to the peak and decline of levels of free, intracellular ^{14}C-leucine, the proportion of A_i^{total} recovered in protein, which is shown in

Figure 3. Recovery of L-[^{14}C]-leucine, as fraction of administered dose, in intracellular free pool of liver, determined by means of equations 1 and 2. Data are presented as standard error bars; total number of animals is 48. Theoretical curves for A_i^{free}, based on equation 8, are shown for the following sets of parameters: (— —) R = 1.0, K_2 = 0.25; (••••••) R = 0.75, K_2 = 0.23; (———) R = 0.5, K_2 = 0.21; (•——•) R = 0.25, K_2 = 0.21; (----) R = 0.0, K_2 = 0.18.. Other rate constants are: K_1 = 2.7, K_3 = 0.9, and K_5 = 0.1, all in units of min^{-1}. K_4 is calculated according to equation 20.

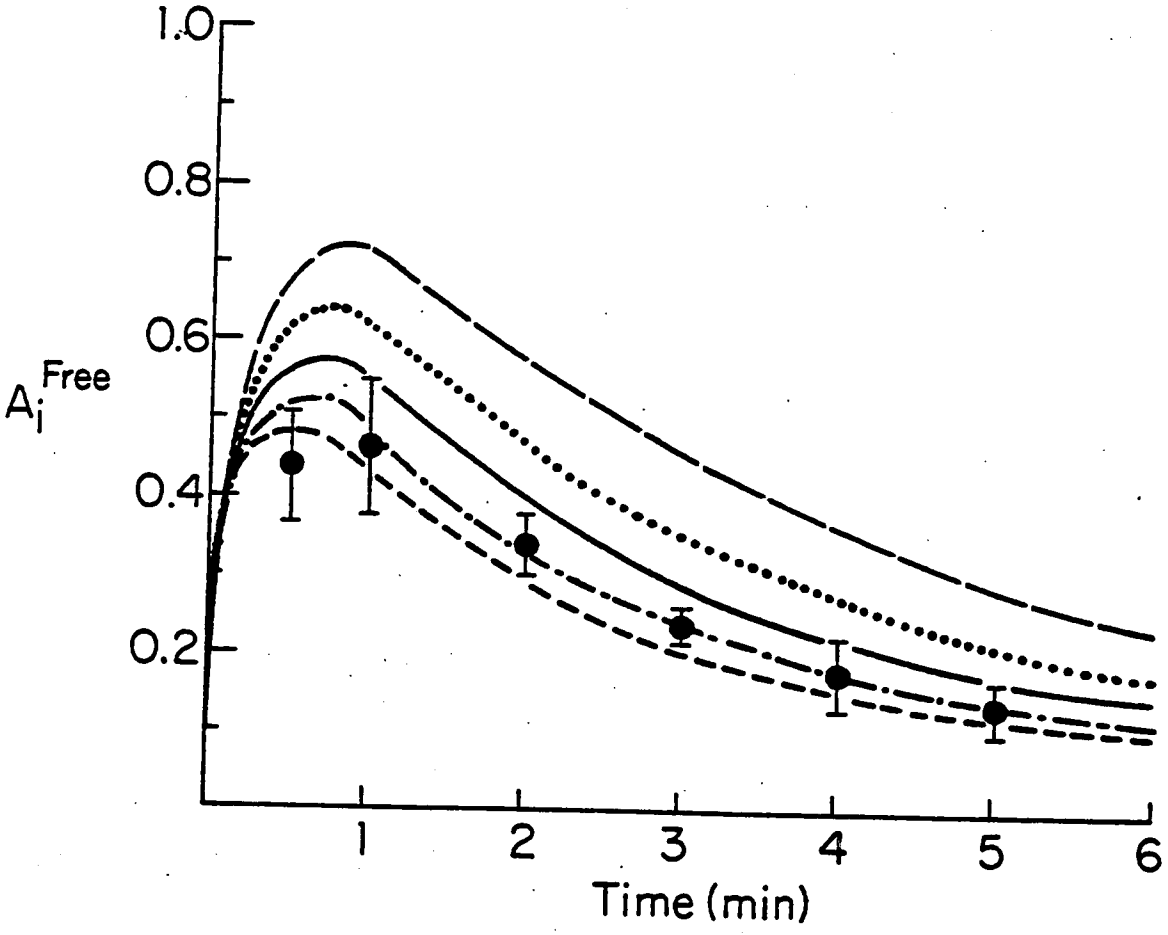

1.0
0.8
0.6
0.4
0.2
A_i^{Free}
1
2
3
4
5
6
Time (min)

Figure 4, continues to increase during the same period. Although the protein synthetic rate as indicated by average polypeptide chain assembly time (4.8 min for the fish in this series acclimated to and measured at 21° as shown in Figure 5) is relatively constant among different individual experimental animals, the fractional rate of leucine incorporation also depends on the leucine pool specific activity. Fig. 4 data yields an initial estimate for K_2 in equation 8 of 0.20 min^{-1} in this experimental series; however, further analysis requires estimation of K_1 and K_4 in equation 8.

1. Evaluation of K_1/K_4 for simple leucine exchange.

If the observed uptake process is assumed to represent an exchange of radioactive leucine between intracellular and extracellular compartments of the same concentration (i.e. no concentrative uptake occurs), then the theoretical value of K_1/K_4 can be calculated from the ratio of the volumes associated with the two compartments. Since A_i^{free} and A_e are referred to the whole liver, concentration equilibrium is achieved when $A_i^{free}/V_i = A_e/V_e$ where V_i and V_e are the volumes of intracellular and extracellular space, respectively. Thus, $K_1/K_4 = V_i/V_e$ for an exchange diffusion of leucine that does not result in any intracellular

Figure 4. Incorporation of L-[^{14}C]leucine into liver protein, presented as ratio of fraction of dose recovered in protein to total intracellular radioactivity ($A_i^{total} = A_i^{free} + A_i^{protein}$). Theoretical curves are shown for following sets of rate constants: (— —) R = 1.0, K_2 = 0.25; (———) R = 0.5, K_2 = 0.21; (-----) R = 0.0, K_2 = 0.18, all in units min^{-1}.

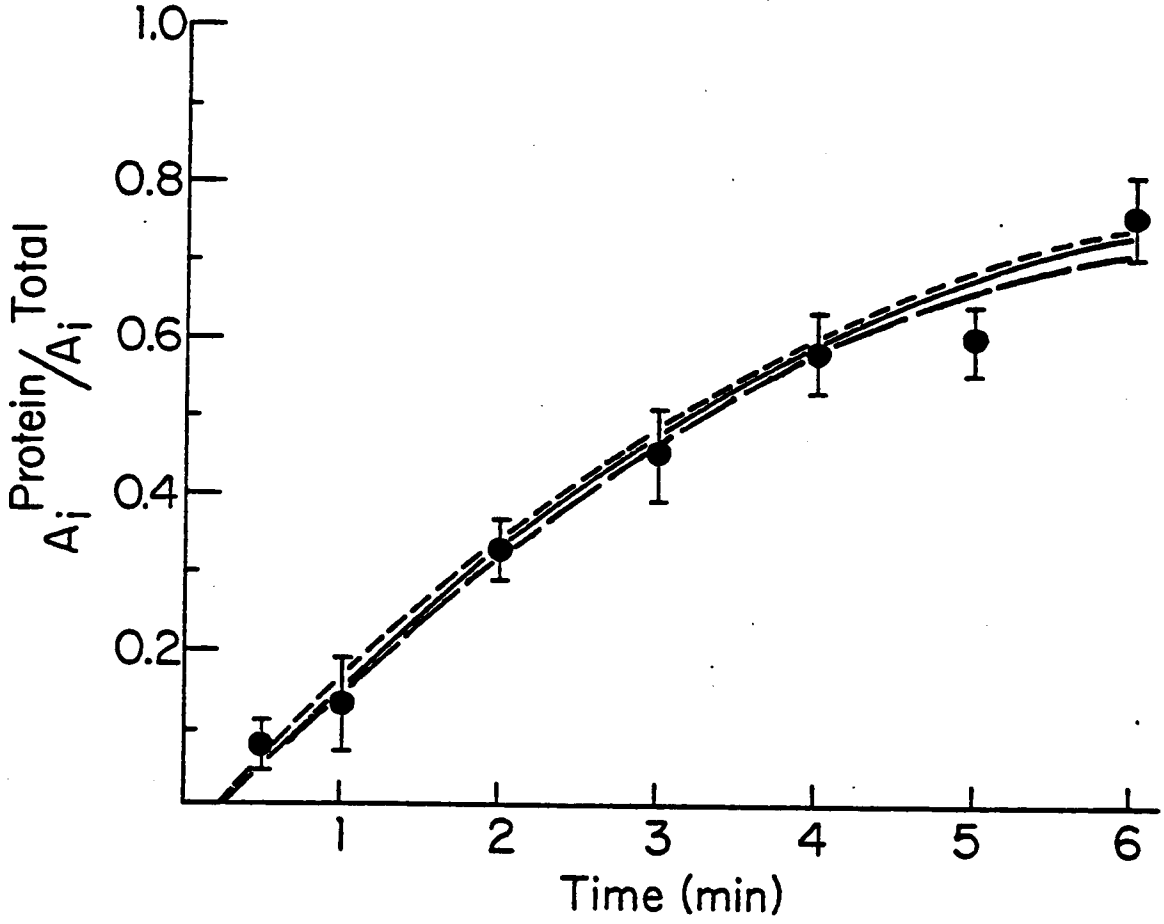
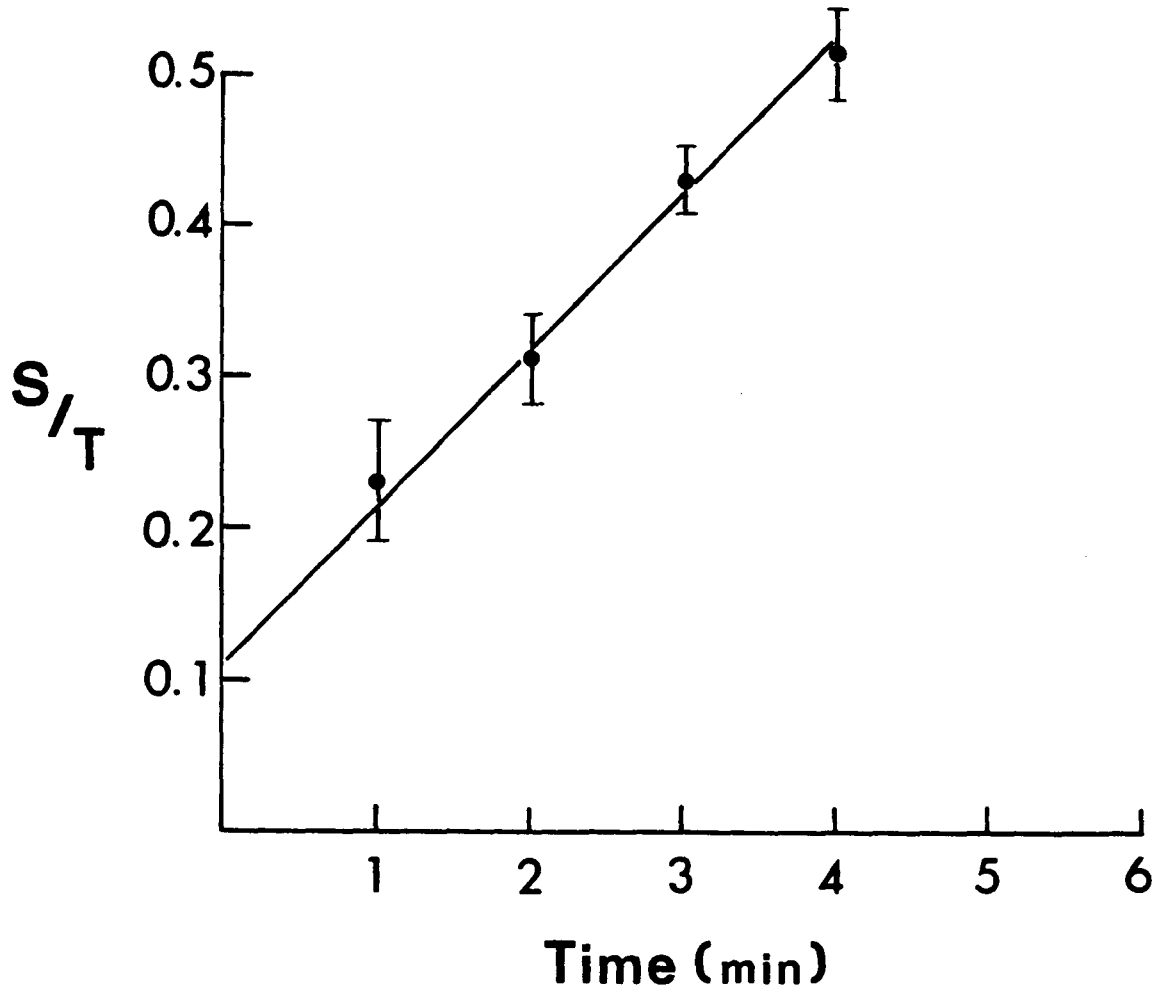

1.0
0.8
0.6
0.4
0.2
Protein A_i / Total A_i
1
2
3
4
5
6
Time (min)

Figure 5. Determination of polypeptide chain assembly time in toadfish liver. For times less than one round of polypeptide chain synthesis, $S/T = (1/2t_c)t$, where S and T refer to the radioactivity incorporated into released, completed protein (100,000 x g supernatant) and into protein in 100 µl of total liver homogenate, respectively. Slope of line is $1/2t_c$ where t_c is the average time for polypeptide chain translation, as previously shown by Haschemeyer (1969a) and Mathews et al., (1973). Data are presented as standard error bars; total number of animals is 21, $T = 21^{o} \pm 1^{o}$. For this series $t_c = 4.8 \pm 0.3$ min.


