

THE ROLE OF AMINO ACIDS IN ALBUMIN SYNTHESIS
AND CATABOLISM.

A Thesis

presented to the University
of Cape Town for the degree
of Doctor of Medicine by
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To Minnie and the late Basil Gibaud.

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I N T R O D U C T I O N

Modern concepts of the structure, function and metabolism of proteins have evolved painstakingly through the centuries. In September, 1772, Rutherford discovered the gas which was later named nitrogen by Chaptal in 1790. The fundamental importance of nitrogen in the body was only appreciated after Gay-Lussac and Thénard had pioneered chemical procedures for organic analysis. Magendie applied this knowledge to dietary constituents and concluded that nitrogen was an essential component of the diet. His early insight into metabolism was depicted in his view that body constituents are continuously being replaced, the rate being dependent on the tissue. In 1838 the term "protein" was coined by Gerard Mulder for substances containing a basic nitrogenous component.

With nitrogen firmly established as a component of body tissues, it became necessary to quantitate nitrogen output relative to intake. The concept of balance studies was pioneered by Boussingault in 1839 in cows. Voit, a pupil of the outstanding chemist Liebig, popularised the use of nitrogen balance techniques in man for the study of protein metabolism. He, together with Bischoff, established that in long-term experiments in healthy experimental subjects, nitrogen intake and output were equal, and the individual in these circumstances was said to be in nitrogen equilibrium.

The next phase of investigation was centred around quantitative protein requirements. Based on nitrogen balance studies, Voit concluded that the average working man required 118 grams of protein per day while Chittenden and others contended that 50-55 grams of protein per day were required to maintain nitrogen equilibrium. The concept of qualitative protein requirements

evolved slowly. In 1810 the first amino acid, cystine, was discovered by Wollaston. Later, it was shown that gelatin, while containing nitrogen, was unable to maintain nitrogen equilibrium on its own (Bischoff and Voit, 1860). While Braconnot in 1820 had released individual amino acids from protein by acid hydrolysis, it was not until 1900 that Kossel and Kutcher observed the variation of amino acid content of different proteins. It then became possible for Kauffmann(1905), Willcock and Hopkins (1906) and eventually Osborne and Mendel (1914) to show that at least certain of the amino acids are indispensable constituents of the diet. The meticulous studies of Rose, utilising purified amino acid diets, led to the distinction between "essential" and "non-essential" amino acids in 1938 in rats and man. This concept provided the information that the nutritive value of a protein is a function of its essential amino acid content.

Amino acids have widespread effects throughout the body apart from the supply of substrate for protein synthesis. Professor S.J. Saunders' laboratory, in which the author worked, had long been interested in the control of albumin synthesis. Analysis of the plasma aminogram in protein calorie malnutrition (PCM) had revealed a specific profile with the depression of the branched chain amino acids being a prominent feature (Holt et al, 1963; Saunders et al, 1967). The association of this depression with a low plasma albumin was instrumental in stimulating the work to be described in this thesis. A fundamental issue arose: do specific amino acids influence albumin metabolism? Such information has tremendous potential in the understanding of PCM and in the management of patients with hepatic and renal failure.

The first part of this thesis describes the basic study in humans relating dietary protein to albumin synthesis, albumin catabolism and the plasma aminogram.

Part II and Part III describe the effect of amino acid supply on albumin synthesis and catabolism respectively in the isolated perfused rat liver. Such a system obviates any confusion created by different pools or hormonal responses which occur in vivo, and allows specific factors to be more easily tested.

While this work was in progress it became apparent that the state of the protein synthetic machinery, namely the ribosomes, should be defined in the system utilised in Part II and Part III. Such basic knowledge would be helpful in understanding any change and at the same time would provide information about protein synthesis as a whole rather than albumin synthesis alone. Part IV therefore contains the work on ribosomal profiles in the isolated rat liver perfusions.

The review of amino acid pools places in perspective current views on the relationship of amino acids to diet, protein, body compartments and hormones. The principles of the methods used and their specific application are described in a separate chapter, while the detailed methodology is described in the Appendix.

REVIEW OF AMINO ACID POOLS .

Introduction

Concepts of amino acid - protein - hormone interrelationships have advanced in the past decade. Reference will be made to various aspects of these relationships in future chapters. To provide perspective for the work to be described, an attempt will be made to review these relationships.

The processes regulating the hepatic intracellular free amino acid content are represented in Figure 1. The pool may be partitioned into "micro pools", which are supplied by amino acids from (i) the diet, (ii) other tissues and plasma, (iii) protein catabolism, and (iv) synthesis of non-essential amino acids. On the other hand, amino acids are drawn from the pool(s) for (a) protein synthesis, (b) amino acid catabolism, and (c) transport of amino acids to the plasma and other tissues. Changes in this equilibrium occur in response both to the internal metabolic demands of the organism and to external environmental influences. Appropriate responses represent co-ordinated and highly evolved controlling mechanisms incorporating changes at the cellular, intercellular and interorgan level. It should be emphasised that isolation of any one pathway is impossible and in practice could be compensated for by changes in other pathways. However, for convenience, components of the system will be considered independently.

Compartmentation.

There is a growing body of evidence in favour of intracellular compartmentation of amino acids in most tissues of the body (Munro and Portugal, 1970; Portugal and Jeffay, 1966; Portugal et al, 1970; Green and Lowther, 1959; Garfinkel and

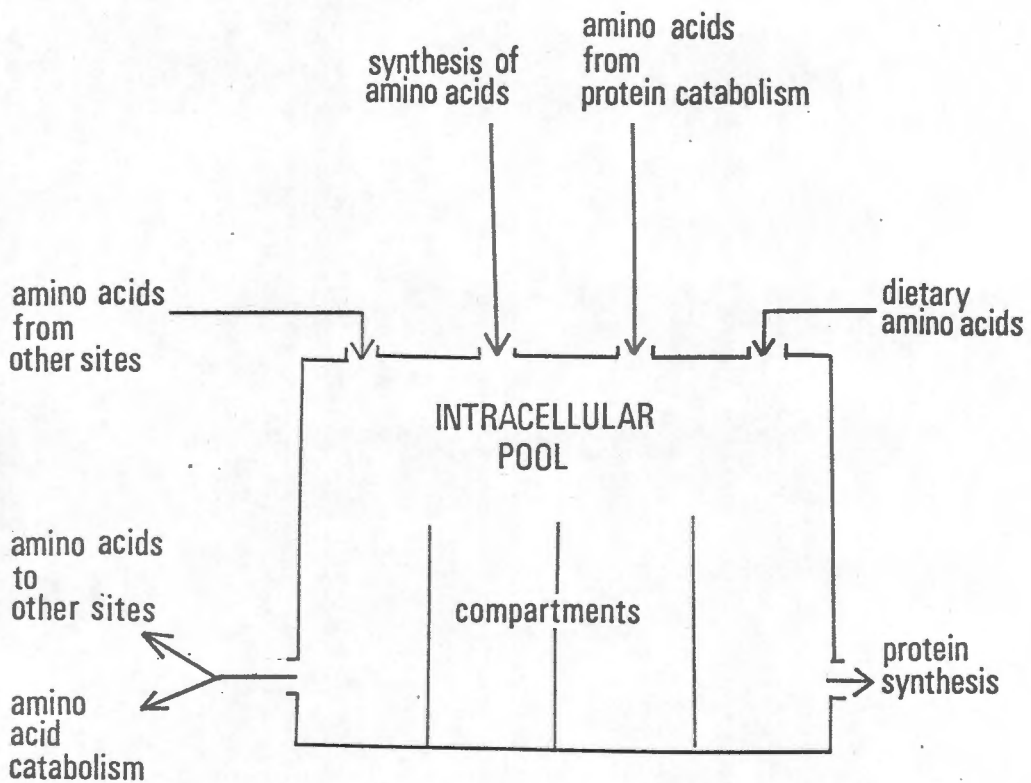


Figure 1: PROCESSES REGULATING THE HEPATIC INTRACELLULAR FREE AMINO ACID CONTENT

Lajtha, 1963; Garfinkel, 1966; Rosenberg et al, 1963; Hider et al, 1969; Kipnis et al, 1961; Henriques et al, 1955; Askonas and Humphrey, 1958; Hendler, 1965 and Ito et al, 1964).

The concept of division of the intracellular amino acid pool into "physical" or "functional" compartments has profound significance. A fundamental application of the heterogeneous intracellular amino acid pool could be the ability of the organism to use this as a biological controlling mechanism. For example, in states of protein depletion or stress, amino acids could be channelled into compartments which supply protein more essential in the particular circumstances. A further application arises from the fact that compartmentation of amino acids is contrary to the basic assumption of nearly all techniques designed to measure protein synthesis by radioactive isotopes. Protein synthesis is frequently estimated from the relationship between the specific activity of the precursor amino acid and the radioactivity incorporated into the relevant protein. However, if compartmentation exists to any degree then it is mandatory for the investigator to measure the relevant precursor specific activity. Technically this represents a formidable task.

The relationship between amino acid supply and protein synthesis.

In 1936, Addis demonstrated the tremendous loss of liver protein on protein deprivation. This has subsequently been confirmed and recently extended to specific proteins. Furthermore, Kirsch et al (1968) and James and Hay (1968) have confirmed the reduction in albumin synthesis during protein restriction inferred by earlier workers (Hoffenberg et al, 1966; Freeman and Gordon, 1964). In vitro, Eagle et al (1961), Riggs and Walker (1963) and Schingoethe et al (1967) have shown that,

within limits, protein synthesis is augmented with increasing amino acid supply. Similarly, in perfusion studies of the isolated rat liver, both Jefferson and Korner (1969) and John and Miller (1969) have shown augmented protein synthesis with increased amino acid supply. In addition, Amenta and Johnston (1963) added hydrazine, which increases free amino acid concentration in both blood and tissues (Korty and Coe, 1968; Cornish and Wilson, 1968), to their perfusions and were able to increase protein synthesis.

If one continues to supply amino acids above requirements, then decreasing proportions of amino acids are incorporated into protein (Meyer et al, 1947; Eckhardt et al, 1948). While amino acids exert some basic control over protein synthesis, there is a great deal still to be learned: do different amino acids influence protein synthesis to different degrees? Is the response protein specific? What is the mechanism of action? While no clearcut answers can be provided, much can be inferred. It is pertinent at this stage to review relevant mechanisms of protein synthesis.

The amino acids to be used for protein synthesis are drawn from the pool of supply (probably extracellular in the case of muscle - Hider, 1969), activated on their carboxyl end and transferred to their respective transfer ribonucleic acid (tRNA) in a series of reactions involving adenosine triphosphate (ATP) and the respective aminoacyl tRNA ligases. While the latter enzymes are specific for their amino acids, more than one tRNA is available for many amino acids. The charged tRNA then places the amino acid in order in the peptide chain according to the code specified by messenger RNA (mRNA).

Two tRNA species with different anticodons for the same amino acid go to different points in the messenger sequence (Weisblum et al, 1965).