0.5
0.4
0.3
0.2
0.1
S/T
1
2
3
4
5
6
Time (min)

concentration, assuming no compartmentalization of leucine within these spaces. On the basis of liver space determinations presented in Table 1, and neglecting the small contribution of trapped blood cells, one obtains

$$K_1/K_4 = (0.47 \text{ ml/g})/(0.31 \text{ ml/g}) = 1.5 \qquad (16)$$

2. Estimation of K_1/K_4 from liver and plasma leucine pools.

Although leucine pools vary widely among individual experiemental animals, particularly in relation to nutrition and time of year, liver concentratons in general slightly exceed those of plasma in untreated fish or those injected at low leucine doses as shown in Table 2. The distribution ratio of $[\text{Leu}]_{in}/[\text{Leu}]_{out}$ of 1.2 ± 0.03 is nearly identical to the value of 1.15 ± 0.21 found by McGivan et al. (1977) for isolated rat hepatocytes.

If leucine is freely exchangeable and if plasma leucine concentration is taken to apply to all liver extracellular space, intracellular free leucine may be calculated:

$$[\text{Leu}]_{in} = \frac{0.12 \text{ µmol/g} - [(0.31 \text{ ml/g})(0.10 \text{ µmol/ml})]}{0.47 \text{ ml intracell. } H_2O/\text{g}}$$

$$= 0.19 \text{ µmol/ml intracell. } H_2O \qquad (17)$$

TABLE 2. Uptake of L-[^{14}C]-leucine by liver in vivo.

L-leucine injected, μmol	Avg A_i^{total}	Avg $\frac{\% A_i^{protein}}{A_i^{total}}$	L-leucine Concentration	
			Liver, μmol/g	Plasma, μmol/ml
0.01	0.43 ± 0.05 (30)	18 ± 9 (30)	0.12 ± 0.04 (8)	0.10 ± 0.02 (4)
1.5	0.22 ± 0.05 (10)	6.0 ± 3.0 (10)	ND	ND
10.0	0.10 ± 0.04 (4)	2.0 ± 0.8 (5)	0.70 ± 0.10 (6)	1.65 ± 0.15 (4)

Values, shown for three concentrations of leucine injected into hepatic portal vein, are calculated as explained in the text for t = 2 min and include corresponding SD and number of samples. Analyses of plasma L-leucine concentrations are made from blood drawn from the heart at t = 2 min after injection without liver excision. T = 20-22°, ND, not determined.

The concentration ratio for intracellular and extracellular space together with the volume ratio permits a new estimate of K_1/K_4:

$$K_1/K_4 = (V_i/V_e)([\text{Leu}]_i/[\text{Leu}]_e) = \frac{1,5(0,19\ \mu\text{mol/ml})}{0,1\ \mu\text{mol/ml}}$$

$$= 2,8 \qquad (18)$$

Net loss of leucine through the synthesis and secretion of plasma proteins by liver has been shown by Haschemeyer (1973) to be small compared to K_1 and K_4 (20% of K_2 or approximately 0,04) and may therefore be neglected in this calculation,

3. Experimental determination of K_1 and K_4.

The earlier published work by Haschemeyer and Persell (1973), upon which this thesis was based, suggested that values for K_1 may range between 1,0 and 5,0 min^{-1} with the value of 2,7 min^{-1} most exactly fitting the graphical solution of the simple differential equation

$$dA_i/dt = K_1A_e - K_4A_i^{free} \qquad (19)$$

Once the value of K_1 was set at 2,7 min^{-1}, the value of K_4 could be calculated for various values of a new

parameter, R, defined by

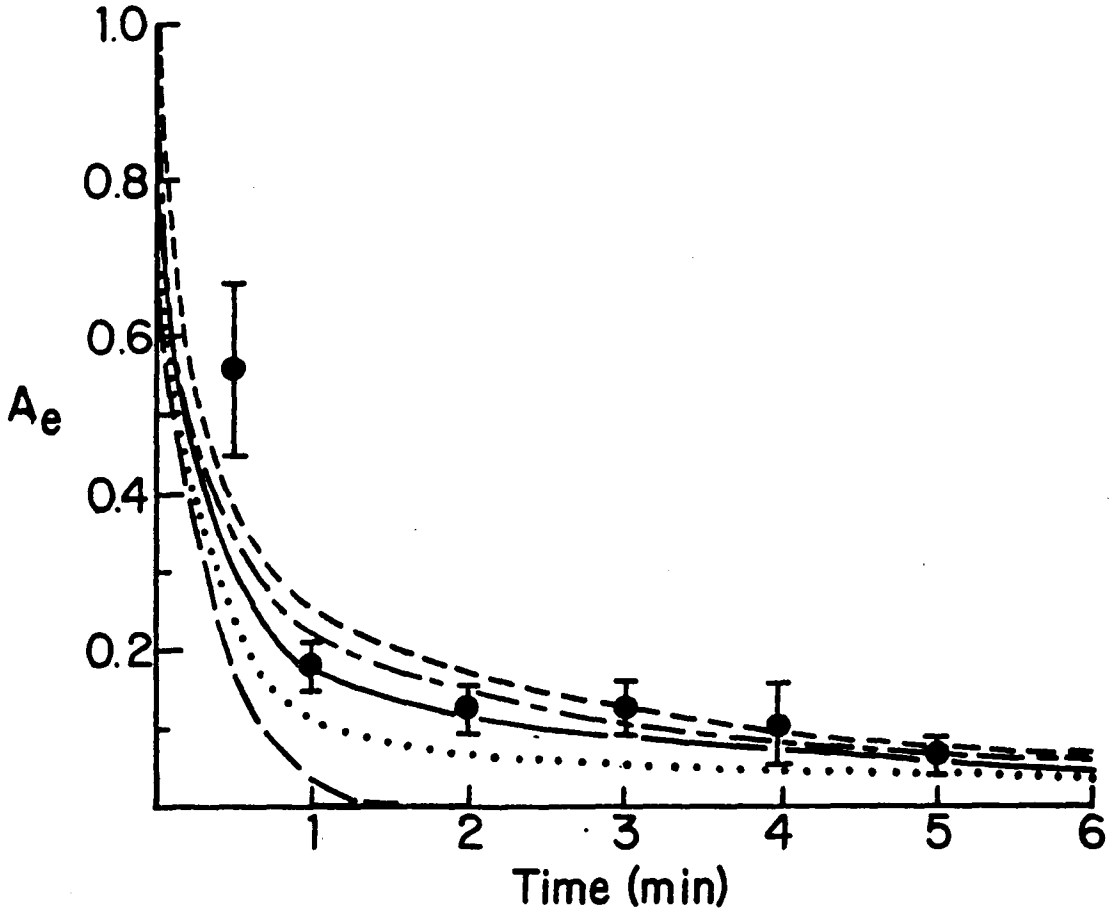
$$K_4 = K_1(1.0 - R)/1.5 \qquad (20)$$

where R represents the fraction of uptake that is concentrative above any contribution due only to exchange diffusion. For a case of pure exchange diffusion, R = 0.0, and R = 1.0 represents concentrative uptake only. Determination of the best fit of the rate constants of equation 8 to the experimental data, then, was carried out by selecting values of K_1 around 2.7 min^{-1} and calculating K_4 from equation 20.

The theoretical curves of Fig. 3 illustrate the effect of the variation of R on the predicted time course of A_i^{free} based on equation 8. No significant improvement in fit was seen throughout the range of $1.0 \leq K_1 \leq 5.0$ min^{-1}; therefore, the original graphical solution of equation 19, from which $K_1 = 2.7$ min^{-1}, was chosen. Values of K_3 and K_5 are from the solution of equation 15, taken from Fig. 2. In each calculation K_2 has been adjusted in order to maintain a fit of the incorporation data, $A_i^{protein}/A_i^{total}$ from Fig. 4. It can be seen that increasing values of R necessitates small increases in K_2 to maintain agreement with the data.

Figure 6 illustrates the theoretical curves for A_e

Figure 6. Recovery of L-[^{14}C]-leucine in liver extracellular space, as fraction of administered dose, calculated according to equation 6. Theoretical curves for A_e are shown based on parameters given in Fig. 3.

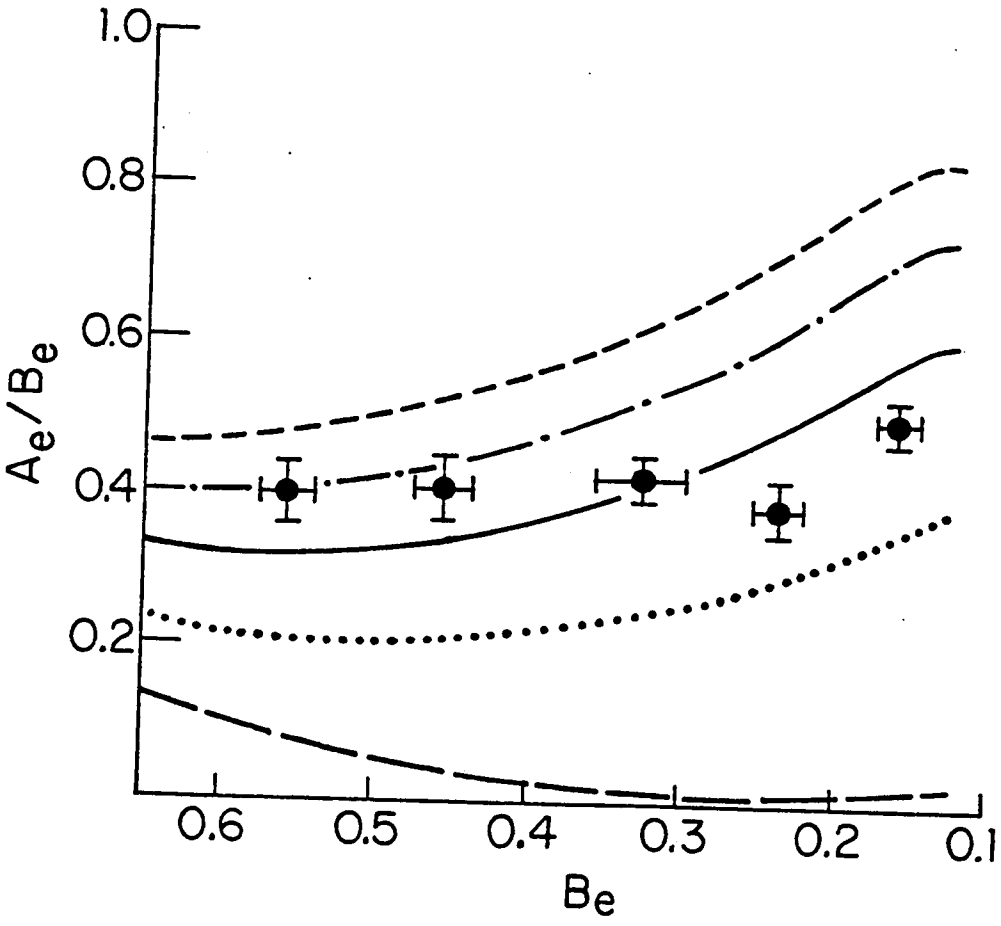

1.0
0.8
0.6
0.4
0.2
A_e
1
2
3
4
5
6
Time (min)

at various values of R, superimposed on the experimental A_e values calculated from equation 6.

A second, independent test for the determination of the K_1/K_4 ratio is provided by the experimental data for $(A/B)_{plasma}$ as a function of B_{liver}, obtained at times less than one full circulation period (2 min). As shown in Figure 7, the theoretical curves of A_e/B_e calculated according to the solutions of equations 10-14 are strongly influenced by the relative proportion of concentrative uptake (R = 1.0) vs. exchange diffusion (R = 0.0). The experimental data fall between the two extremes. These data show that during the time mannitol is washed out of the liver, the movement of L-leucine is such that a constant plasma ratio is maintained in blood leaving the liver. A value of R = 0.4 or K_1/K_4 = 2.5, very similar to the result suggested by the leucine pool data (equation 18), gives satisfactory agreement and falls within the range of R indicated by Figs. 3 and 6.

The value of 2.7 min^{-1} for K_1 represents a leucine influx rate of 0.6 µmol/min for the liver as a whole at normal plasma leucine concentration (0.1 mM) or 0.18 µmol/min per ml intracellular H_2O at 21°. With a liver protein concentration for toadfish of 115 ± 23 mg/g (Nielsen et al., 1977), the influx rate becomes 0.75 nmol/min/mg protein at 21°. These values are compared

Figure 7. Plasma ratio of L-[^{14}C]-leucine:D-[^{3}H]-mannitol (normalized) as a function of mannitol recovery in liver (B_e) obtained at times up to 2 min after injection. Six animals have been averaged for each point, and standared error bars are shown. Theoretical curves of A_e/B_e vs. B_e based on rate constants given in Fig. 3 are shown.


1.0
0.8
0.6
0.4
0.2
A_e/B_e
0.6
0.5
0.4
0.3
0.2
0.1
B_e

with published values for leucine influx rates in Table 3.

C. Saturation of leucine uptake.

Increasing the concentration of L-leucine in the injection pulse produces a continuous decline in the recovery of total radioactive leucine in the liver as shown in Figure 8. A concomitant increase in the ratio of leucine to mannitol, $(A/B)_{plasma}$, in blood leaving the hepatic circulation is observed (e.g., from 0.40 at the lowest dose to 0.65 at 1.5 μmole). A double reciprocal plot of uptake at t = 2 min as a function of leucine dose, treated as a one-way process only, yields a straight line corresponding to a Michaelis-Menten relationship, shown in Figure 9. From the axis intercept at infinite substrate concentration, maximal uptake is 1.0/1.7 or 0.6 μmole per 7-g liver; half-maximal uptake is achieved at a dose of 1.5 μmole. The exact concentration of substrate which reaches the hepatocyte, however, may only be estimated by taking into account the dilution of the injected bolus by both portal blood and hepatic arterial blood. Quantitative measurements of portal dilution have not been made, but observation of the toadfish portal vein during injection suggests a 50% dilution of the incoming bolus by portal blood alone. Dilution of portal blood by hepatic arterial blood has been estimated at 30% in several

TABLE 3. L-leucine influx rates.

Tissue	Temperature	Leucine Influx Rate		reference*
		μmol/min/ml H_2O	nmol/min/mg protein	
Toadfish liver in vivo	21°	0.18	0.75	
Ehrlich ascites cells	37°	0.33	—	1.
Isolated rat hepatocytes	37°	—	2.6	2.
Rat liver in vivo	37°	—	0.27	3.

Reported values for leucine influx at normal physiological extracellular concentration. In vivo values are an average for the whole organ. The value for rat liver in vivo has been calculated from the author's data on the basis of 190 mg protein/g liver reported previously for rat liver (Mathews, 1974).

*Refs. 1. Oxender and Christensen, 1963

2. McGivan et al., 1977

3. Pardridge, 1977

Figure 8. Dose dependency of total intracellular L-[^{14}C]-leucine recovery, in toadfish liver at t = 2 min, temperature = 20°. Data presented as fraction of injected radioactivity with standard error bars. Each point represents data from 4-10 animals.

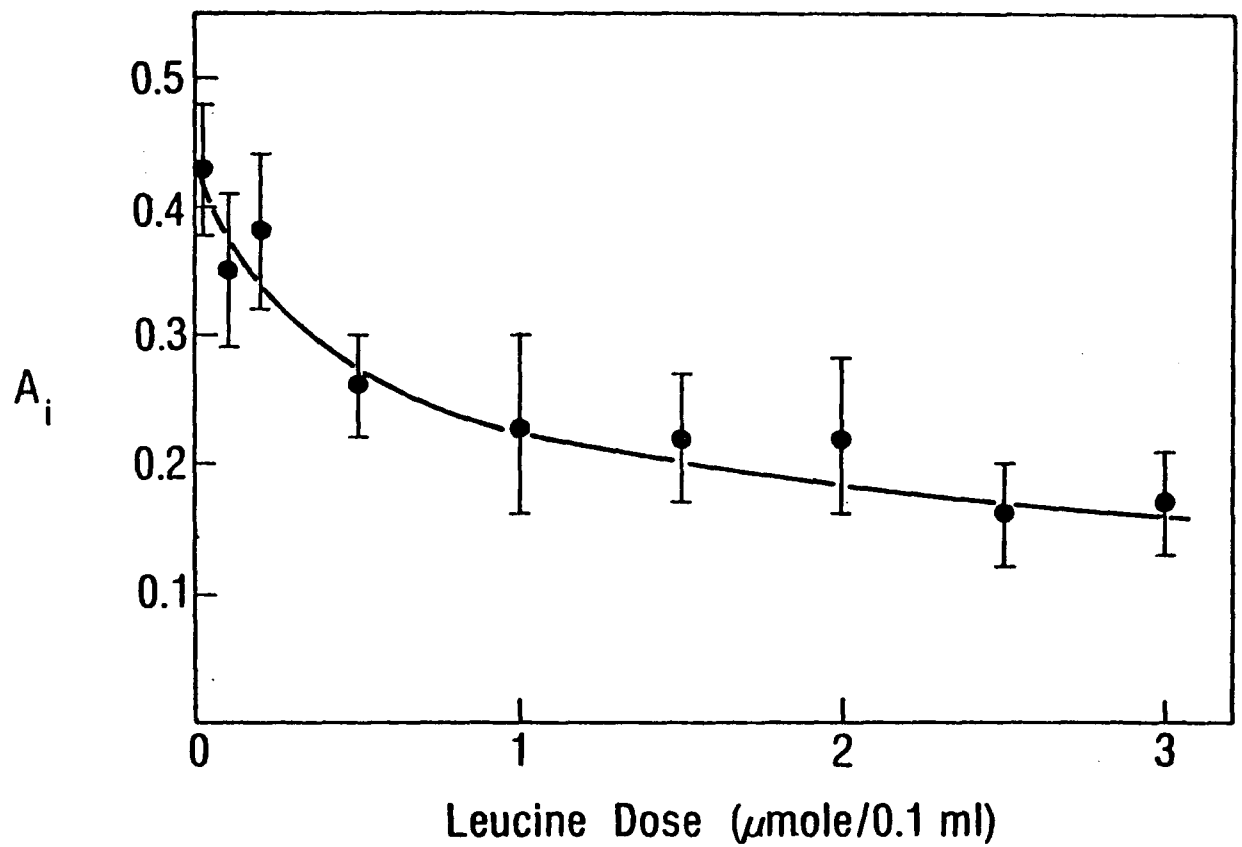
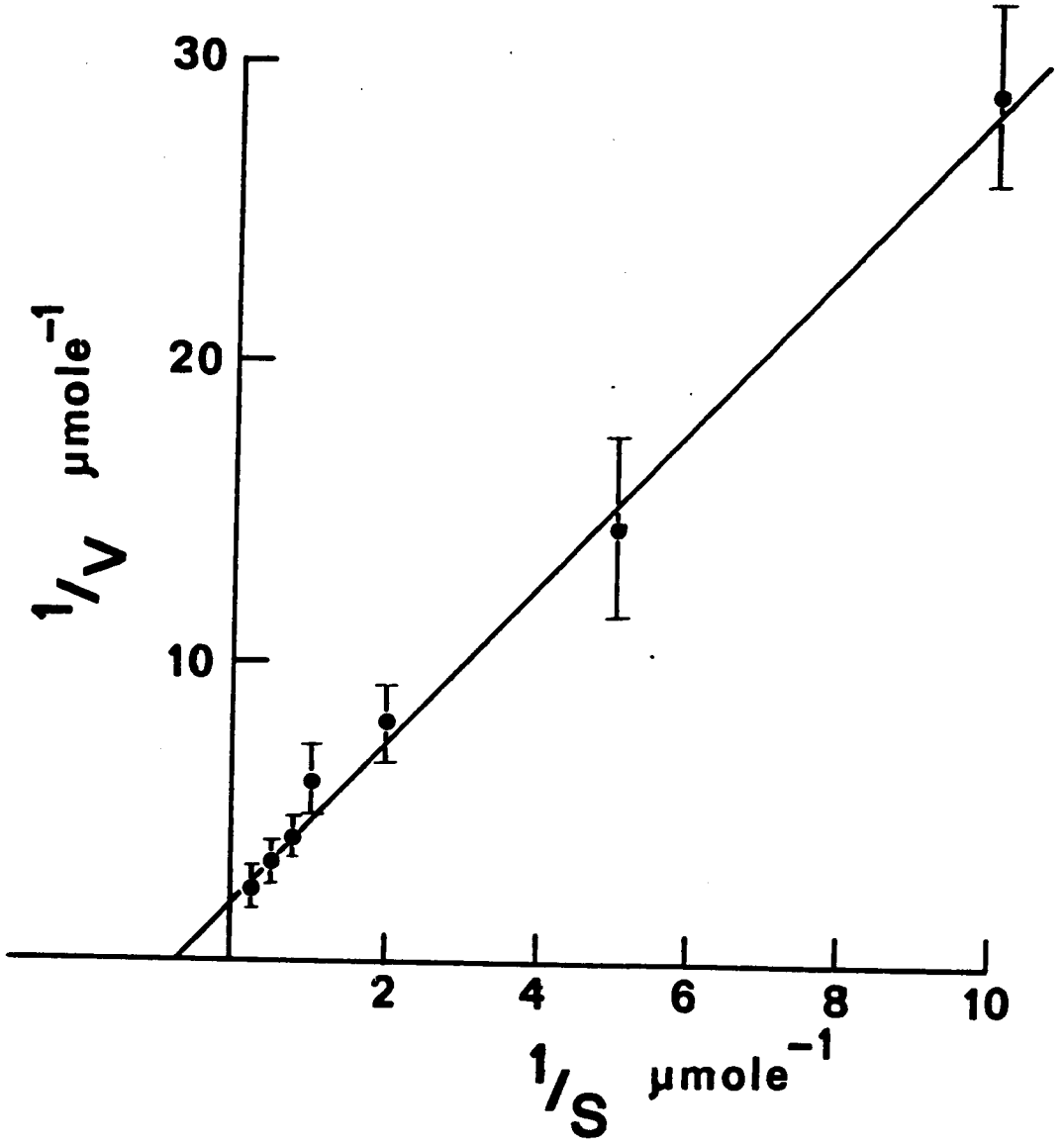

0.5
0.4
0.3
0.2
0.1
A_i
0
1
2
3
Leucine Dose (μmole/0.1 ml)

Figure 9. A double reciprocal plot of data from Fig. 8, showing uptake at t = 2 min as a function of leucine dose. At infinite concentration, maximal uptake is 0.6 μmole/7-g liver; half-maximal uptake is achieved at a dose of 1.5 μmole.


30
20
10
1/V μmole⁻¹
2
4
6
8
10
1/S μmole⁻¹

mammalian species (Greenway and Stark, 1971). Total dilution of the injected bolus, then, may be estimated to be 65%. Half-maximal uptake would, therefore, be reached at a substrate concentration at the hepatocyte membrane of 0.6 mM.

Chemical analysis of liver leucine levels at the highest injection dose confirmed the results of radioactivity measurement. Data for livers analyzed at t = 2 min after injection are summarized in Table 2. Utilizing equation 17 and the data from Table 2, one may calculate the intracellular free leucine concentration after a 10 μmole dose: at the high dose, total liver leucine concentration is 0.7 μmole/g and extracellular leucine concentration (equal to plasma leucine) is 1.65 μmole/ml. Total extracellular leucine is given by (1.65 μmole/ml) x (0.31 ml/g), or 0.5 μmole/g. Intracellular leucine, then, is (0.7 - 0.5) μmole/g, which, when divided by the intracellular water content of the liver (0.47 ml/g), yields an intracellular free leucine concentration of about 0.40 μmole/ml intracellular H_2O. At physiological doses of leucine, intracellular leucine concentration was calculated to be 0.19 μmole/ml H_2O (equation 17). When considered for a 7-g liver (7 x 0.47 ml H_2O/g), the difference in concentration between the high and physiological doses, (0.40 - 0.19) μmole/ml, yields a net uptake at the high dose of 0.7 μmole/liver or 7% of the 10 μmole dose. From

Table 2, uptake of radioactivity (A_i^{total}) is 10% of the dose.

Although full kinetic analysis of the saturation behavior is difficult, an estimate of maximal flux (V_{max}) can be made from the extrapolated uptake value (0.6 μmole per liver) in conjunction with the data at 0.1 mM and at the high (10 μmole) dose. If saturation did not occur, uptake at the latter dose, representing an average extracellular concentration of 4.9 mM (after dilution with 2.0 ml extracellular fluid and plasma at 0.1 mM), would be about 50 times that at the lower dose, or 30 μmole/min/7-g liver, based on the 0.1 mM flux rate calculated from K_1. Saturation, however, reduces uptake by the factor 0.06/0.43 compared to the 0.1 mM level, hence V_{max} (taken here as identical to uptake at the 10 μmole dose) is found to be 4.1 μmole/min/7-g liver or 1.2 μmole/min/ml intracellular H_2O. Calculation of K_m from the 0.1 mM uptake data by means of the equation

$$v \text{ (leucine flux)} = \frac{V_{max}\ [\text{Leu}]_{plasma}}{K_m + [\text{Leu}]_{plasma}} \qquad (21)$$

yields a value of 0.58 mM, similar to that estimated above for the effective extracellular concentration at the half-maximal dose of 1.5 μmole.