The advances in ultracentrifugation, electron microscopy and time sequence tracer studies have clarified the basic mechanisms of protein synthesis. Claude (1937, 1940) first introduced the term "microsomes" which were shown to be widespread in nature (Claude, 1941) and to consist of RNA and phospholipid (Claude, 1943). Electron microscopy by Porter and Kallman (1952) and Porter (1953) led to the definition and naming of the endoplasmic reticulum with its tubules and vesicles. Palade and Siekevitz (1956-1960) described two types of endoplasmic reticulum according to the presence or absence of electron dense particles. In 1958 the term "ribosomes" was introduced by Roberts "to designate ribonucleo protein particles in the size range 20s to 100s". Ribosomes have become established as the structural support for the series of enzymic reactions involved in peptide bond formation and as a carrier of the enzymes involved. The ribosome has a protein content of 55-65% with a high concentration of basic amino acids (Cohn and Butler, 1958; Butler et al, 1960) but little cystine or tryptophan (Crampton and Petermann, 1959) and 35-45% RNA. It was found that the incorporation of radioactive amino acids was concentrated in the 170s fraction rather than the 70-80s fraction while aggregations of ribosomes were demonstrated by numerous workers after gentler methods of cell fractionation were evolved (Warner et al, 1963; Wettstein et al, 1963; Noll et al, 1963; Gierer, 1963; Spyrides and Lipmann, 1962). These aggregates were named "polysomes" by

Warner et al (1962) or "ergosomes" by Wettstein et al (1963). The term polysome is in common usage and is applied to groups of five or more ribosomes on a mRNA strand. Polysomes are regarded as indicators of active protein synthesis although Munro et al (1964) showed that single ribosomes are probably active if attached to mRNA.

Ribosomes have two sites to which tRNA binds, an "A" site (aminoacyl) which is responsible for the initial binding, and a "P" site (peptidyl) to which the tRNA peptide chain is transferred. In mammalian cells the presence of chain initiation factors (Galper and Darnell, 1969; Reboud, 1969; Arnstein and Rahamimoff, 1968; Mosteller et al, 1968; Smith and Marcker, 1970; and Prichard et al, 1970) is debated, while the presence of termination factors is even more uncertain. Peptidyl transferase and translocase are found within the ribosome. Peptidyl transferase catalyzes the reaction between the free carboxyl group of the nascent peptide chain on the "P" site and an amino group of the entering aminoacyl tRNA on the "A" site. Translocase catalyzes the transference of newly made peptidyl tRNA from the "A" site to the "P" site.

In 1961, Birbeck and Mercer suggested that endoplasmic reticulum-bound ribosomes and free ribosomes manufacture export and intracellular protein respectively. Considerable support for this concept has emerged. Thus Redman (1968), Takagi and Ogata (1968); and Hicks et al (1969) found that albumin was synthesized chiefly on the endoplasmic reticulum-bound ribosomes. Ganoza and Williams (1969) reported similar findings for all plasma proteins. Ganoza and Williams (1969) and Hicks et al (1969) found that free and bound ribosomes

were responsible for liver cell sap protein and ferritin synthesis respectively. However, Ragnotti et al (1969) found that both populations were able to incorporate labelled amino acids into cytochrome C reductase of liver microsomes. The latter findings have yet to be confirmed. A rat liver cell contains 60% bound ribosomes and 40% free ribosomes.

Ribosomes are synthesized within the nucleolus (Brown, 1966). However, ribosomal proteins are found in the cytoplasm (Lindsay, 1966; Vincent et al, 1966; Mundell, 1967) and are probably synthesized on cytoplasmic polysomes (Ogata et al, 1967 and Terao et al, 1968). The complete ribosome is probably assembled within the nucleolus and matures progressively as it reaches the cytoplasm (Darnell, 1968).

The total amount of deoxyribonucleic acid (DNA) per diploid nucleus is considered to be essentially constant at 7×10^{-12} grams from one mammal to another and from one tissue to another in the same animal (Vendrelly, 1955). This contrasts with the RNA constituent where an increase in body size is associated with increased RNA synthesis and an increased rate of protein synthesis. Therefore with regard to DNA, tissue differences are achieved by suppressing part of the information contained in the structural gene of the cell so that only appropriate proteins are produced. Gurdon and Uehlinger (1966) have shown that even in specialised cells this information is potentially available. They showed that when the nuclei from the intestinal epithelium of the frog are transferred to an enucleated frog ovum, embryonic development still occurred. This suggests that cytoplasmic mechanisms regulate the expression of nuclear information. It is probably the histone

fraction of the chromosomes which is responsible for masking the remaining DNA template activity. Histones are probably synthesized on cytoplasmic polysomes and subsequently transported into the nucleus (Robbins and Borun, 1967; Gallwitz and Mueller, 1969). Histones are basic proteins that have been fractionated into three major types : (1) lysine rich, (2) moderately lysine rich, and (3) arginine rich (Bonner et al, 1968; Kinkade and Cole, 1966; Stellwagen and Cole, 1968; Hnilica, 1967). Their suppressive role can be modified by their interaction with non-histone protein, (Marushige et al, 1968; Paul and Gilmour, 1968) by phosphorylation and by acetylation (Allfrey et al, 1966; Allfrey, 1968). Phosphorylation of histones by cyclic adenosine monophosphate has been described (Langan, 1968).

The role of RNA polymerase in the DNA- directed synthesis of RNA is well established (Reviews by Hayes, 1967; Martin and Tocchini-Valentini, 1967; Chambon, 1968; Geiduschek and Haselkorn, 1969). 70-80% of this rapidly labelled RNA is broken down in the nucleus and the remainder probably constitutes mRNA. While Goldstein and Prescott (1968) have shown the rapid migration of protein into and out of the nucleus, protein particles have been identified in the nucleus associated with rapidly labelled RNA which is probably mRNA. This protein, synthesized on cytoplasmic polysomes, could perform the dual function of transport of mRNA out of the nucleus and directing its association with the appropriate ribosomal population.

Thus, while the nucleus undoubtedly contains the primary information for protein synthesis, the process appears to be subject to cytoplasmic feedback control mechanisms which may be

of greater significance than is generally appreciated.

Protein catabolism.

The hepatic intracellular amino acid pool is supplied mainly by the diet. Buchanan (1961) showed that 70% of rat liver protein is replaced every four to five days from dietary sources. Reduction of dietary amino acid supply is compensated for by an increased utilisation of amino acids from other sources. The response by the organism to maintain its hepatic amino acid pool size during protein deprivation is a fascinating phenomenon. The immediate response appears to be an attempt to maintain the status quo with the channelling of amino acids from protein by catabolism, decreased catabolism of essential amino acids and transport of amino acids from other tissues to the liver. The major early compensating mechanism is the increase in hepatic protein catabolism. Gan and Jeffay (1967), using a constant infusion technique in rats, showed that recycling of amino acids from liver protein degradation provides 50% of the free amino acid pool of fed rats but 90% of the pool in fasted rats. In addition, Stephen and Waterlow (1966), using the differential decay rate of the carboxyl relative to the guanido carbon of arginine, concluded that normally 50% of arginine liberated is re-utilised whereas about 70% is re-utilised for protein synthesis on a low protein diet. However, protein degradation is poorly understood, not only at the control level but even the basic mechanisms - that is the site, and the physico-chemical reactions involved. Current concepts will be reviewed briefly at this stage.

Most proteins are degraded according to first order kinetics. Once formed they have a random chance of degradation.

The molecule could be considered to be in equilibrium in a number of forms and only certain configurations are liable to degradation. Normally, the majority of molecules are in a state resistant to degradation. Increased susceptibility to degradation could be induced "by interaction with small molecules, similar or dissimilar peptides and subunits or by association with various intracellular organelles" (Schimke, 1970). A degradation system(s) with its relevant features is shown in Figure 2.

Altered or denatured proteins are more readily degraded than are "native" proteins. (Green and Neurath, 1954; Linderstrøm-Lang, 1950). The conformational change involved would depend on the type of peptide bond exposed, specificity of the system or interaction with other molecules. This is exemplified by the following examples : Markus (1965) has shown that, while the binding of methyl orange to albumin causes no change in sedimentation or optical rotatory dispersion properties, it materially inhibits the proteolytic degradation of albumin. Similarly, Mazur and Shorr (1950) have shown that purified ferritin with a high iron content is less susceptible to inactivation by proteolysis than is ferritin with low iron content. Also, tryptophan stabilizes tryptophan pyrolase to trypsin inactivation particularly when the enzyme is completely conjugated with haematin (Schimke et al, 1965a). According to Schimke (1970) "the concept that the rate of degradation of a protein is determined by the properties as a substrate for a non-specific degradation system is supported by studies with various enzymes and cell organelles". Thus, irrespective of dietary intake (8-70% protein), degradation (expressed as a first order constant or half-life) is constant for many proteins, e.g. arginine

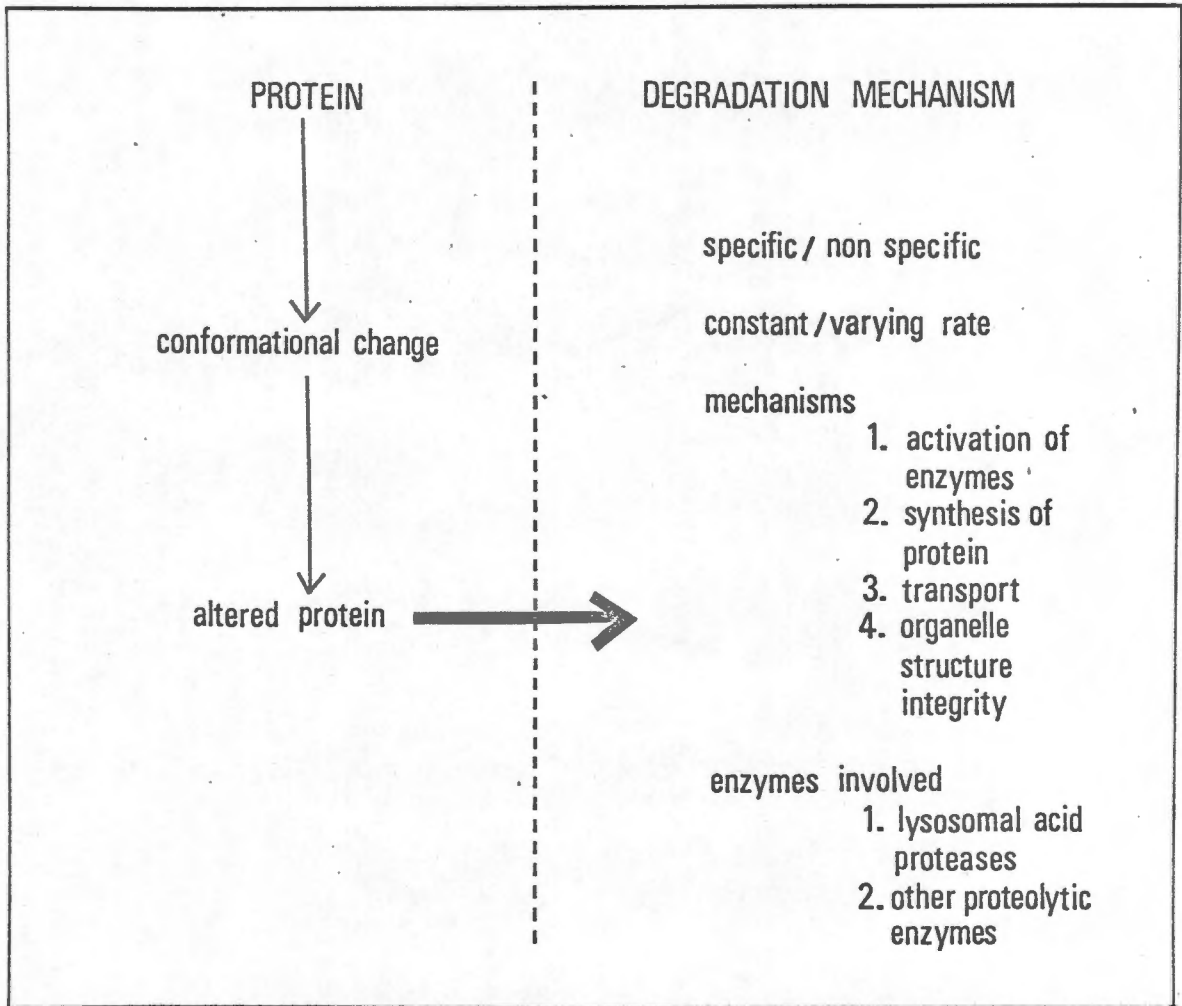


Figure 2: PROTEIN DEGRADATION SYSTEM

(Schimke, 1964), xanthine oxidase (Rowe and Wyngaarden, 1966), total mitochondrial protein (Swick et al, 1968) and total liver protein (Schimke et al, 1964).