D. Incorporation into protein at high leucine concentrations.

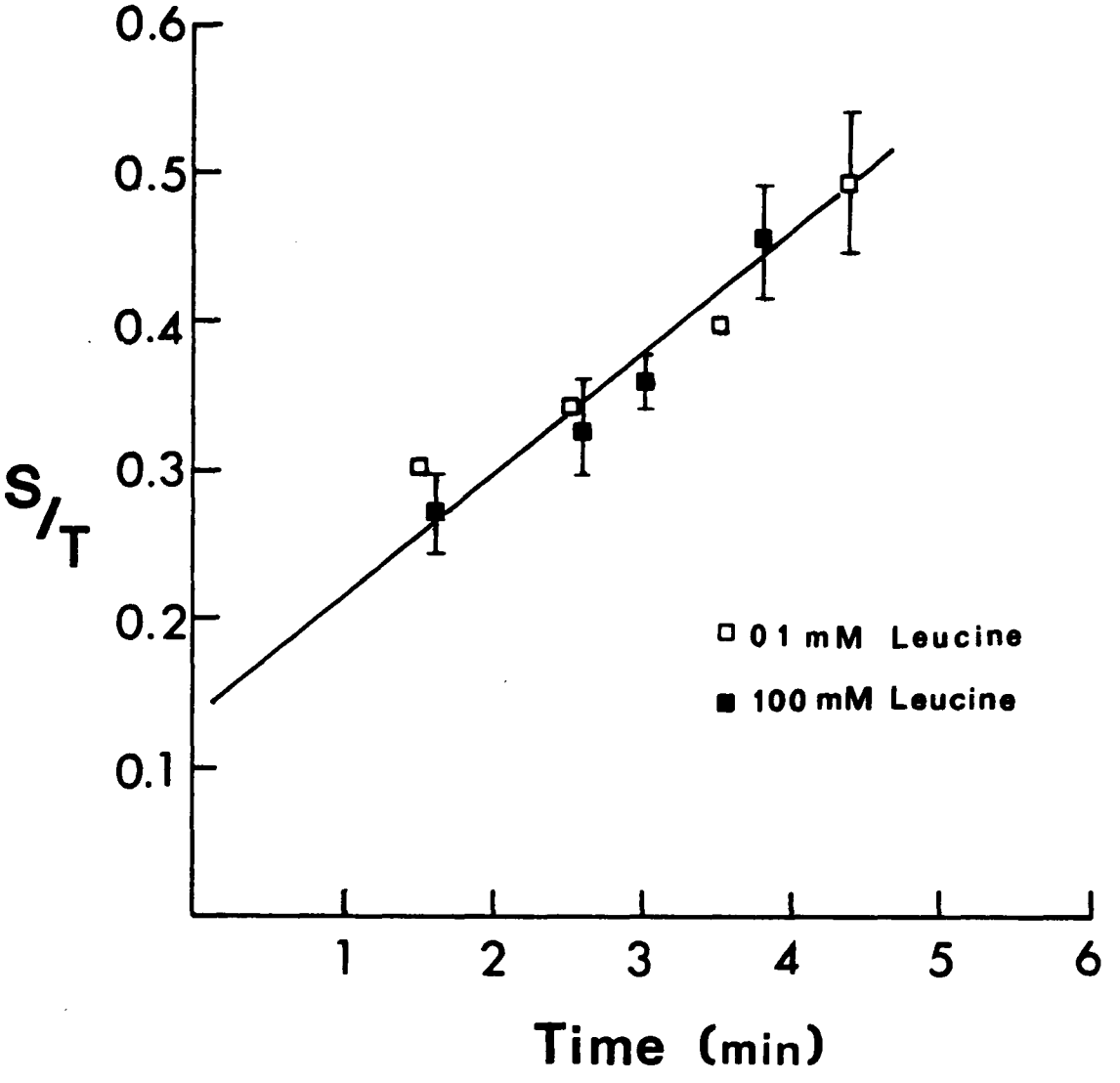
Examination of the proportion of intracellular radioactivity incorporated into protein at various leucine doses provides a picture of the fate of the injected amino acid relative to pre-existing intracellular pools. With hepatic portal vein injection, uptake would not be expected to be uniform throughout the entire tissue, particularly at low doses. Dissection of the lobes before analysis has indicated variations in uptake per unit weight of tissue of about 5-fold at the 0.01 μmole dose about 2-fold variation at the 1,5 μmole dose (Hudson, 1977). The uptake pattern generally follows the circulatory anatomy of the toadfish liver; the highest levels occur in the distal portions of the two larger lobes.

High levels of incorporation were obtained at tracer doses (Table 2). This observation indicates good tissue viability since it has been demonstrated that hepatic protein synthesis decreases with hepatic ATP depletion caused by metabolic disturbances (Hardwick, 1973). Previous studies of liver protein synthesis in toadfish by Haschemeyer (1969) have also shown that, although polypeptide chain assembly time is relatively invariant,

levels of amino acid incorporation into protein are strongly affected by oxygen supply to the animal. The latter observation has also been made in hypoxic rat (Surks and Berkowitz, 1971). This factor is important in transport studies, since an energy-requiring component of the transport process is likely to be similarly affected by hypoxia.

At increased leucine doses ^{14}C-L-leucine incorporation into protein relative to radioactivity taken up is reduced (Table 2). No effect, however, was observed on the incorporation of ^{3}H-L-phenylalanine administered simultaneously, nor on polypeptide chain assembly time measured with ^{14}C-leucine at doses up to 1.5 µmole (see Figure 10). Thus, protein synthesis was not affected within this time period by the leucine load. The change in incorporation rate, therefore, may be attributed to reduced specific radioactivity resulting from leucine uptake and consequent expansion of the intracellular pool. Compared to the results for the tracer-level leucine dose, the data for $A_i^{protein}/A_i^{total}$ in Table 2 show a 3-fold and 9-fold effect of pool dilution at the 1.5 µmole and 10 µmole doses, respectively. Assuming the full volume of the liver is involved in the uptake process, one may calculate the uptake of leucine that is required to achieve these pool dilutions. The normal leucine total for the

Figure 10. Determination of toadfish liver polypeptide chain assembly time in the presence of 0,1 mM physiological concentration of L-leucine (□) and 100 mM L-leucine load (■). Incorporation of L-[^{3}H]-phenylalanine into released, completed polypeptide chains (S) and into protein in total liver homogenate (T) is measured as described in Fig. 5. Data are presented as standard error bars; total number of animals is 18. T = 22° ± 1°. For this series, t_c = 5,1 ± 0,5 min.


0.6
0.5
0.4
0.3
0.2
0.1
S/T
0 1 mM Leucine
100 mM Leucine
1
2
3
4
5
6
Time (min)

entire 7-g liver is given by the intracellular concentration, 0.19 μmole/ml, multiplied by the total intracellular water space, 3.3 ml. (Both values are from equation 17.) The result is 0.63 μmole leucine. A 3-fold and a 9-fold dilution of the leucine pool would be achieved, respectively, by an additional uptake over normal liver leucine of 1.3 (total 1.9 μmole) and 5.0 (total 5.6) μmoles of leucine for the entire liver. The data for A_i^{total} from Table 2, however, indicate that net uptake cannot be more than 0.33 (22% of dose) and 1.0 (10% of dose) μmole at the two doses. The assumption, then, that the full liver volume is occupied is probably incorrect. An alternative explanation is that the uptake values determined from A_i^{total} reflect the actual occupied volume multiplied by the change in leucine concentration calculated from the dilution of the pool for protein synthesis. By dividing the uptake value by the change in concentration, one may obtain the actual liver volume occupied by the incoming amino acid dose. At the 1.5 μmole dose, the volume occupied is 0.87 ml or 26% of liver intracellular space, and at the 10 μmole dose, the volume occupied is 0.67 ml or 20% of the intracellular space.

E. Competition experiments.

Table 4 presents the effect of various L-amino acids at 15 mM concentration (1.5 μmole/0.1 ml) on the fractional uptake at 2 min of ^{14}C-L-leucine into liver intracellular space and on the observed leucine: mannitol ratio of plasma. Both isoleucine and phenylalanine produced reductions of leucine uptake comparable to that found with lueicne itself at 15 mM concentration. No effect was found with alanine, glycine, histidine, lysine and proline. Variable effects ranging from 15-40% were found with the other six amino acids tested. Levels of incorporation of ^{14}C-leucine into protein ($A_i^{protein}/A_i^{total}$) were not significantly affected by the presence of the other amino acids.

The experiment was reversed in the case of phenylalanine in order to evaluate the effect of leucine load on ^{3}H-phenylalanine uptake and incorporation into protein. At a leucine dose of 1.5 μmole phenylalanine uptake was reduced to one-half the level observed in the absence

TABLE 4. Uptake of ^{14}C-L-leucine in the presence of other L-amino acids at 15 mM

Amino Acid	Avg Body Weight, g	Avg Liver Weight, g	Avg $(A/B)_{plasma}$	Avg A_i^{total}
ALA	203	7.0	0.40	0.46 ± 0.10 (6)
ASP	190	6.8	0.48	0.28 ± 0.08 (5)
CYS	213	5.3	0.46	0.31 ± 0.07 (2)
GLY	265	6.8	0.36	0.51 ± 0.07 (3)
HIS	191	7.8	0.43	0.45 ± 0.03 (2)
ILE	220	5.8	0.56	0.20 ± 0.06 (4)
LEU	209	6.8	0.65	0.21 ± 0.07 (10)
LYS	233	6.6	0.36	0.43 ± 0.03 (2)
MET	214	5.5	0.46	0.35 ± 0.06 (3)
PHE	202	7.2	0.61	0.21 ± 0.10 (10)
PRO	288	5.3	0.42	0.52 ± 0.04 (2)
THR	211	5.7	0.48	0.35 ± 0.06 (2)
TYR	232	5.6	0.36	0.26 ± 0.06 (3)
VAL	206	8.2	0.54	0.29 ± 0.04 (3)

All results are mean values for the number of animals given in the last column. Values of A_i^{total} include SD.

of leucine, an effect similar to the phenylalanine inhibition of leucine uptake. In spite of reduced uptake, the proportion of phenylalanine radioactivity incorporated into protein remained high and was not affected by leucine doses up to 10 μmoles.

F. Temperature dependency of leucine transport.

The effect of acute reduction of body temperature from 21° to 10° on the recovery of D-mannitol as a function of time after hepatic portal vein injection of a 0.1 ml bolus is presented in Figure 11. Loss of marker follows an exponential decline after acute temperature reduction, permitting the use of equation 15 to solve for the rate constants K_3 and K_5, as shown in Figure 12. These constants show only a slight temperature effect in the 10-21° range. Approximation of the temperature effect can be made by using the Q_{10} temperature coefficient (Prosser, 1973), defined as

$$Q_{10} = \left(\frac{K_a}{K_b} \right)^{10/(T_a - T_b)} \qquad (22)$$

where K_a and K_b represent rate constants obtained at temperatures T_a and T_b, respectively. At 10° K_3 = 0.9 min^{-1} and K_5 = 0.1 min^{-1}, equivalent to a Q_{10} of 1.3 for blood flow in this temperature range.

Figure 11. Effect of acute temperature reduction from 21° (O) to 10° (●) on recovery of D-[^{3}H]-mannitol from toadfish liver as a function of time. Injection time = 5-10 s; t = 0 represents end of injection. Data from 21° experiments are shown on an expanded scale in Fig. 1.