The site of conformational change may differ from the site of degradation. This may be in a different organ or organelle depending on the protein or the conditions, and thus transport may constitute another regulatory site. The degradation system may be specific for a particular protein or non-specific. In normal circumstances it would operate at a constant rate so that the fractional catabolic rate would remain constant but the absolute rate would depend on the magnitude of the protein pool. However, as will be discussed in a later chapter, with respect to albumin, the fractional rate can change as well in certain conditions. The factor involved in this change is unknown but it is probably mediated by hormonal changes and the response dependent on the tissue involved.

The mediator of the conformational change could operate by activation of an enzyme, increasing enzyme protein synthesis, transport of the substrate (protein) as already mentioned or change in the structural integrity of the organelle involved. It is possible that all these mechanisms may be operative to different degrees depending on the protein. In the tadpole, inhibitors of protein synthesis were shown to prevent a response of acid protease to thyroxin which normally occurs in the process of metamorphosis (Weber, 1967), while the glucocorticoid-induced collagenolytic and proteolytic activity in the skin were blocked by puromycin and actinomycin (Houck et al, 1968). Degradation of intracellular protein is inhibited by agents that inhibit energy production (Simpson, 1953; Steinberg and Vaughan,

1956). This has been re-evaluated by Brostrom and Jeffay (1970) who showed that the action of several types of inhibitors, including energy or respiratory inhibitors, is dependent on the level of tissue integrity. They concluded that "there may be a structural component involved in protein catabolism, the integrity of which requires metabolic energy, while the enzymes involved in the actual degradation of proteins probably do not have an energy requirement". The lysosome system has been implicated in a number of catabolic processes. While steroids stabilise lysosomes, progesterone increases their breakdown (Weissman and Thomas, 1964). Lysosomal acid proteases are probably involved in tail metamorphosis in amphibians (Weber, 1967), the increased turnover in muscle proteins in hereditary muscular dystrophy of mice (Tappel et al, 1962), the increased degradation involved in vivo (Ashford and Porter, 1962; Deter and de Duve, 1967) and the increased protein degradation obtained when glucagon is added to fluid perfusing rat livers (Green and Miller, 1960; Miller, 1961). However, proteolytic enzymes with properties, pH optima and distribution different from the cathepsins of lysosomes have been described in the brain, (Marks and Lajtha, 1963, 1965), kidney and liver (Fruton, 1960; Green and Neurath, 1954; Smith, 1951.) On the other hand, tissue inhibitors of protease activity have been reported by Finkenstaedt (1957) and Blackwood and Mandl (1964). The mechanism and control of degradation can be seen to be highly speculative at this stage. It has been common to draw conclusions from "liver protein" experiments which may be different from changes in other tissues. Furthermore, examination of large populations of proteins may mask changes

in individual proteins. Degrees of degradation undoubtedly exist and not only protein molecular integrity but also functional activity and possibly immunological reactivity should be assessed. Thus Schimke et al (1965b) showed that enzyme activity and immunological reactivity may not necessarily be lost together. He found that tryptophan pyrrolase activity was lost, while immunological properties were retained following degradation in crude homogenates.

Despite the lack of basic knowledge in this field, the contribution of protein catabolism to the amino acid pool is established. This is hardly surprising when one considers that the total free amino acid content of tissues is 15-30 millimoles while the protein-bound amino acid concentration is 60-120 times this quantity. A small change in protein catabolism can therefore bring about a vast change in the intracellular free amino acid concentration. Interplay of protein catabolism and diet is well illustrated by the work of Munro and his colleagues. In vitro all amino acids (except isoleucine) were shown by Baliga et al (1968) to be essential for ribosomal aggregation. In vivo only tryptophan became limiting, the other amino acids being supplied by protein catabolism. However, when recycling of amino acids is reduced by protein restriction, other amino acids as well as tryptophan were shown by Pronczuk et al (1969) to be limiting to ribosomal aggregation. An important corollary arises : to what extent can control of protein catabolism be a regulating mechanism of a protein synthesis and, more basically, what controls protein catabolism ?

Amino acid synthesis and catabolism:

In 1937 Braunstein and Kritzman discovered the transaminases which play a major role in the transfer of nitrogen between compounds. On the other hand, the work of Rose (1938) established that the body is dependent on the diet for certain "essential" amino acids. Those amino acids found to be "essential" for man are tryptophan, lysine, leucine, isoleucine, valine, phenylalanine, threonine and methionine. The rat has similar requirements although, in addition, histidine and arginine are required. The "non essential" amino acids can be synthesized by the body if substrate is provided.

Degradation of amino acid offers a rapid means of controlling tissue amino acid concentrations. The effect of hydrazine in raising tissue levels of amino acid by inhibiting transamination has already been described. Miller (1962) has shown in perfusion studies that the liver is required for degradation of arginine, histidine, lysine, methionine, phenylalanine, threonine, tryptophan and tyrosine. The branched chain amino acids are degraded chiefly at extra hepatic sites. Thus Ichihara and Koyama (1966) found high concentrations of specific transaminases in heart, kidney and skeletal muscle and very much smaller concentrations in liver. In addition, perfusion of the isolated rat liver has shown a fall or no change in most amino acids while the branched chain amino acid concentrations have risen progressively (Schimassek and Gerok, 1965; Fisher and Kerly, 1964).

Hankes et al (1967a, 1967b) and Daniel and Waisman (1969)

demonstrated that an increased amino acid intake is associated with increased degradation of that amino acid. By measuring rat liver enzymes involved in amino acid metabolism under increasing protein intakes, Harper (1968) has concluded that enzymes involved in the metabolism of non-essential amino acids undergo adaptation related to the amount of protein consumed, while enzymes for catabolism of essential amino acids adapt to dietary protein levels as they relate to the needs of the body. In support of this concept is the work of McFarlane and von Holt (1969) who found that the oxidative degradation of leucine and phenylalanine markedly decreased on a 2% casein diet whereas glutamate and alanine were unaffected.

The unique role of dietary tryptophan in vivo in relation to protein synthesis is possibly related to the complex changes in its degradative enzyme, tryptophan pyrrolase, brought about by a meal. Thus the work of Knox (1951), Knox and Mehler (1951), Civen and Knox (1959), Lee (1956), Dubnoff and Dimick (1959), Schimke et al (1964 and 1965a), Knox et al (1966) and Greengard and Feigelson (1961a, 1961b, and 1962) have established that tryptophan pyrrolase is stabilised by its substrate tryptophan and induced by glucocorticoids. This once again highlights the basic amino acid-protein-hormone relationships.

Amino acid transport and interorgan flux.

The work of Christensen (1966, 1968) has done much to clarify amino acid transport across the cell. Using Ehrlich ascites tumour cells, he has shown that amino acids are transported in groups according to their structure. Each group probably has a specific carrier protein. Amino acids from the same group may inhibit one another or in some cases

stimulate transport of another group by an exchange mechanism. This is contrary to the data of Schafer and Jacquez (1967) who showed competitive stimulation of the transport of some free amino acids into ascites tumour cells by others probably unrelated to the "exchange mechanism". Similar mechanisms are probably responsible for transport out of cells and the net effect probably depends on the balance between influx and efflux in relation to other intracellular processes already described.

Twenty amino acids are commonly found in proteins. However, alanine, glutamine and glutamic acid, and glycine constitute 80% of the total free amino acid pool. While these four amino acids are probably chiefly responsible for amino nitrogen transport between organs, the plasma proteins may play a limited role by virtue of their widespread degradation. Felig et al (1969) consider that the amino nitrogen resulting from peripheral degradation reactions is probably transported to the liver mainly in the form of alanine.

One of the most striking actions of some hormones is their effect on plasma and tissue amino acid levels. It is frequently difficult to determine their specific action on transport as the net effect could well be a result of the action on transport, synthesis of amino acids and protein and degradation of amino acids and protein. Furthermore, one process may trigger another : elevated amino acids definitely increase certain hormonal levels (Cochrane et al, 1956; Floyd et al, 1966; Knopf et al, 1965; Parker et al, 1967; Cremer et al, 1968; Munro and Mukerji, 1962; Munro et al, 1965). On the other hand, Noall et al (1957) suggested that hormones

trigger protein synthesis in target organs by increasing flow of amino acids to that organ. No attempt will be made to review the effect of hormones on protein synthesis but instead, keeping to the central theme of this review, the effect of hormones on interorgan amino acid fluxes are illustrated in Figure 3.

Cortisone or cortisol increases the levels of amino acids in muscle (Kaplan and Shimizu, 1963; Ryan and Carver, 1963; Bethell et al, 1965) but this appears to be due to decreased muscle protein synthesis (Kostyo and Redmond, 1966). Noall et al (1957) found that a few hours after intravenous administration of hydrocortisone, the transport of alpha amino isobutyric acid into the liver was increased. Weber et al (1965) found that steroids increased the level of free amino nitrogen in the liver of feeding rats, while Bethell et al (1965) found that this increased only glutamic acid, aspartic acid and alanine.

Thyroxine increased the levels of all amino acids in the liver whereas in the carcass only the concentration of histidine, lysine and tryptophan were significantly increased. (Wellers and Leblanc, 1966). Both cortisol and thyroxine mobilise amino acids from muscle to the liver but the difference in amino acid concentrations finally achieved is probably due to differing actions of cortisone and thyroxine on the amino acid pathways. Noall et al (1957) found that growth hormone increased the penetration of alpha amino isobutyric acid in all tissues except cardiac muscle.