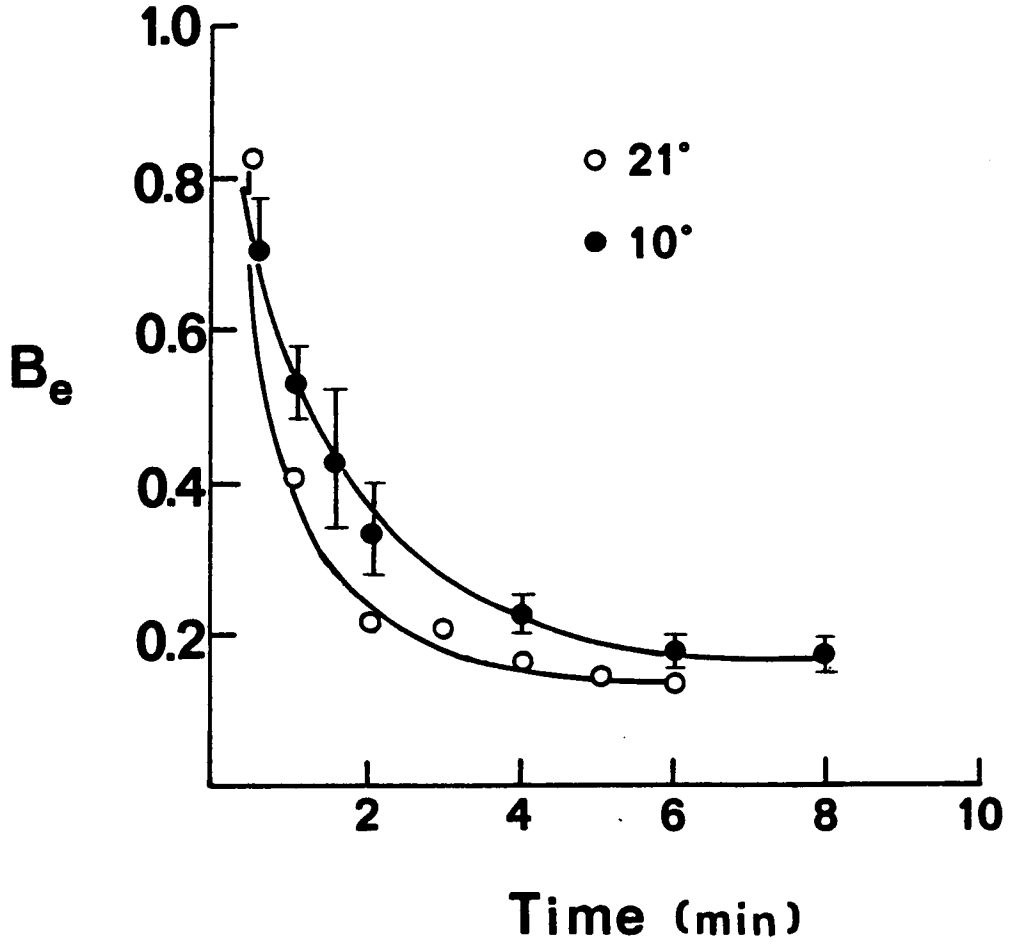
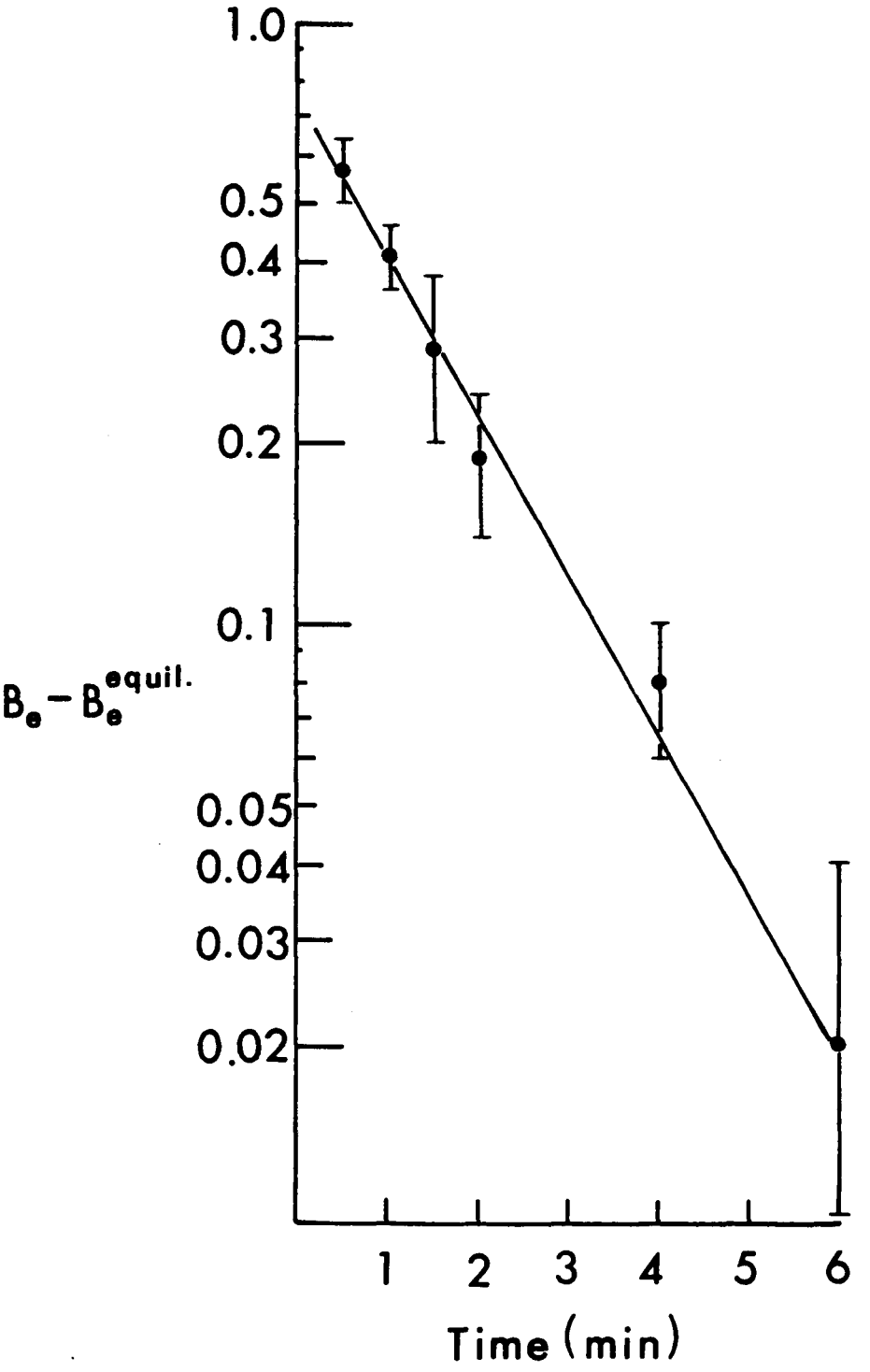

1.0
0.8
0.6
0.4
0.2
B_e
○ 21°
● 10°
2
4
6
8
10
Time (min)

Figure 12. Recovery of mannitol extracellular space marker presented in Fig. 11 for 10^{o}, plotted according to equation 15, with slope equal to K_3 in equation 9. From this graph $K_3 = 0.7\ min^{-1}$ at 10^{o}.


1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
0.03
0.02
$B_e - B_e^{equil.}$
1
2
3
4
5
6
Time (min)

Uptake of ^{14}C-L-leucine into intracellular space is strongly temperature dependent as shown in Figure 13. The levels of free intracellular radioactive leucine, A_i^{free}, determined by means of equations 1 and 2, reach a maximum level at about 2 min and decline slowly over the next 8 min. The proportion of A_i^{total} recovered in protein, shown in Figure 14, continues to increase during the same period, but at a significantly reduced rate relative to incorporation at 21°. Figure 15 shows the effect of acute temperature reduction on the loss of radioactive leucine from the extracellular space (A_e) calculated from equation 6.

1. Evaluation of incorporation and transport rate constants at 10°.

Figure 14 data yields an initial estimate for K_2 in equation 8 of 0.02 min^{-1} (Q_{10} = 10) in this low-temperature experimental series. Determination of the transport constants K_1 and K_4 was based on the best fit of the solutions of equations 10-14 to the experimental data in Figs. 13 and 15. With an assumed Q_{10} for transport of 2.5 (Prosser, 1973), values for K_1 at 10° would be around 1.1 min^{-1}. Using this initial estimate for K_1, the value of K_4 may be calculated from equation 20 for all values of R, $0.0 \leq R \leq 1.0$. No satisfactory fit could be obtained for Fig. 13 data with an inital estimate of

Figure 13. Effect of acute temperature reduction from 21^{o} (O) to 10^{o} (●) on the recovery of L-[^{14}C]-leucine, as fraction of administered dose, in intracellular free pool of liver, determined by means of equations 1 and 2. n = 52 animals at 10^{o}. Theoretical curves for A_i^{free}, based on equation 8, are shown for the rate constants determined for each experimental series shown in Table 5. Data from 21^{o} series are shown on an expanded scale in Fig. 3.

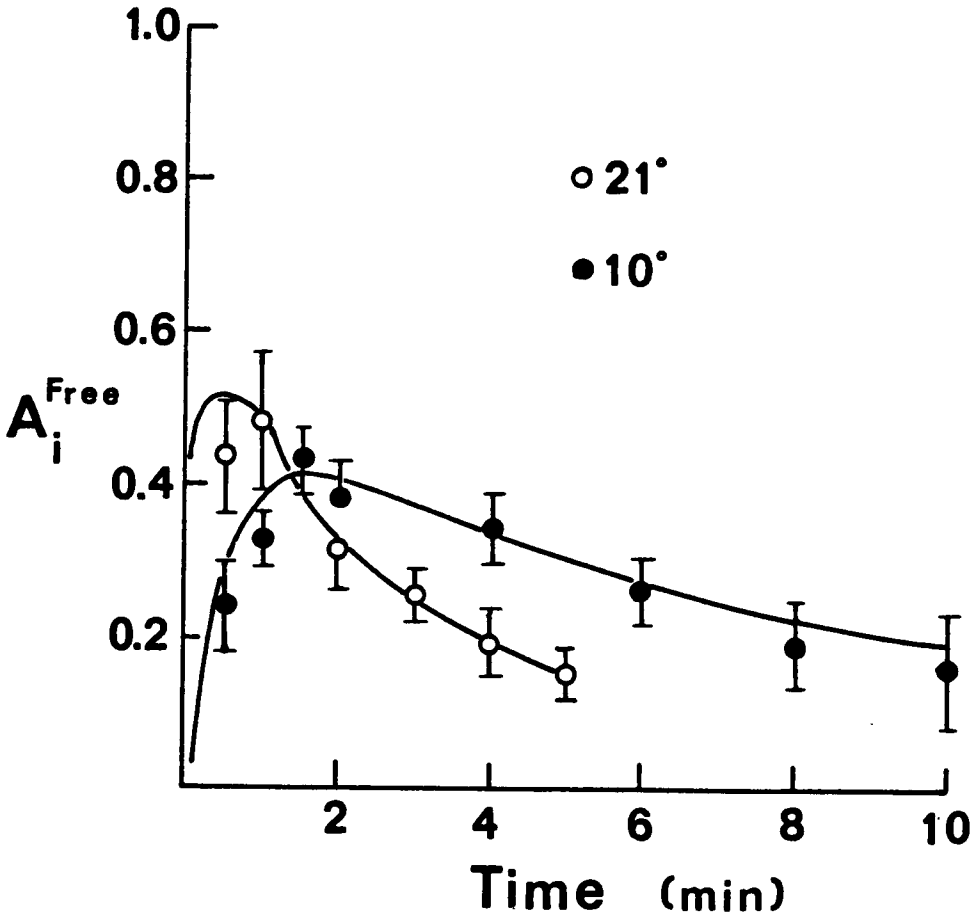

1.0
0.8
0.6
0.4
0.2
A_i^{Free}
21°
10°
2
4
6
8
10
Time (min)

Figure 14. Effect of acute temperature reduction from 21^{o} (O) to 10^{o} (●) on incorporation of L-[^{14}C]-leucine into liver protein, presented as ratio of fraction of dose recovered in protein to total intracellular radioactivity ($A_i^{total} = A_i^{free} + A_i^{protein}$). Theoretical curves are shown for rate constants listed in Table 5. Data from 21^{o} series are shown on an expanded scale in Fig. 4.

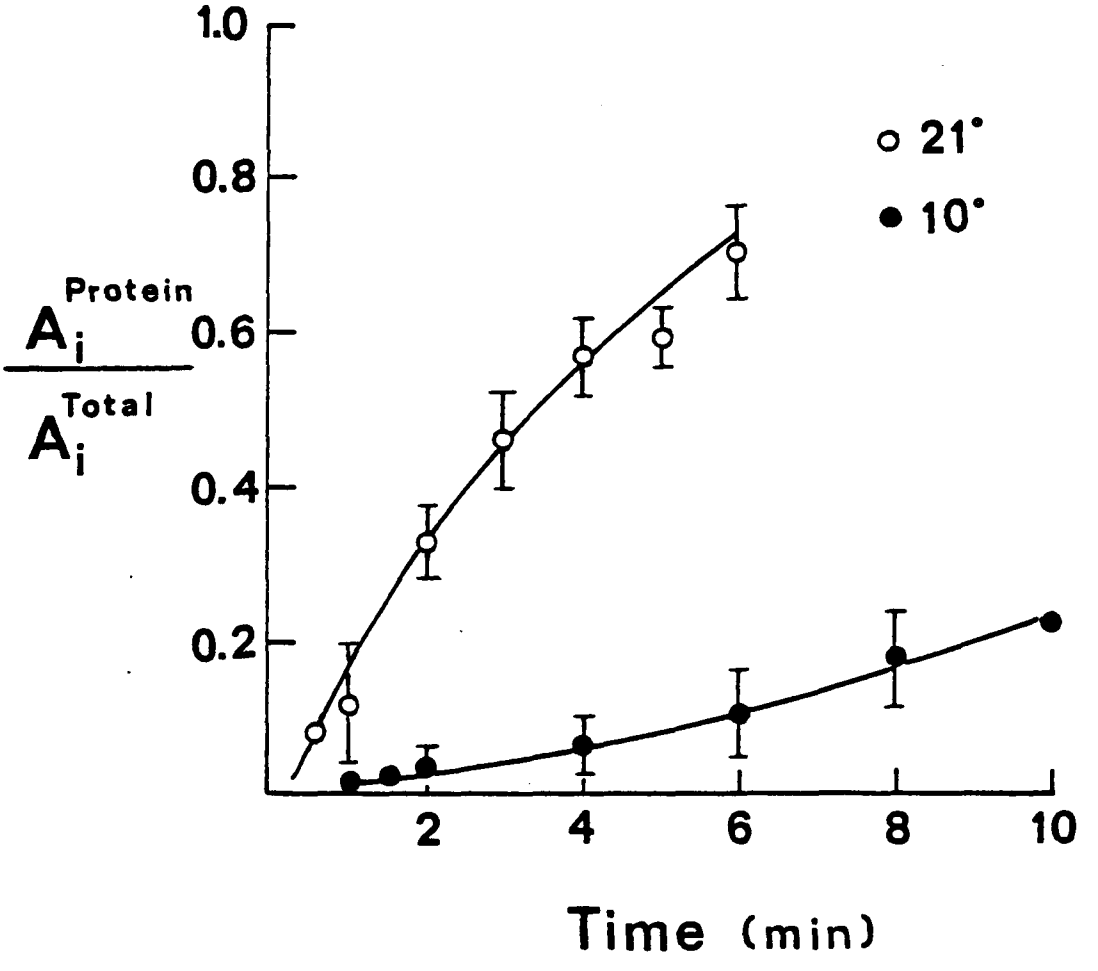
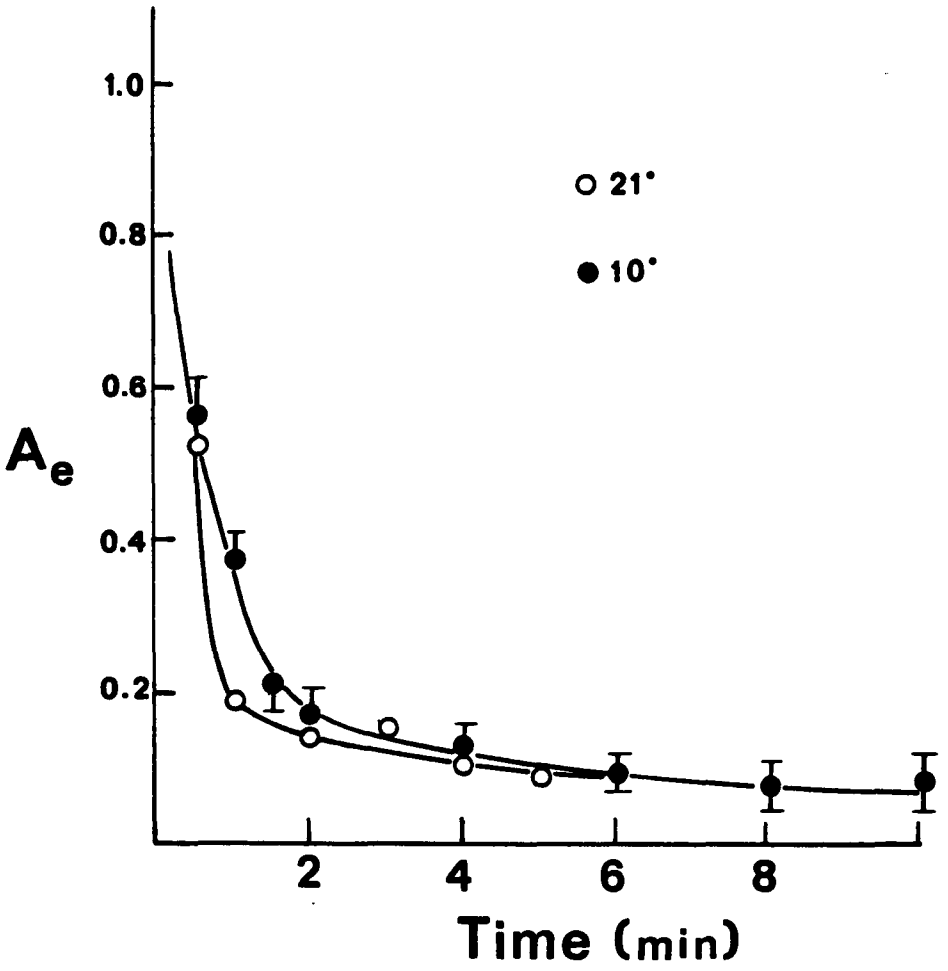

1.0
0.8
0.6
0.4
0.2
$\frac{A_i^{Protein}}{A_i^{Total}}$
21°
10°
2
4
6
8
10
Time (min)

Figure 15. Effect of acute temperature reduction from 21° (O) to 10° (●) on recovery of L-[^{14}C]-leucine in liver extracellular space, as fraction of dose, calculated according to equation 6. Theoretical curves for A_e are shown for rate constants listed in Table 5. Data from 21° series are shown on an expanded scale in Fig. 6.


1.0
0.8
0.6
0.4
0.2
A_e
○ 21°
● 10°
2
4
6
8
Time (min)

K_1 = 1.1 min^{-1}; maximal levels of A_i^{free} would be reached at approximately 1.5 min, sooner than the 2 min maximum indicated by the experimental data. Subsequent estimates of K_1 were based on incremental increases in Q_{10} from 2.5 to 4.0, with the best fit obtained for K_1 = 0.7 min^{-1}, or Q_{10} = 3.8. Calculation of K_4 from equation 20 requires an estimate of the parameter R. Results from experiments done at 21° (Fig. 7) indicate that the ratio of amino acid:marker leaving the liver, $(A/B)_{plasma}$, as a function of B_{liver} is the most sensitive measure of the relative proportion of concentrative uptake (R = 1.0) vs. exchange diffusion (R = 0.0). The best fit of the theoretical curve of A_e/B_e vs. B_e, calculated according to equations 10-14, is found for R = 0.2 as shown in Figure 16. From equation 20 K_4 at 10° is then calculated to be 0.4 min^{-1}, equivalent to a Q_{10} for efflux of 2.8. Kinetic constants derived from experiments at 10° and 21° are summarized in Table 5.

2. Polypeptide chain assembly time at 10°.

Concomitant measurement of average polypeptide chain assembly time during actue body temperature reduction revealed an increase in translation time from 4.8 ± 0.3 min at 21° (Fig. 5) to 20 ± 1.8 min at 10° (Fig. 17), equivalent to a Q_{10} of 3.8 for polypeptide chain assembly.

Figure 16. Effect of acute temperature reduction from 21° (O) to 10° (●) on plasma ratio of L-[^{14}C]-leucine: D-[^{3}H]-mannitol (normalized) as a function of mannitol recovery in liver (B_e) obtained at times up to 2 min after injection. Six animals have been averaged for each point, with standard error bars shown. Theoretical curves are based on rate constants listed in Table 5. Data from 21° series are shown on an expanded scale in Fig. 7.

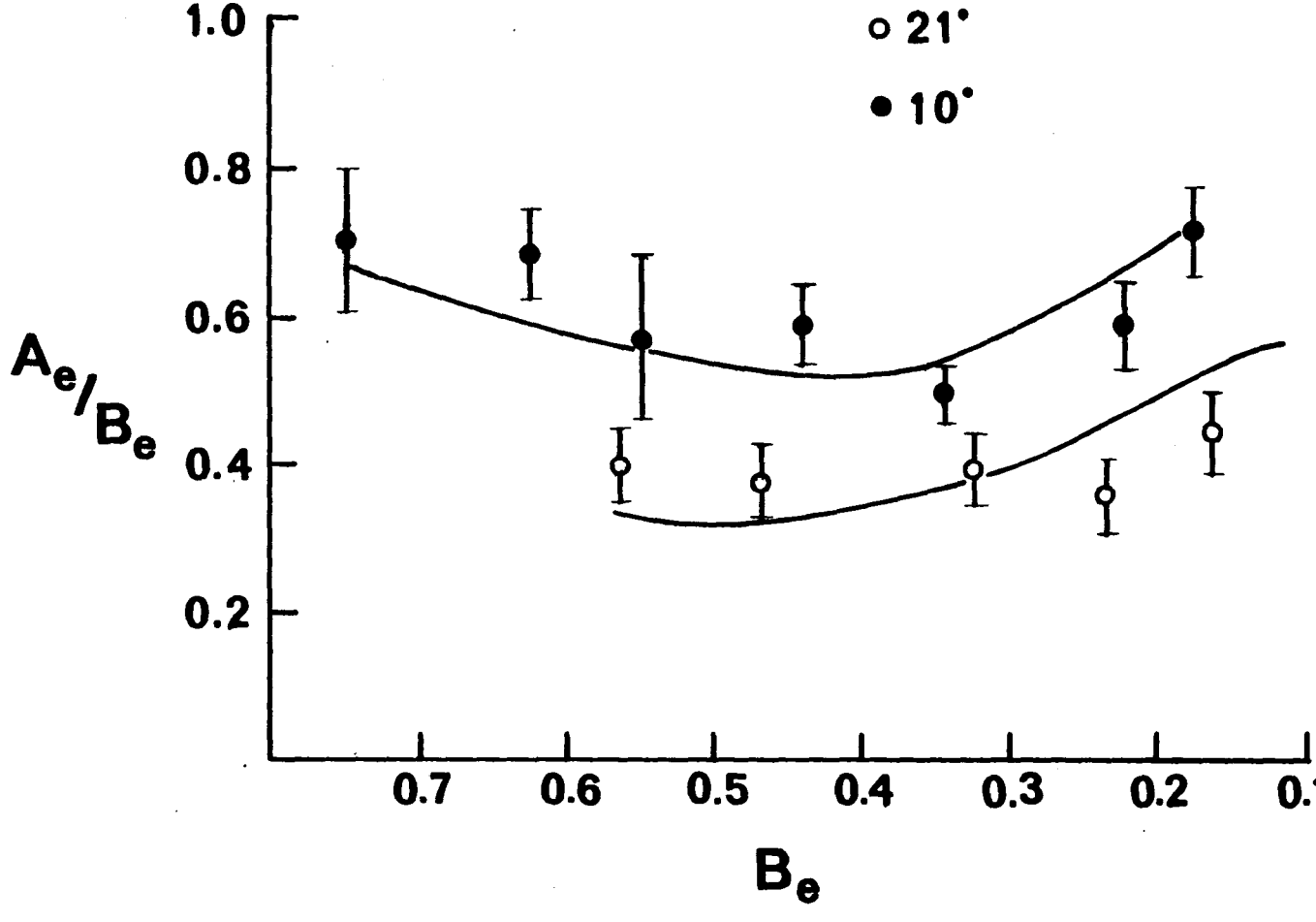
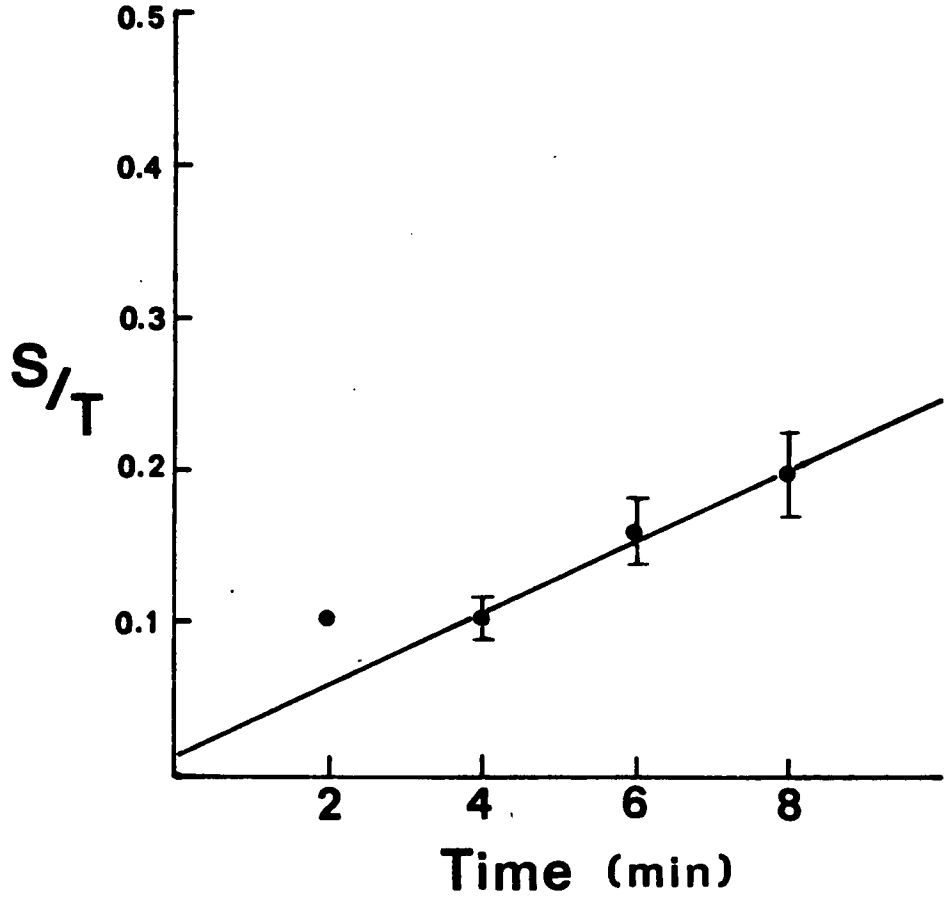

1.0
0.8
0.6
0.4
0.2
A_e/B_e
○ 21°
● 10°
0.7
0.6
0.5
0.4
0.3
0.2
0.1
B_e

TABLE 5. Temperature dependency of L-leucine transport in toadfish liver in vivo.

Kinetic Constants (min^{-1})		Temperature 21°	Temperature 10°	Q_{10}	E* (kJ/mol)
Circulation	K_3	0.9	0.7	1.3	17
	K_5	0.1	0.08	1.3	17
Transport	K_1	2.7	0.7	3.8	84
	K_4	1.1	0.4	2.8	65
Incorporation	K_2	0.2	0.02	10	145

Kinetic rate constants at 21° and 10°, determined according to the best fit of theoretical curves (equations 10-14) to experimental data as explained in text. At 21° R = 0.4 and at 10° R = 0.2 for the determination of K_4 from equation 20. Temperature coefficient (Q_{10}) is calculated according to equation 22. Apparent energy of activation (E*) is calculated according to $\ln(K_a/K_b) = -(E^*/R)(1/T_a - 1/T_b)$, where R is the Gas Constant, 8.3143 J $^{o}K^{-1}$ mol^{-1}.

Figure 17. Determination of average polypeptide chain assembly time in toadfish liver at 10^{o}. Time is calculated according to the equation $S/T = (1/2t_c)t$ as described for Fig. 5, where t_c is the average time for one round of polypeptide chain synthesis (Haschemeyer, 1969a). For this experimental series $t_c = 20 \pm 1,8$ min. Total number of animals is 13. Standard error bars are shown.