Mondon and Mortimer (1967) have shown that during

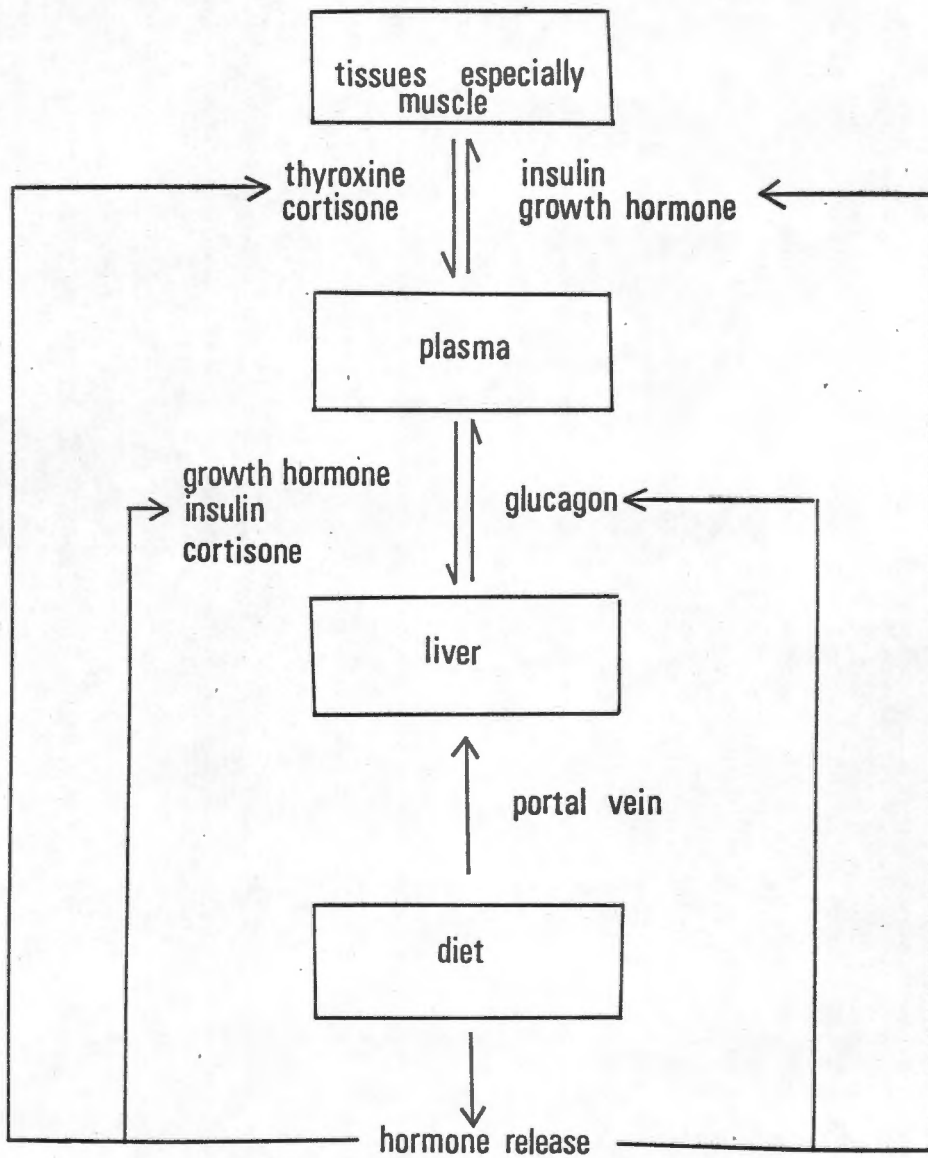


Figure 3: INFLUENCE OF HORMONES ON INTERORGAN AMINO ACID FLUX

perfusion of the isolated rat liver, insulin decreased the release of amino acids into the perfusate, probably due to decreased protein degradation. Sanders and Riggs (1967) have demonstrated increased penetration of model amino acids into the liver following insulin administration. This appears to be secondary to other changes. Wool (1964) found that insulin results in accumulation of only six amino acids within muscle despite the reduction in all plasma amino acids. Wool and co-workers (Castles and Wool, 1964; Scharff and Wool, 1965; Wool and Scharff, 1968) have suggested that protein synthesis removes the others too rapidly to allow their accumulation.

While thyroxine and cortisone mobilise amino acids from the muscle (Fig.3), glucagon appears to have this effect on liver. On the other hand, cortisone, insulin and growth hormone increase amino acid transport into the liver while the latter two hormones have the same effect on muscle.

The response of the body is probably a sequential response with initially glucagon increasing hepatic protein catabolism and augmenting the diet by increasing the intracellular hepatic amino acid pool. Insulin then decreases catabolism and increases penetration of amino acids into muscle and, more sluggishly, into the liver. In a post-absorptive phase cortisone probably mobilises amino acids from the carcass to the liver. In a fasted state glucagon levels are increased and this may represent the attempt by the body to maintain the hepatic intracellular amino acid pool. However, tryptophan is still limiting to protein synthesis (see before) in the circumstances. The increased amino acid

levels in the liver are wasted in the absence of sufficient tryptophan; glucagon levels therefore decline after forty-eight hours of fasting. The balance between the hormones and their interaction with diet may be fundamental in controlling basic aspects of protein metabolism. It is interesting to note the degradation rates of hormones by the liver (Figure 4), which in conjunction with other tissues, further modifies hormone levels. From this Figure it can be seen that insulin and glucagon are rapidly degraded by the isolated rat liver (Kelman et al, 1971) while parathyroid hormone, proinsulin and growth hormone are degraded very slowly. Albumin degradation is shown for comparison. This proposed response is probably an oversimplification but it is an attempt to emphasise the complex control of the hepatic intracellular amino acid pool. Emphasis has been placed on the liver but control takes place at all levels of cellular and organ function.

The influence of diet.

The daily intake of each amino acid in dietary protein is many times greater than the pool of the corresponding free amino acid. In 1912 van Slyke and Meyer showed that the response to a meal of protein was characterised by an increase in the plasma amino nitrogen. If one essential amino acid is present in relatively low concentration in the dietary protein, it will tend to be removed by the tissues as rapidly as it is absorbed. In these circumstances, the limiting amino acid of dietary protein is indicated by the lowest rise of an amino acid in the plasma relative to requirements of the animal (Longenecker and Hause, 1959, 1961; Longenecker, 1963). This was confirmed

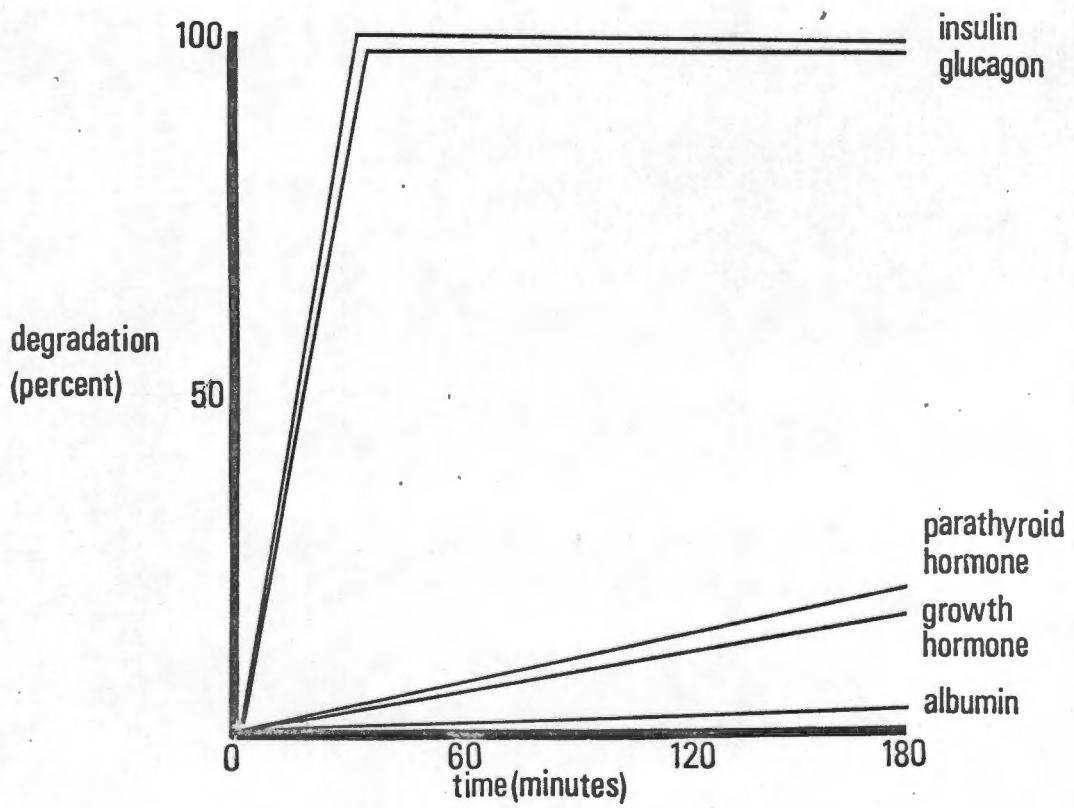


Figure 4: DEGRADATION OF GLUCAGON, INSULIN, GROWTH HORMONE, PARATHYROID HORMONE AND ALBUMIN BY THE ISOLATED, PERFUSED RAT LIVER

in man by Yearick and Nadeau (1967). The portal blood response to dietary protein is up to five times that of the peripheral blood (Denton et al, 1953; Denton and Elvehjem, 1954; Ganapathy and Nasset, 1962; Porter and Williams, 1963; Peraino and Harper, 1963; Elwyn, 1966). Similarly, the portal blood amino acid concentration in general reflects that of the dietary amino acids (Denton and Elvehjem, 1954; Goldberg and Guggenheim, 1962; McLaughlan et al, 1963; Pion et al, 1964; Buraczewski et al, 1967). However, Elwyn (1968) has shown that the exchange of nitrogen between aspartate, glutamate, glutamic acid and alanine takes place at gut level. Thus very little glutamine or aspartate but high concentration of alanine and ammonia are present in portal blood. In addition, while glutathione is absent in the diet, large amounts of it appear in portal blood and this accounts for more cystine and glutamic acid than appears as free amino acids. Thompson et al (1950) have shown that most essential amino acids in liver, muscle and spleen are decreased in the rat on a protein-free diet. In all tissues histidine and arginine were increased, while in liver and muscle threonine was increased. Wiss and Krueger (1949) and Wiss (1949b) also showed the reduction of the essential amino acids and the increased histidine levels. Under conditions of protein restriction in rats (Wiss, 1948, 1949a; Henderson et al, 1949), dogs (Longenecker, 1961) and pigs (Richardson et al, 1965) the plasma essential amino acids are reduced, except for histidine and lysine. In man (Tuttle et al, 1962; Swendseid et al, 1966, 1968; Adibi, 1968) similar changes occurred. In rats (Wiss, 1949a) and man (Swendseid et

al, 1963, 1966, 1968; Young and Scrimshaw, 1968) the non-essential amino acids were increased or remained constant, while in kwashiorkor in children (Holt et al, 1968) similar changes were found. The study of the effects of different diets on amino acid pools is fraught with problems, apart from trying to separate the part played by the different contributions to the pool. Decreased amino acid intake or the administration of imbalanced amino acid diets leads to falling off of appetite which can be reversed by supplying the missing amino acid directly to the blood supply of the brain (Leung and Rogers, 1969). Pair feeding would seem the logical control. Unfortunately this results in the control animals consuming all their food in a short space of time and one is therefore dealing with a fasted control and a fed experimental animal. Forced feeding, which is the other alternative, is associated with metabolic changes (Sidransky and Farber, 1958; Sidransky and Verney, 1964; Sidransky et al, 1964; Staehelen et al, 1967). These include increase in liver size, uptake of amino acid into protein and increased RNA content with the shift of polysomes towards heavier classes. The reverse occurs in muscle. Wagle and Sidransky (1965) have shown that adrenalectomised rats on cortisone still show this response and Leon et al (1965) have shown that physical stress applied to adrenalectomised rats still results in an increased capacity for protein synthesis.

The administration of high protein diets is associated in infants with elevated plasma amino acids, especially the branched chain amino acids, with no change in growth rates (Holt et al, 1968; Snyderman et al, 1968). In rats, high protein

diets led to increased levels on the first day of the diet but thereafter levels decreased to normal range, except for the branched chain amino acids (Anderson et al, 1968).

PRINCIPLES OF METHODOLOGY.

ALBUMIN SYNTHESISIntroduction

The introduction of radioactive isotopes offered new opportunities for quantitative measurements of protein metabolism. Nitrogen balance studies were gradually superseded by kinetic measurements of the individual protein.