0.5
0.4
0.3
S/T
0.2
0.1
2
4
6
8
Time (min)

DISCUSSION

A. The study of amino acid movement in liver in vivo.

Quantitative characterization of transport systems for naturally-occuring amino acids has been hampered by two factors discussed most recently by Pardridge and Jefferson (1975). First, transport is extremely rapid (equilibration time between liver and blood, for example, is as short as 0.5 min), and second, labeled compounds enter various metabolic pathways subsequent to transport. As a result, relatively little is known about transport systems of liver, although this organ is one of the major sites of amino acid transport and metabolism (McGivan et al., 1977). The results presented in this thesis indicate the feasibility of a pulse injection technique for study of amino acid transport by liver in vivo. The method permits determination of both quantitative fluxes and transport characteristics as they operate in the living animal. Analysis is based on a set of monomolecular reactions descring the movement of amino acid between three compartments: first, movement between extrahepatic space and liver, measured by the flow-dependent movement of an extracellular space marker; second, transport between hepatic extracellular and intracellular spaces; and third, intracellular sequestration by incorporation

into protein. These reactions serve to identify the significant in vivo kinetic barriers which are potential sites of physiological regulation of amino acid and protein metabolism. Results are based on recovery of radioactivity in plasma and liver compartments following pulse injection of labeled amino acid, and evaluated according to the summary reaction scheme of equation 6.

The model provides a basis from which to pursue studies of the regulation of liver amino acid metabolism in relation to both environmental factors, in particular temperature, and physiological factors, including hormones, diet, or disease conditions. Furthermore, study is not limited to non-metabolizable amino acids since protein synthesis is readily measured at the same time. Limitations of the model and of in vivo experiments in general, to be discussed in greater detail in the following sections, also point to areas of future research in amino acid transport. These include the question of distribution of radioisotope in the liver at very short times after a bolus injection and the possibility that kinetic characteristics of hepatocytes may differ in different regions of the liver. The method is currently limited to amino acids which are not involved to a significant extent in reactions other than protein synthesis. In order to extend these studies to substrates with a greater number of intracellular metabolic

pathways than leucine, further analytical procedures would need to be included.

B. Leucine transport.

Saturation. This thesis provides evidence of a leucine transport system in toadfish liver in vivo with many characteristics of System L, first described in Ehrlich cells (Oxender and Christensen, 1963; Christensen, 1977a). The observed saturation of uptake is consistent with a carrier-mediated process, as demonstrated for eukaryotic cells in culture (Heinz, 1972; Hoare, 1972a; Oxender and Christensen, 1963). The results in toadfish, however, differ from those in isolated rat hepatocytes, where saturation of leucine uptake was not obtained (McGivan et al., 1977). The authors concluded that at physiological concentrations of leucine, nearly 15% of uptake is due to a free diffusion component. Significant free diffusion also occurs in rat liver in vivo, as indicated by studies of phenylalanine and valine uptake (Pardridge and Jefferson, 1975). The presence of a non-saturable pathway for leucine is still at issue in erythrocytes. Winter and Christensen (1964) attributed 26% of total leucine uptake to this pathway in rabbit erythrocytes, whereas Hoare (1972a), working with human erythrocytes, concluded that no more than 1% is non-saturable at normal physiological leucine concentration.

Affinity. The effective concentration (K_m) for half-maximal uptake in toadfish liver in vivo has been estimated in this thesis by two methods. First, the decline in recovery of radioactive leucine in liver as a function of the concentration of injected leucine (Figs 8 and 9) yields a concentration of 1.5 mM. Allowing for the dilution of substrate by incoming portal and arterial blood, one obtains an apparent K_m of 0.6 mM. Second, calculation of K_m is possible by means of the Michaelis-Menton equation (equation 21) after chemical analysis of liver and plasma leucine pools at both physioloigcal (0.1 mM) and high (100 mM) leucine levels. Pool data reveal that uptake is lower than what would be expected from an extrapolation of the tracer-level influx rate. The leucine pool data lead to an estimation of V_{max} at 4.1 µmol/min/7-g liver and, from equation 21, a K_m value of 0.58 mM. This value is similar to that reported for Ehrlich cells (0.5 mM) at 37° (Oxender and Christensen, 1963), suggesting that carrier molecules in both the fish and mammalian system have similar leucine-binding properties, at least those properties reflected by K_m. As pointed out by Farmanfarmaian et al. (1972) in a review of glucose transport, similar K_m values in different species for natural substances such as sugars may reflect the evolutionary conservation of fundamental transport processes. One might also expect K_m values for amino

acid transport to be within a narrow range among different species and tissues.

No comparable data are available for leucine uptake in rat liver in vivo, although apparent K_m values have been reported for glutamate (4.8 mM) and aspartate (2.7 mM) (Pardridge and Jefferson, 1975). These values, however, are overestimated since they are based on the assumption that substrate concentration reaching the hepatocyte membrane is identical to the injected concentration. A similar approach in rat brain in vivo, where little dilution of substrate occurs, yielded K_m = 0.15 mM for leucine (Pardridge and Oldendorf, 1975). The authors found that K_m values for transport of neutral amino acids across the blood-brain barrier are of the same order of magnitude, ranging from 0.12 mM for phenylalanine to 0.73 for threonine. Comparing these results, Pardridge (1977) characterized amino acid transport in brain as high-affinity and low-capacity, whereas that in liver is lower affinity but high capacity. Consideration of substrate dilution in the studies on transport by rat liver, however, may make the differences between liver and brain less striking. Studies on other vertebrate classes are neede to provide insight into the evolution of amino acid transport systems, particularly with respect to the conservation of binding-site characteristics.

Capacity. The magnitude of leucine influx in

toadfish liver at normal body temperature (21°) and plasma leucine concentration (0.1 mM) may be compared with influx rates in other tissues and species in Table 3. Even if only 20% of the liver participates in the uptake of the pulse-injected leucine, as the present studies suggest, leucine influx is 0.12 μmol/min/7-g liver. This rate is twice the maximum rate of leucine incorporation into protein, based on polypeptide chain assembly time and ribosome concentration from Haschemeyer (1969), and 10 times the rate of net utilization of leucine through plasma protein synthesis (Haschemeyer, 1973) and growth (Smith and Haschemeyer, 1974). At high leucine doses, maximal uptake is 1.2 μmol/min/ml intracellular H_2O or 5.1 nmol/min/mg protein, about one-half the value (2 μmol/min/ml) found in Ehrlich cells at 37° (Oxender and Christensen, 1963), and one-third the value (15.1 nmol/min/mg) found in isolated rat hepatocytes at 37° (McGivan et al., 1977). A V_{max} of 0.32 μmol/min/g has been reported for leucine uptake by perfused dog brain (Betz et al., 1975).

In contrast to the narrow range for K_m, actual transport rates are highly variable since they reflect the number of functional sites and depend of experimental conditions (Farmanfarmaian et al., 1972). In the case of glucose transport, data also indicate that capacity depends on tissue and species (Farmanfarmaian et al., 1972).

Comparable data on maximal amino uptake rates at high plasma concentrations have not been reported for mammalian liver in vivo, although Pardridge (1977) has estimated unidirectional flux of glutamine and leucine in rat liver in vivo at normal physiological concentrations. Such studies are needed to distinguish how amino acid transport capacity is associated with the physiological role of different tissues and with evolutionary factors.

Active transport. In addition to determination of uptake levels and examination of saturation phenomena, the present analysis of the time dependency of transfer among compartments permits an estimation of influx and efflux rates operating in vivo. This is of particular interest with regard to the mechanism of leucine transporrt. It has been considered that eukaryotic leucine transport functions by carrier-mediated exchange or facilitated diffusion only (Heinz, 1972), even though early results (Oxender and Christensen, 1963) suggested concentrative uptake. Leucine uptake in perfused brain is not affected by up to 10 min of anoxia, suggesting that the process is not energy-dependent (Betz et al., 1975). The present results for leucine transport in toadfish liver indicate that as much as 40% of uptake at 21°, and 20% of uptake at 10°, is attributable to concentrative or active transport. This conclusion follows primarily from the analysis of

the leucine:mannitol ratio in blood leaving the liver, a parameter highly sensitive to relative rates of influx and efflux of leucine within the liver (Fig 7). These data yield a ratio of influx to efflux rate constants (K_1/K_4) of about 2.5 at 21°, significantly higher than the theoretical value of 1.5 for pure exchange diffusion or facilitated diffusion (equation 16). This result falls within the range indicated by the absolute values of A_1^{free} and A_e (Figs 3 and 6), although it must be noted that these quantities are less sensitive to K_1/K_4 than the leucine:mannitol ratio between R = 0.0 and R = 0.5. The estimate of R = 0.4 at 21°, derived from Fig 7, represents a component of influx amounting to 40% of K_1 that is not balanced by efflux. Such a component will concentrate leucine in the liver intracellular space relative to plasma and, therefore, may be considered uphill or active transport. The results yield a predicted steady-state concentration ratio for liver and plasma very close to that calculated from amino acid pool measurements (equations 17 and 18), providing a degree of confirmation of the transport data. The error associated with small differences in measured leucine concentrations of liver and plasma (Table 2), however, is recognized.

Competition. The effect of high doses of other amino acids on leucine transport (Table 4) indicates the degree to which these amino acids may compete for the same carrier molecules. The finding of isoleucine and phenylalanine as principal competitors is consistent with results in other organisms (Yudilevich et al., 1972; Betz et al., 1975; Christensen, 1975). Lesser effects were found for aspartic acid, cysteine, methionine, threonine, tyrosine, and valine. This behavior is characteristic of System L transport, as described by Christensen (1975). McGivan et al. (1977) found cross-inhibition for the group leucine, isoleucine, valine, and methionine in isolated rat hepatocytes. Although more comparative data are needed, it appears that discrimination of substrate structure by System L carrier molecules has been conserved through evolution, at least among vertebrates.

The competition results also have some bearing on the question of possible heteroexchange as a driving force for leucine transport. An amino acid which is concentrated by energy-dependent transport (for example, by System A) could drive the uptake of another amino acid through exchange (Heinz, 1972), provided both can use the same carrier. The major System A amino acid (alanine), however, does not compete with leucine in toadfish liver (Table 4) and may be excluded from taking part in any significant

heteroexchange. Other neutral amino acids which may utilize the System A pathway (for example, methionine and valine) have a small competitive effect on leucine transport. In view of the low intracellular concentrations of these amino acids in toadfish liver (unpublished results), it is uncertain whether their flux rates via System A and their affinity for the leucine carrier are adequate to account for the 40% of uptake attributed in this thesis to active transport. Christensen (1977a) has reviewed evidence for net uptake of System L amino acids under conditions in which heteroexchange was minimized, and he concludes that System L is capable of active transport via an energy source, as yet uncharacterized, that is distinct from that of System A. More definitive experiments in vivo are necessary in order to describe the relationship between System A and System L transport, as well as to characterize their energizing reactions.

C. Leucine distribution and protein synthesis.

At tracer levels, retention by liver in the first few minutes after a pulse injection is up to 75% of the administered dose for a mixture of 15 ^{14}C-labeled amino acids (Haschemeyer and Persell, 1973) and 43% for ^{14}C-leucine alone (Table 2). These high levels of uptake establish a high specific radioactivity in the liver and, considered together with subsequent uptake by other body

tissues (Smith and Haschemeyer, 1975), minimize the uncertainty associated with isotope recirculation in the whole animal. The use of a rapid pulse system, however, introduces questions about the distribution of radioisotope in the liver at very short times. It is likely that at tracer doses early uptake and incorporation is concentrated in cells with earliest access to portal blood. In the toadfish liver some nonuniformity of amino acid distribution over different regions of the liver has been observed (Hudson, 1977). A concentration gradient from liver entrance to exit, and within each liver sinusoid, has been described for galactose uptake (Goresky, 1973a). The author has suggested that these concentration gradients are typical of substrates transported by liver, especially when they can be irreversibly sequestered in the intracellular space, as in leucine incorporation into protein (Goresky, 1973b). In addition, the liver system in vivo may exhibit heterogeneity as a result of streamlining of portal flow, leading to unequal delivery of circulating amino acids to different lobes. (LeBouton and Hoffman, 1969). In the liver in vivo, then, it is clear that caution must be used in interpreting hepatocyte function based on values which are averaged for the entire organ. Some of these uncertainties may be cleared up by extending these studies to toadfish

hepatocyte systems in vitro, where the concentration of substrate reaching the hepatocyte membrane may be measured with greater certainty.

The measurement of protein synthesis in these experiments permits an analysis of the volume of liver occupied by the incoming bolus of injected leucine. This analysis is possible since it has been demonstrated (Fig 10) that the rate of protein synthesis, as measured by polypeptide chain assembly time, is not affected by the concentration of injected leucine, an observation also reported for perfused rat liver (Peary and Hansen, 1976). The observed (Table 2) decrease in ^{14}C-leucine incorporation into protein at high leucine doses, therefore, must be due to a lower specific radioactivity resulting from the expansion of the intracellular pool. The results indicate that 20-30% of the liver is occupied by the incoming leucine pulse. This volume probably represents the volume of the terminal venule cells which have greatest access to incoming portal blood.