Until recently, the standard method of measuring albumin synthesis was to derive synthesis from catabolic studies in nutritional equilibrium, when synthesis was presumed to equal catabolism. This suffered from the drawback of having to derive synthesis over long periods of time and, more limiting, to be dependent on steady state conditions. Another approach was that of Grossman et al (1960): total body half-life of (^{131}I) albumin provides a measure of the fraction of total body pool catabolised per day. The extra- to intra-vascular ration of the protein applied to total body catabolism provides a measure of the true fraction of intra-vascular protein catabolised per day. However, plasma half-life represents both catabolism of labelled molecules and dilution of labelled pools by unlabelled, newly synthesized molecules. The difference between the two fractional values provides a measure of synthesis. Problems arise with the accurate measurement of pool sizes, the time required and the fact that the pool must either remain constant or be remeasured frequently. Matthews (1961) proposed a method for measuring albumin synthesis and transfer rates of albumin molecules from the extra-vascular compartment. However, the individual contributions to the "synthesis plus transfer rate" cannot be accurately quantitated

although approximate values can be obtained by means of an analogue counter.

Most estimates of the synthesis rate of proteins are based on the precursor - product principle. Labelled amino acids are used and an estimate of synthesis is obtained by the ratio of protein-bound radioactivity relative to free labelled amino acid at the site of protein synthesis, i.e.

$$M = \frac{R}{a} \dots \dots \dots \text{Equation 1.}$$

where

M = Mass of protein synthesized.

R = Radioactivity incorporated into protein.

a = Mean specific activity of the amino acid.

The main problem is the accurate measurement of the precursor amino acid specific radioactivity at the site of protein synthesis. The radioactivity achieved in the protein is dependent on the rate of incorporation of the labelled amino acid into the protein. This in turn depends on the proportion of labelled amino acid relative to the total concentration of that amino acid in the precursor pool. The latter is subject to numerous variables: the dose of labelled amino acid administered, the blood flow to the organ, transport across the cell membrane, intracellular compartmentation, synthesis and degradation of amino acids, catabolism of protein and supply of unlabelled amino acids (Figure 5). The variables are enormous - not only from condition to condition, but also from individual to individual and from one period to another. It is therefore clear that the precursor specific activity must be measured. Plasma amino acid specific activities are worthless, while serial biopsies for the precursor specific activity are impractical. The next logical development was

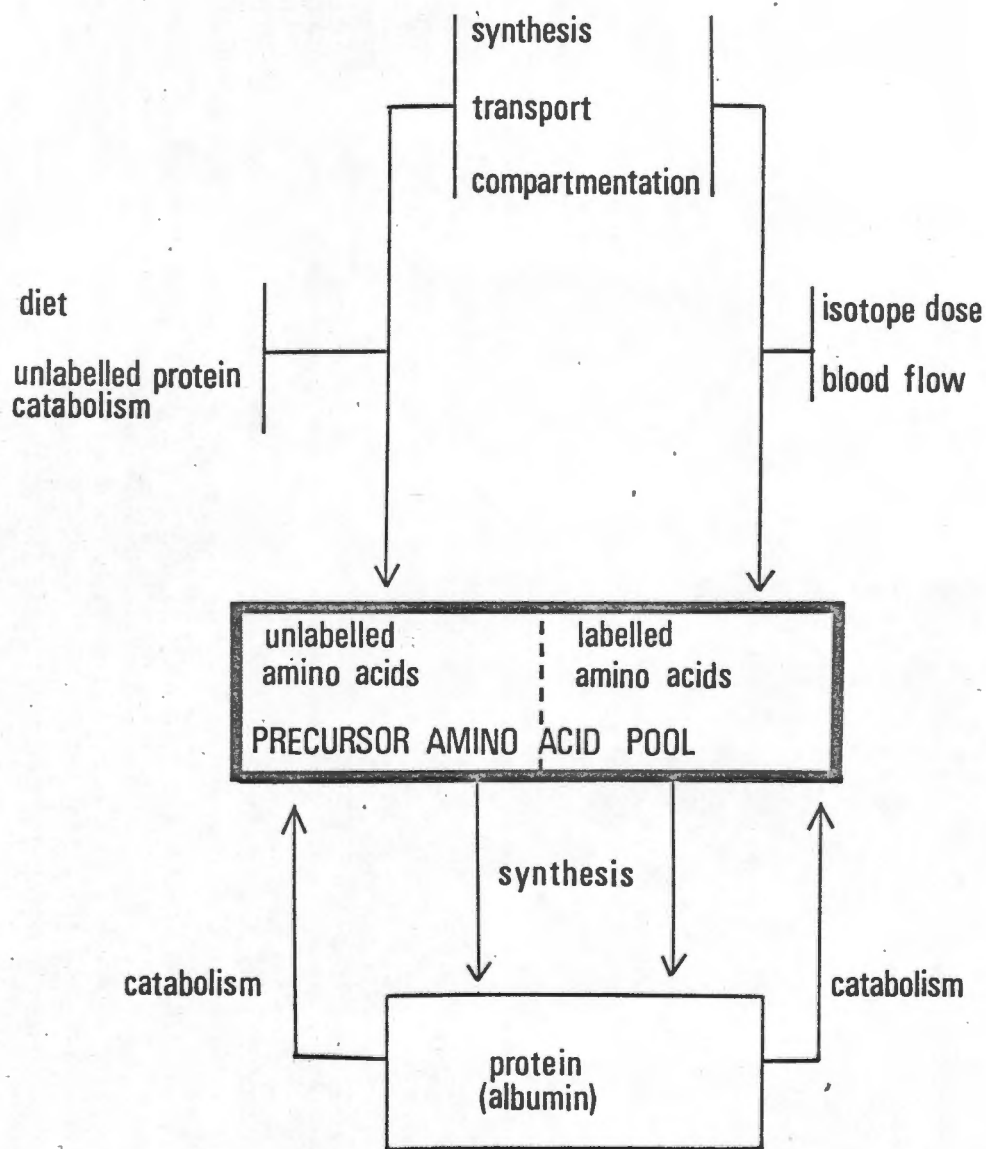


Figure 5: FACTORS INFLUENCING THE PRECURSOR AMINO ACID SPECIFIC ACTIVITY

a means of estimating the intracellular precursor activity. However, to be effective, it had to be possible to sample this easily and at least at two points in time to obtain a measurement of rate of synthesis. This is only practical in tissue culture or cell free systems.

It therefore became imperative to find an extracellular indicator of intracellular events. Hippurate was tried as an indicator of glycine precursor pool specific radioactivity but this approach proved to be fraught with problems (Weismann et al, 1961).

In 1946, Delluva and Wilson demonstrated that $^{14}\text{CO}_2$ combines with endogenously produced ammonia and ornithine in the liver to form arginine labelled only in the guanidine carbon atom. The arginine may then be used for albumin synthesis while the guanidine carbon of arginine may be cleaved off by the action of arginase to provide the urea carbon atom (see diagram of Krebs-Henseleit cycle, Figure 6). Swick (1958) fed rats a diet containing calcium (^{14}C) carbonate and compared the final specific activity of urea and of liver protein arginine guanidine carbon. Because of the close relationship between guanidine carbon of arginine and the carbon of urea, he suggested that urea released from the liver be used as an indicator of the specific activity of the guanidine carbon of intracellular arginine. Both McFarlane (1963a) and Reeve et al (1963) independently defined the principles of the system and established the technique known as the (^{14}C) carbonate technique for liver-produced plasma protein synthesis.

The impetus given to the investigation of albumin metabolism by the work of Delluva and Wilson, Swick, McFarlane

and Reeve et al has been enormous. Equipped with tools for simultaneously measuring synthesis and catabolism independently, the investigator is able to separate and identify the respective controlling mechanisms.

An alternative method of measuring protein synthesis directly is based on the principle that if a labelled amino acid is infused continuously, a constant plateau precursor specific radioactivity will be achieved, the level depending on conditions and the tissue examined (Waterlow and Stephen, 1968 and Gan and Jeffay, 1967). This technique has a number of disadvantages: the plateau specific radioactivity must be defined for each condition and tissue, the tissue must be sampled, albeit only once, after the plateau has been defined and relatively large doses of isotope must be used. This technique is therefore unlikely to achieve application in humans.

Principles of the method.

Essentially, the method depends on a comparison of total activities and total masses of carbon incorporated into two products of the same precursor in a given time.

Equation 1 can be rewritten :

$$a = \frac{R}{M} \quad \dots \quad \text{Equation 2.}$$

If more than one product is derived from the same precursor then :-

$$a = \frac{R_1}{M_1} = \frac{R_2}{M_2} = \frac{R_3}{M_3} \quad \dots \quad \frac{R_a}{M_a} \quad \dots \quad \text{Equation 3.}$$

In the case of the (^{14}C) carbonate technique

$$\frac{R \text{ albumin}}{M \text{ albumin}} = \frac{R \text{ urea}}{M \text{ urea}} \quad \dots \quad \text{Equation 4.}$$

where M albumin = the mass of albumin guanidine carbon synthesized

M urea = the mass of urea carbon synthesized

R albumin = total activity in albumin arginine guanidine carbon

R urea = total activity in urea carbon.

The method has been mathematically defined by Reeve et al (1963) and Reeve (1965). Figure 7 depicts the system in a simplified form. The common precursor pool is shown to consist of units each emitting 10 counts per minute. For each molecule of arginine in albumin or of urea synthesized, one unit of precursor is depicted as being utilized.

Therefore

$$\frac{\text{mass of albumin}}{\text{mass of urea}} = \frac{\text{radioactivity of albumin}}{\text{radioactivity of urea}} \text{ i.e. } \frac{50}{20} = \frac{5}{2}$$

Thus the absolute rate of albumin synthesis may be obtained if the total activity incorporated into the two products and the absolute rate of urea synthesis are known.

This system is shown in Figure 8. The four basic

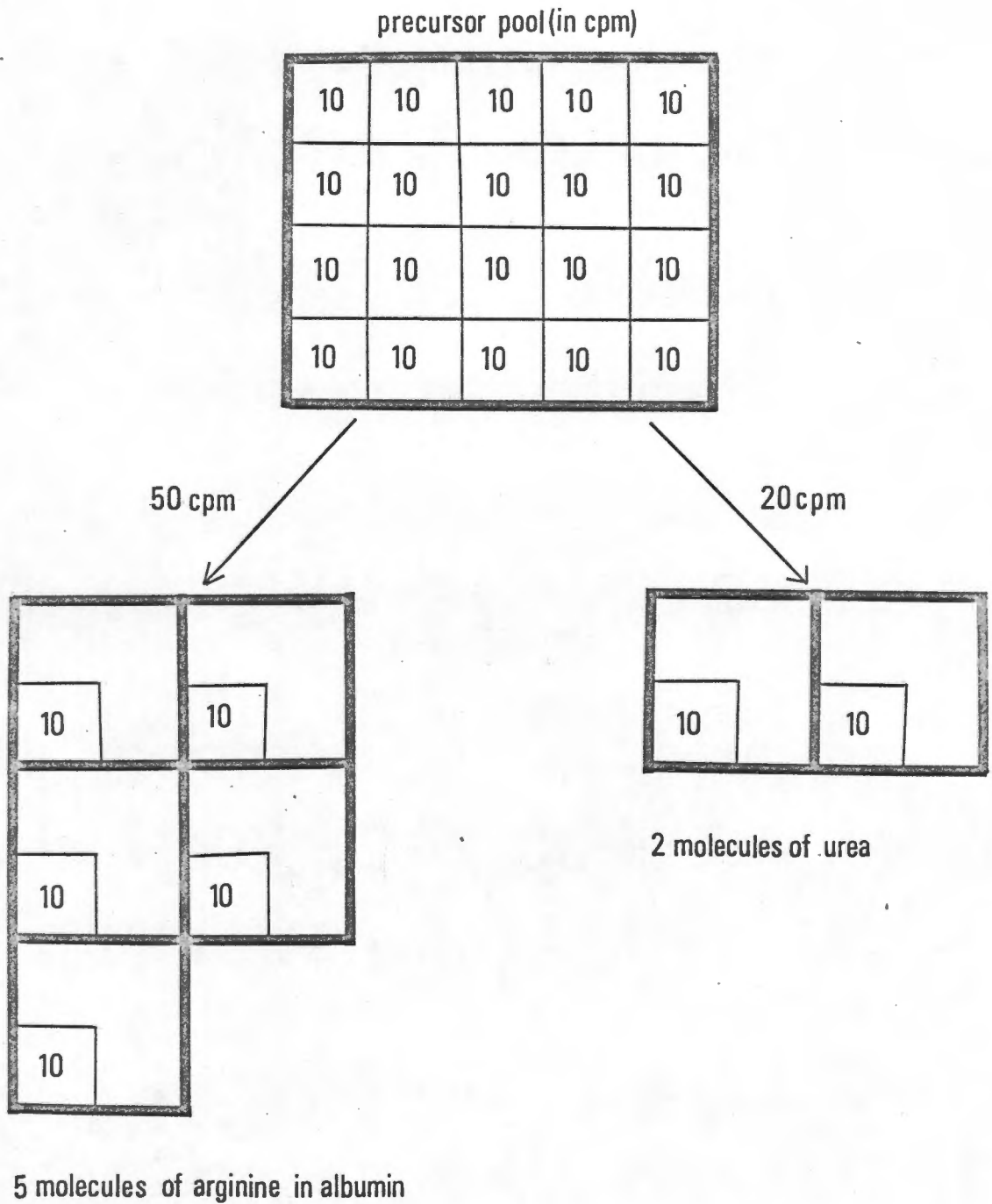


Figure 7: PRINCIPLE OF THE CARBONATE METHOD

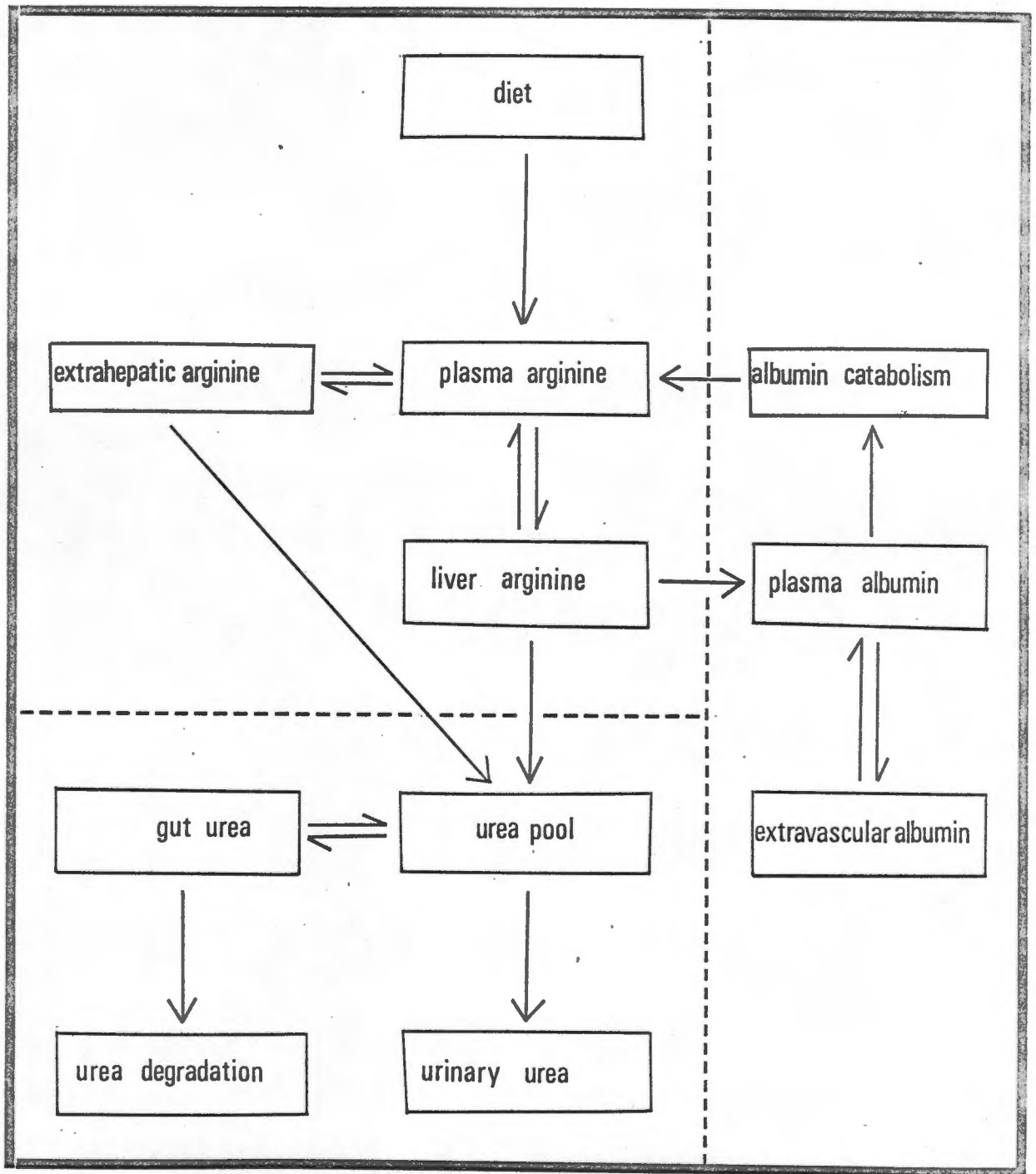


Figure 8 : THE ARGININE, ALBUMIN AND UREA POOLS OF THE CARBONATE METHOD

components of the system include (1) the urea system, (2) the arginine system, (3) the albumin system, and (4) the (^{14}C) label.

The urea system.

Bollman et al (1924) demonstrated that urea is largely synthesized in the liver. The finding of arginase activity in the brain, skin, plasma, kidney and intestine implied that urea could be formed at a site other than the liver. This would negate one of the basic prerequisites of the method that both urea and albumin synthesis occur at the same site.

Tavill et al (1968) found essentially no urea synthesis extrahepatically while Kornberg et al (1952) concluded that in cats 2% of total urea synthesis could result from extrahepatic urea synthesis. For practical purposes therefore, urea is synthesized exclusively in the liver.

After synthesis in the liver, urea diffuses rapidly into the plasma and then throughout the body water. The body water urea is either broken down by the gut or excreted in the urine. From Equation 4 it is apparent that two measurements in the urea system are required :

- (1) the mass of urea produced, and
- (2) the radioactivity achieved in urea.

Koj et al (1964) suggested that the mass of urea synthesized could be obtained from the product of the fractional rate of urea synthesis and the body urea pool. However, Regoeczi et al (1965) showed that inaccuracies existed in the measurement of the size of the body urea pool, while the assumption, on which this method depends, that the urea is

present throughout the body water at the same concentration as in the plasma, is invalid. McFarlane et al (1965) therefore suggested a modified formula substituting specific activities for total activities by dividing both sides of Equation 4 by the sizes of the urea carbon and guanidine carbon pools. The following formula is thus obtained :

$$\frac{\text{FSR albumin}}{\text{FSR urea}} = \frac{\text{SA albumin at time } t}{\text{SA urea at time } t} \dots \text{Equation 5.}$$

This can be rearranged :

$$\text{FSR albumin} = \frac{\text{FSR urea} \times \text{SA albumin at } t}{\text{SA urea at time } t} \dots \text{Equation 6.}$$

Where FSR = fractional synthesis rate

SA = specific activity.

In a closed system this specific activity at time t can be replaced by the maximum specific activity reached. However, in vivo losses of urea occur during measurement as shown in Figure 8. By extrapolating the curve of plasma urea specific activity to zero time, a hypothetical maximum value for (^{14}C) urea specific activity can be obtained before losses have time to occur. However, extrapolation does not give an exact result as neither slow arrival of (^{14}C) labelled molecules in the plasma nor the continuing synthesis of (^{14}C) urea is taken into account. Therefore Koj and McFarlane (1968) have suggested the use of (^{13}C) urea slopes. The (^{13}C) urea curve is essentially a single exponential and the hypothetical maximum urea specific activity can be obtained by multiplying the area of a plasma slope of the (^{13}C) urea by the area measured under the (^{14}C) urea specific activity curve plotted on linear paper. However, this "area method" assumes that equilibration between plasma and extra-vascular urea is so rapid that newly synthesized (^{14}C) urea can be regarded as

being almost instantaneously distributed throughout the body urea pool. Jones et al (1968) have shown that this is not the case and that the plasma slope consists of two exponentials indicating at least two body compartments. In spite of this, more accurate corrections have yet to be proposed in this system.

Similarly, the fractional synthesis rate of urea is more truly represented by the (^{13}C) urea curve than by the (^{14}C) urea curve due to the continued incorporation of the (^{14}C) label into hepatic arginine with subsequent continuous release of labelled urea resulting in reduced slopes. The use of the endogenously produced (^{14}C) urea therefore results in underestimation of the fractional synthesis rate and maximum specific activity of urea.

The arginine system.

While it is clear that hepatic intracellular arginine can be derived from numerous sources such as diet, plasma, de novo synthesis and protein catabolism, little is known of the origin of arginine used for urea and protein synthesis. It is conceivable that significant compartmentation exists (see review of amino acid pools) and that arginine specific activity at the ribosomal level may differ from that at a level of the Krebs-Henseleit cycle activity. It is unlikely that subcellular fractionation will solve this problem and it remains as a basic assumption of the method.

The albumin system.

Albumin is synthesized exclusively in the liver. However, unlike urea which appears in the blood almost

immediately after labelling, labelled albumin only appears in the blood after twenty to thirty minutes due to the time taken for peptide chain synthesis and release. Release continues for some time, depending on the system. In the meantime, labelled albumin, like the unlabelled albumin, is subject to both distribution throughout the extravascular space and to catabolism, probably at numerous sites (see review on amino acid pools.) Labelled albumin then starts to return to the intravascular space while the labelled arginine derived from albumin catabolism can either be reincorporated into protein throughout the body or be broken down to urea by arginase. This complex dynamic system presents problems to the application of the (^{14}C) carbonate technique :

(1) The asynchronous appearance in the plasma of urea and albumin would suggest that a correction factor should be applied. For example, final urea radioactivity should be measured at time t while final albumin activity should be measured at $t + 30$ minutes. In practice, provided the time for release is short relative to total experimental time, this adjustment can be ignored.

(2) Originally, ad hoc corrections were applied for the distribution and catabolism of albumin. However, this fails to take into account individual variation. Instead (^{131}I) labelled albumin is given with the (^{14}C) label and the fall in iodoalbumin is used to correct the ultimate (^{14}C) activity. However, this correction ignores the fact that (^{14}C) albumin is continuously being released from the liver during the early part of the experiment. A more valid correction would

therefore utilize an area method similar to urea correction or the use of a computer which would simultaneously take into account release, distribution and degradation.