The results do not support the notion that extracellular amino acids are preferentially used in protein synthesis (Introduction, Section B) since incorporation of labeled leucine into protein (Table 2) does not reflect the specific radioactivity of extracellular leucine. A combination, however, of direct utilization and equilibration with

intracellular pools cannot be excluded. Seglen and Solheim (1978) have suggested that incorporation of valine into rat hepatocyte protein may occur directly from extracellular valine pools at high external concentrations, but at physiological concentrations incorporation reflects the rapid equilibration of exogenous valine with the intracellular pool.

D. Temperature dependency of leucine uptake.

An acute drop in body temperature from 21° to 10° results in a decrease in all of the measured kinetic parameters (Table 5). The temperature coefficient (Q_{10}) from equation 22) for circulation through the hepatic vascular bed is low, as measured by the disappearance of extracellular marker from the liver. This small temperature effect is consistent with the relative temperature independency of complex rate functions such as circadian rhythm in otherwise temperature-conforming animals (Prosser, 1973). The toadfish appears to be capable of rapid cardiovascular compensation in the face of sudden temperature drops, similar to the response reported for the rainbow trout, Salmo gairdnerii, which shows a Q_{10} between 1 and 2 for cardiac output between 12° and 18° (Holeton and Randall, 1967).

The temperature dependency of the specific influx and efflux parameters is near the range ($Q_{10} = 2.5 \pm 0.5$)

typically found for metabolic processes in poikilotherms at temperatures close to their natural habitats (Prosser, 1973; Scholander at al., 1953). Leucine influx (Q_{10} = 3.8) exhibits a greater temperature dependency than efflux (Q_{10} = 2.8). Leucine uptake between 10° and 20° in isolated human erythrocytes also exhibits a greater temperature dependency (Q_{10} = 11) than leucine efflux (Q_{10} = 5) (Hoare, 1972b). McGivan et al. (1977) found that leucine uptake in isolated rat hepatocytes shows a Q_{10} of 3.4 in the temperature range between 4° and 18°, and in a similar system valine transport below 25° exhibits a temperature coefficient of 4.2 (Seglen and Solheim, 1978).

The system presented here permits a direct comparison of in vivo and in vitro data on the relative contribution of exchange diffusion to total leucine entry as temperatures are lowered. In the toadfish at 10° only 20% of uptake can be considered active or concentrative, while 40% can be attributed to active transport at 21° (Fig 7). These results confirm Hoare's (1972b) conclusion that exchange diffusion assumes a relatively greater role in leucine uptake in human erythrocytes at lower temperatures. As Hoare points out, changes in balance between exchange and net transport could represent a mechanism for control of amino acid flux. A differential response in rates of

efflux and influx to decreased temperatures has also been demonstrated in rat brain slices for valine and phenylalanine (Banay-Schwartz et al., 1977) and in Ehrlich ascites cells for phenylalanine (Im et al., 1978).

Protein synthesis at low temperatures. The present results yield a Q_{10} of 3.8 for elongation in protein synthesis and 10 for amino acid incorporation, but the results probably represent an average temperature effect over the 10-21° range. These observations have recently been extended by Mathews and Haschemeyer (1978) who show a break in the Arrhenius plot at 17°. Above that temperature Q_{10} = 2.5, while below 17° the Q_{10} increases to about 5. Seglen and Solheim (1978) have reported a Q_{10} of 4.2 for protein synthesis in isolated rat hepatocytes. Craig (1976) found values of Q_{10} between 7 and 8 for elongation alone and for total protein synthesis below 24° in mouse L-cells.

The high Q_{10} for amino acid incorporation reported here is consistent with the observation that toadfish liver protein synthesis appears to be almost completely inhibited below 7° (Mathews and Haschemeyer, 1978). Partial inhibition is likely to occur at 10°. Low-temperature inhibition of protein synthesis in the toadfish may be related to cold dormancy, which the toadfish exhibits below an ambient temperature of 5° $\pm$ 1° as part

of its normal winter response to low water temperatures (Gray and Winn, 1961).

APPENDIX I

Leucine metabolism in liver.

Since the early experiments of Rose (1949), it has been accepted that leucine is an essential amino acid for rats and humans and probably for all vertebrates (Meister, 1965). After intestinal absorption, dietary leucine is first exposed to metabolic action by the liver. In higher organisms leucine metabolism is confined to either catabolic oxidation, yielding acetyl-SCoA and acetoacetate, or to ligation to tRNA and subsequent incorporation into polypeptide chains (Connelly et al., 1968). No intermediates are available for carbohydrate synthesis (Bender, 1975).

Two enzymes have been identified which can initiate leucine oxidation by deamination. The first is the general branched-amino acid transferase (EC 2.6.1.6) which transfers an amino group from leucine to α-oxo-glutarate, resulting in the formation of glutamate and α-oxo-isocaproic acid (Bender, 1975). Valine and iso-leucine serve as substrates as well as leucine. A second, leucine-specific enzyme which catalyzes the same reaction is L-leucine:α-ketoglutarate aminotransferase (EC 2.6.1.42). Activity for both enzymes has been

demonstrated in liver, but overall aminotransferase activity is very low relative to the activity in muscle and kidney (Miller, 1962; Mimura et al., 1968; Ichihara and Takahashi, 1968)

Miller (1962) first demonstrated that liver is not an important site of leucine deamination by showing that hepatectomized animals exhibited no significant decline in leucine deamination over control animals. Mimura et al. (1968) and Yamamoto et al. (1974) have demonstrated that oxidative deamination of leucine occurs for the most part in kidney and muscle, which exhibit a total amino-transferase activity up to 10-fold greater than that of liver per unit weight (Krebs and Lund, 1977). Krebs (1972) has suggested that this particular distribution of branched-chain catabolic enzymes may be correlated with the need to allow essential amino acids to pass through the liver and circulate to the rest of the body.

Although adult liver exhibits the lowest activity of the deamination isozymes, fetal rat liver possesses considerable enzyme activity which declines rapidly only after birth (Ichihara and Takahashi, 1968). Moreover, only the liver branched-chain amino transferase varies in activity in response to the protein content of the diet, showing up to a five-fold increase with high-protein diet (Krebs and Lund, 1977; Crabb and Harris, 1978).

Deamination is the first step in leucine catabolism, and it is clear that this step occurs primarily in peripheral tissues. The liver, however, is the site of the greatest activity of the second step in leucine oxidation, catalyzed by the α-ketoisocaproate dehydrogenase complex (EC 1.2.4.3), also known as the ketoleucine dehydrogenase complex (Crabb and Harris, 1978). This enzyme complex catalyzes the decarboxylation of ketoleucine to form isovaleryl-SCoA.

In their recent review on the metabolism of the branched amino acids, Krebs and Lund (1977) summarize the distribution of leucine catabolic enzymes by noting that muscle and kidney are the main sites of deamination, and the liver is the main site of subsequent oxidation of the leucine carbon skeleton. For the short time experiments reported in this thesis, leucine oxidation by liver may be ignored.

APPENDIX II

Listed below is a program written in FORTRAN IV for use with the interactive CALLOS system on an IBM 370 computer at the City University of New York. The program solves by numerical integration the set of simultaneous differential equations listed in the thesis text as equations 10-14. Output is in the form of percent injected dose of radioactively-labeled leucine and mannitol appearing in the liver compartments described by equation 8 as a function of time. The program listed is for analysis of rate constants derived for experiments done at 21°; for the experimental series done at 10°, different rate constants were used as shown in Table 5.

```
TRANS
10  DIMENSION YAF(350),YAE(350),YAI(350),YAP(350),XT(350),%
11  YATOT(350),YRAT(350)
15  REAL K1,K2,K3,K4,K5,K3B,K5B
20  WRITE (6,25)
21  25 FORMAT (' ',T5,"INITIAL R?  INCREMENT R BY?'/)
22  READ (5,*) R,DELTA
40  WRITE (6,600)
41  600 FORMAT ('0',T4,'INITIAL K2?',T18,'FLOW THRU%
42  TIME', T38,'K1?',T45,'K2 INCREMENT?'/)
```

```
43  READ (5,*) K2,TM,K1,DK2
50  18 K3=0.0
60  K4=K1*(1-R)/1.5
70  K5=0.0
100  K3B=0.9
120  K5B=0.1
130  TT=0.0
140  YAF(1)=0.0
150  YAP(1)=0.0
160  YAE(1)=1.0
170  YAI(1)=0.0
180  XT(1)=0.0
185  N=310
190  DT=0.02
201  DO 400 I=1,N
210  TT=TT+DT
220  IF (TT.GE.TM) GO TO 410
230  415 DE=DT*(-(K1+K3)*YAE(I)+K4*YAI(I)+K5*YAF(I))
240  DI=DT*(K1*YAE(I)-(K2+K4)*YAI(I))
250  DF=DT*(I3*YAE(I)-K5*YAF(I))
255  DP=DT*K2*YAI(I)
260  YAE(I+1)=YAE(I)+DE
270  YAI(I+1)=YAI(I)+DI
280  YAP(I+1)=YAP(I)+DP
290  YAF(I+1)=YAF(I)+DF
```

```
292  YATOT(I)=YAI(I)+YAP(I)
293  YRAT(I)=YAP(I)/YATOT(I)
300  XT(I+1)=XT(I)+DT
310  GO TO 400
320  410 K3=K3B
360  K5=K5B
370  GO TO 415
380  400 CONTINUE
384  WRITE (6,525) K1,K2,K3B,K4,K5B,TM
386  525 FORMAT ('0',T5,'K1=',F4.2,T15,'K2=',F4.2,T25,%
387  'K3=',F4.2,T35,'K4=',F4.2,T45,'K5=',F4.2,T53,'DELT%
388   T=',F4.2,T65,'MIN'//)
390  WRITE (6,505)R
400  WRITE (6,515)(YAE(I),YAI(I),YAF(I),YAP(I),YATOT(I),%
401  YRAT(I),XT(I),I=1,N,20)
410  505 FORMAT (T3,'AE',T12,'AI',T21,'AF',T30,'AP',T39,%
411  'ATOT',T48,'AP/ATOT',T57,'TIME',T63,'R=',F4.2/)
420  515 FORMAT (T3,F5.3,T12,F5.3,T21,F5.3,T30,F5.3,T39,%
421  F5.3,T48,F5.3,T57,F5.2)
422  R=R+DELTA
423  K2=K2+DK2
430  IF (R,GE.1.1) GO TO 450
440  GO TO 18
450  450 STOP
460  END
```

The program listed below solves by numerical integration the set of simultaneous differential equations listed in the thesis text as equations 10-14. Output is in the form of the ratio of radioactivity found in amino acid and marker leaving the liver (A_e/B_e) as a function of the marker in the liver extracellular space (B_e). Values for rate constants are found in Table 5.

```
FLOW
10  DIMENSION YAF(500),YAE(500),YAI(500),YAP(500),XT(500)%
11  ,BE(500),PR(500),BB(500)
20  REAL K1,K2,K3,K4,K5,K3B,K5B,
21  WRITE (6,600)
22  600 FORMAT (' ',T5,'VALUES OF K1,K2,K3,K5?')
23  READ (5,*) K1,K2,K3B,K5B
24  WRITE (6,610)
25  610 FORMAT (' ',T5,'THE INITIAL VALUE OF R EQUALS')
26  READ (5,*) R
27  WRITE (6,620)
28  620 FORMAT (' ',T5,'R WILL BE INCREMENTED BY')
29  READ (5,*) RX
30  WRITE (6,630)
31  630 FORMAT (' ',T5,'FLOW THROUGH TIME IS')
32  READ (5,*) TX
```

```
50  40 K3=0.0
60  K4=K1*(1-R)/1.5
70  K5=0.0
130  TT=0.0
135  BE(1)=1.0
136  BB(1)=0.0
140  YAF(1)=0.0
150  YAP(1)=0.0
160  YAE(1)=1.0
170  YAI(1)=0.0
180  XT(1)=0.0
185  N=200
190  DT=0.02
200  DO 400 I=1,N
210  TT=TT+DT
220  IF (TT.GE.TX) GO TO 410
230  415 DE=DT*(-(K1+K3)*YAI(I)+K4*YAI(I)+K5YAF(I))
240  DI=DT*(-(K4+K2)*YAI(I)+K1*YAE(I))
241  DBE=DT*(K5*BB(I)-K3*BE(I))
245  DF=DT*(K3*YAE(I)-K5*YAF(I))
250  DP=DT*(K2*YAI(I))
260  YAE(I+1)=YAE(I)+DE
270  YAI(I+1)=YAI(I)+DI
271  BE(I+1)=BE(I)+DBE
280  YAP(I+1)=YAP(I)+DP
```

```
281   BB(I+1)=BB(I)-DBE
290   YAF(I+1)=YAF(I)+DF
291   PR(I)=YAE(I)/BE(I)
300 XT(I+1)=XT(I)+DT
310   GO TO 400
320   410 K3=K3B
360   K5=K5B
370   GO TO 415
380   400 CONTINUE
390   WRITE (6,510) R
400   WRITE (6,520)(BE(I),PR(I),I=1,N,10)
410 510 FORMAT ('0',T5,'BE',T15,'AE/BE',T25,'R=',F4.2/)
420 520 FORMAT (' ',T5,F5.3,T15,F5.3)
421   R=R+RX
422   IF(R.GE.1.05) GO TO 450
423   GO TO 40
430   450 STOP
440   END
```

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