(3) The release of labelled arginine as a result of albumin degradation appears to be of little significance despite the potential for reincorporation of (^{14}C) arginine into albumin or the production of labelled urea. Swick has stated that "the precursor pool of free arginine is always composed of newly synthesized material" and is small relative to the rate of protein synthesis, while it has been shown that extrahepatic urea production is negligible, and the quantity of arginine returning to the liver and potentially available for urea production must be minute.

The (^{14}C) label.

The arginine precursor pool can be labelled either by the injection of (^{14}C) carbonate or (6- ^{14}C) arginine.

The former has a number of advantages :

(1) Gamma globulin is less highly labelled and therefore the potential problems of contamination are less during isolation of albumin.

(2) A longer half-life of (^{14}C) albumin relative to (^{131}I) albumin occurs with (6- ^{14}C) arginine but not with (^{14}C) carbonate administration. This implies that the (^{14}C) carbonate method avoids the recycling of guanidine carbon or arginine in liver cells which occurs with the alternative method.

(3) The (^{14}C) carbonate label excludes radiation hazard by

minimal labelling of carcass protein.

(4) It also reduces potential errors introduced by extra-hepatic arginase, and most important

(5) the effective synthesis interval of protein is shorter and errors which arise in synchronising the synthesis of labelled protein with that of labelled urea in the liver tend to be reduced.

The major disadvantage of (^{14}C) carbonate is the lower activities achieved in the albumin labelling.

Reeve and McKinley (1970) recently proposed the use of the arterial (^{14}C) CO_2 as an indicator of intracellular arginine specific activity. They based this proposal on the rapid equilibration of intracellular CO_2 and the observation by Kornberg et al (1952) that after intravenous injection of (^{14}C) bicarbonate, the average specific activity of newly synthesized urea was equal to the average specific activity of arterial total (^{14}C) CO_2 and to the expired (^{14}C) CO_2 . The chief advantage of the arterial total (^{14}C) CO_2 method is the avoidance of problems of measuring maximum (^{14}C) urea specific activities and the urea fractional synthesis rate and also the problem of a constant rate of urea synthesis.

The assumptions of the method as used by the author may be summarised as follows :

- (1) The arginine specific activity at the sites of urea and albumin synthesis is identical.
- (2) Urea and albumin synthesis remain constant throughout the experimental period.
- (3) Corrections for continued liberation, distribution and degradation of labelled products are accurate.

The first two assumptions are common to all investigators.

The (^{14}C) carbonate technique has been utilised by the author for (a) a human study, and (b) a liver perfusion study.

Human study.

The formula used in this study is as follows :

$$\text{F.S.R. Albumin} = \frac{\text{F.S.R. urea} \times \text{albumin S.A. at } t_0}{\text{urea S.A. at } t_0} \dots \text{Equation 7}$$

Where albumin S.A. at t_0 = Specific Activity of the guanidine carbon of arginine in albumin at zero time.

The absolute synthesis rate of albumin (mg/24 hrs) formula :

$$\text{Albumin synthesis rate} = \text{F.S.R. albumin} \times \text{intravascular albumin pool} \dots \text{Equation 8}$$

The fractional synthesis rate of urea was obtained from the semilog plot of the prelabelled (^{14}C) urea administered the day following the experiment. The author used this technique for three reasons :

- (1) (^{13}C) urea injection on the day of experiment was precluded through lack of facilities.
- (2) To overcome the defect of the reduced slopes obtained with endogenously produced (^{14}C) urea due to continued incorporation of (^{14}C) label into urea.
- (3) To enable a slope to be obtained accurately under conditions of minimal urea production as in protein depletion. To obtain meaningful results by conventional methods of using 5-6 samples over 6 hours, the larger volumes of blood required would seriously have influenced the intravascular pool size.

The author is aware that utilising (^{14}C) urea the day following the experiment is not ideal and, while probably providing a reasonably accurate assessment of urea synthesis, suffers from the assumptions that the same metabolic conditions prevail on both days and that the labelled urea is not subject to continuous release from the liver and distribution throughout the body water. Both of these assumptions are not entirely tenable, but the degree of error introduced by them is probably not great. In all experimental circumstances the same conditions were maintained.

The albumin specific activity at t_0 was obtained by measuring the value at 6 hours and applying a correction factor derived from the loss of (^{125}I) labelled albumin injected at the beginning of the experiment. In doing this the author is aware that the more accurate technique would be the use of a computer to embrace all the factors previously mentioned.

Specific activity of urea at t_0 is derived from the 6 hour sample on the day of the experiment and extrapolated to zero time by applying the slope of exogenously administered (^{14}C) urea injected on day 2. The author is aware that the area method previously described is more accurate, but both the lack of facilities for measuring (^{13}C) urea and the inability to obtain an accurate endogenous (^{14}C) urea slope under conditions of protein restriction precluded its use.

The technique proposed by Reeve and McKinley (1970) would appear to be ideal under the experimental conditions

applied in this study. However, because of the radiation hazard it is doubtful if this method of constant (^{14}C) bicarbonate infusion will be used in humans.

Radiation hazard.

Morgan (1947) and Marinelli et al, (1948) have calculated that if a total dose of 100 μc of $\text{Na}_2^{14}\text{CO}_3$ were uniformly distributed throughout the body of a 70 kg man, approximately 4.5 microcuries per day would be delivered to the tissues. The maximum permissible level of 40 microcuries per day was suggested by the U.S. Atomic Energy Commission (Manove and Lintz, 1949). Three important points require expansion: the fraction of the total dose retained, the question of a selective tissue concentration of the isotope and the form in which the isotope is retained.

The data of Armstrong et al (1948), Greenburg and Winnick (1949), Gould et al (1949) and Skipper et al (1949a,b) in rats and mice suggest that 95% of the administered dose of $\text{Na}_2^{14}\text{CO}_3$, or $\text{NaH}^{14}\text{CO}_3$ was lost in the expired air within 4 hours, and that less than 2% of the dose persisted in the animal for more than twenty-four hours after intraperitoneal injection.

In humans, Hellman et al (1951), after giving 200 μc of (2- ^{14}C) glycine or (2- ^{14}C) acetate orally to patients with incurable disease, found that in two patients who died one month and eight months later the body retention of the dose was 12% and 1.2% respectively. The work of McFarlane (1963a) with totally labelled (^{14}C) arginine and (^{14}C) carbonate suggests that not more than 1% of the administered radioactivity would be retained in protein 24 hours later.

Retained radioactivity is probably all in the protein fraction

as radioactivity in non-protein products is associated with materials of short biological half-life.

Concentration of the isotope has been examined in both animals and man. While the work in cats (Kornberg et al, 1951, 1952), mice (Skipper et al, 1951), and rats (Bloom et al, 1947) suggested high concentrations in bone, Berlin et al (1952) found the bone concentration in a patient who died 526 days after receiving 2-¹⁴C glycine not greatly different from other tissues.

In summary, it appears that about 2% of the administered dose is retained and that this is reasonably evenly distributed throughout the body. The author has given 200 µc to subjects on a normal diet and then 200 µc to the same subject on a 10 gram per day protein diet which was shown to decrease muscle and plasma protein synthesis (Waterlow and Stephen, 1968) thereby decreasing isotope incorporation. Apparently the British Medical Research Council is permitting 2 doses each of 200 µc of (¹⁴C) carbonate to normal patients on normal diets. Rothschild et al (1969a) reported the use of similar doses in America. In view of this, the dose used appears acceptable, particularly under the conditions of study.

Validation of technique.

This has been approached in two ways :

(1) the measurement of synthesis and catabolism independently in the same animal or man in a steady state. From the work of McFarlane (1963a), Rosender (1967), Kirsch et al (1968), Tavill et al (1968) and Jeejeebhoy et al (1969) it appears that reasonable matching between the two methods has been obtained.

(2) the measurement of synthesis by the (^{14}C) carbonate and immunological techniques simultaneously in the isolated perfused liver. Both Hoffenberg et al (1971) and Katz et al (1967) obtained fair approximation between the two methods.

From these two approaches it appears that the method is acceptable in principle, the assumptions reasonable and the technical problems mentioned not limiting for meaningful results. However, it is possible that small differences in experimental conditions could be missed and, in fact, Tavill et al (1968) found albumin synthesis rates to vary by as much as 20% in the same patient under the same conditions.

Liver perfusion.

The system for the isolated perfused rat liver is depicted in Figure 9. The assumption is made that, for practical purposes, the entire system is closed. Therefore a modification of the original formula (Equation 4) can be utilised :

$$\begin{array}{l} \text{Mass of albumin synthesized in time } t = \\ \frac{\text{Radioactivity incorporated into guanidine carbon of arginine in albumin in } t}{\text{Radioactivity incorporated into urea in } t} \times \frac{\text{Mass of urea synthesized in } t}{\text{}} = \text{Equation 10.} \end{array}$$

The modifications to the four components of the system are outlined below :-

(1) The urea system.

A closed system is assumed due to the absence of the gut bacterial degradation and the renal excretion of urea. However, urea can theoretically be lost in the bile or by degradation caused by urea-splitting organisms in the blood.

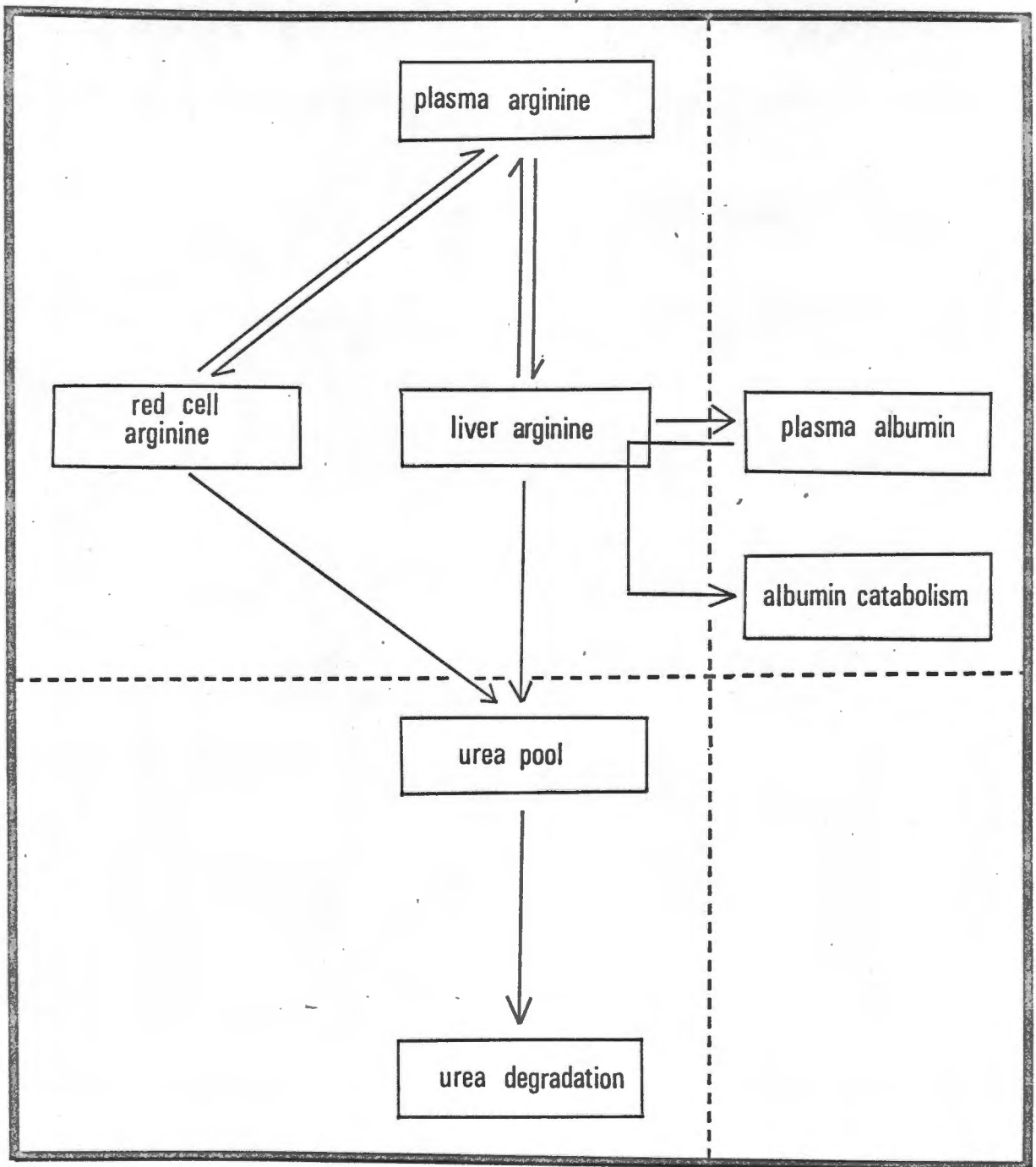


Figure 9: THE ARGININE; ALBUMIN AND UREA POOLS OF THE CARBONATE METHOD IN THE PERFUSION SYSTEM

Biliary excretion of urea can be discounted, but degradation of urea by bacteria is a real possibility as the liver perfusion technique is a non-sterile technique and blood cultures performed by the author have revealed some bacterial growth, including urea-splitting organisms. The author has excluded significant urea degradation by demonstrating constant urea radioactivities during perfusion when exogenously labelled (^{14}C) urea was administered. The assumption of a closed urea system is therefore probably justifiable.

Urea synthesis rates were obtained from the difference between the plasma stable urea before and after perfusion and assuming distribution throughout the blood volume. While the urea synthesis rate varied under different experimental conditions, it nevertheless remained constant throughout any one particular perfusion.

(2) The arginine system.

The same assumption is made that the precursor specific activity of arginine is identical at the sites of urea and albumin synthesis.

Red blood cell haemolysis, which released most amino acids and arginase, occurred to some extent and was constant for all perfusions. The released arginase would have had no influence on the arginine system because of the absence of extra-hepatic protein catabolism and the use of (^{14}C) carbonate rather than the ($6\text{-}^{14}\text{C}$) arginine.

(3) The albumin system.

The assumptions introduced are that the albumin system

is a closed one in that the distribution of albumin is limited to the plasma, and that albumin catabolism by the liver is negligible. While albumin is known not to penetrate red blood cells, distribution within the liver has been ignored by the author. A correction factor is difficult to apply. The usual expedient of using the (^{125}I) albumin correction factor suffers from the same problems previously mentioned, while the percentage change is small enough to be almost meaningless. Blood volumes and liver weights remained relatively constant in the different perfusions and the distribution correction can probably be ignored. On the other hand, albumin catabolism by the isolated liver certainly occurs, albeit at a relatively slow rate of 0.1% per hour of the intravascular pool. For this to be ignored it is essential that albumin catabolism remain constant under all experimental conditions. The author has undertaken to show this later in this thesis. In spite of omitting these corrections both Hoffenberg et al (1971) and Katz et al (1967) obtained reasonable agreement between this method and the simultaneous measurement of albumin synthesis using an immunological technique.

(4) The (^{14}C) label.

The labelling of the guanidine carbon of arginine was achieved by an injection of (^{14}C) carbonate into the reservoir. ($6\text{-}^{14}\text{C}$) arginine suffers from the problems of red blood cell arginase previously mentioned. Administration of the label into the portal vein is a more direct and efficient method. Injection into the reservoir is technically simpler but presents the hazards of continuous

labelling of the hepatic arginine pool instead of a "pulse-like" method. However, the author has found that within a few minutes the (^{14}C) carbonate is lost from the system, analogous to peripheral vein injection in the intact animal. Katz et al (1967) have also shown that the constant infusion of (^{14}C) carbonate gives similar results to those obtained by using a single injection.

The (^{14}C) bicarbonate infusion method proposed by Reeve and McKinley (1970) would offer a more direct method and would enable the urea problems to be avoided.

ALBUMIN CATABOLISM.

Techniques for the measurement of albumin catabolism have become well established. Initial attempts employing labelled amino acids suffered from the disadvantages of the slow rate of incorporation of the labelled amino acid into protein and, more fundamentally, from the re-utilisation of the labelled amino acid resulting from protein catabolism.

Iodine-labelled albumin was introduced in the 1950's as a means of measuring catabolism. When the (^{131}I) albumin molecule is degraded, the iodide attached to tyrosine is released. The behaviour of both the (^{131}I) iodide and the (^{131}I) albumin has been used as an index of albumin catabolism. The assumption that (^{131}I) is liberated only during reactions involving rupture of peptide bonds was validated by the work of Cohen et al (1956) and Campbell et al (1956), who showed the half-life of protein labelled with iodine to be almost identical with that of the corresponding protein labelled with (^{14}C) by a biosynthetic procedure. Cohen et al (1956) excluded reutilisation of (^{131}I) iodide on the grounds that they failed to detect plasma protein labelling, and achieved almost complete recovery of the label in the urine within two days after feeding animals (^{131}I) proteins.

After injection of the (^{131}I) albumin it is rapidly distributed throughout the intravascular space, the extent of its dilution after ten minutes providing a measure of the plasma volume. (^{131}I) albumin may leave the intravascular space by catabolism, migration to the extravascular space or pathological losses from the kidney, gut or other sites.

For the following three days distribution throughout the extracellular space occurs. After three days the semi-log plot of the plasma radioactivity against time is found to be linear. Provided no losses occur in the urine, gut or other sites, this slope has been used to calculate the albumin catabolic rate (Sterling, 1951). However, as depicted in Figure 10, there is a bidirectional extra- intravascular movement of labelled albumin and therefore as the plasma (^{131}I) albumin starts to fall it will receive some (^{131}I) albumin from the extravascular space. Catabolic rates obtained by this procedure are therefore underestimated. The degree of error will be determined by the extravascular protein mass, the rate of protein catabolism and the degree of vascular permeability. Similarly, the slope of the total body curve, after preliminary equilibration, reflects partly catabolism and partly net transfer of labelled molecules into the plasma compartments. Therefore, for plasma data to be accurate, there must be no net transfer of labelled protein from the extravascular to the intravascular space (so-called "equilibrium time"). Campbell et al (1956) proposed that at that point in time, a tangent to the plasma (^{131}I) albumin semi-log plot against time would provide a measure of the albumin catabolic rate as a fraction of the plasma protein pool, while the Y intercept of this line would provide the ratio of intra- to extra-vascular pool size. In practice, the extra-vascular radioactivity required shows a broad plateau and the maximum point of the curve is difficult to define.

Labelled iodide, released by catabolism, is distributed throughout the body water, excreted in the urine, re-utilised by the thyroid gland and secreted and reabsorbed by the gut (see

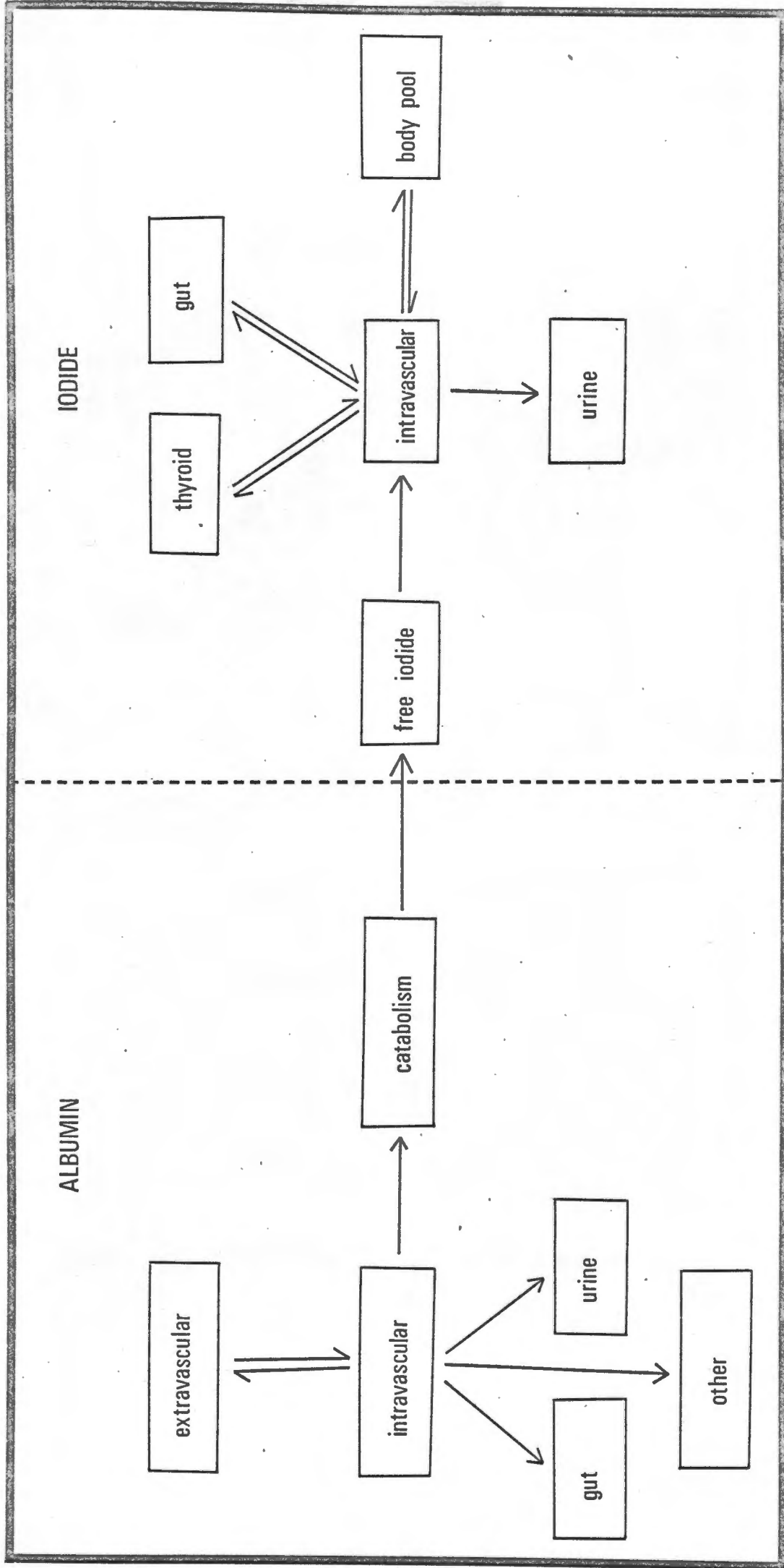


Figure 10: THE POTENTIAL PATHWAYS OF IODOALBUMIN

Figure 10). Thyroid uptake can be blocked by administration of stable iodine while gut secretion, because of virtually complete reabsorption of iodide, can be ignored. Zizza et al (1959), Lewaller et al (1959) and McFarlane (1963b) have established that the majority of non-protein-bound radioactivity is free iodide. Therefore, the total iodide released from the labelled albumin, expressed as a fraction of the mean albumin-bound activity in the plasma, integrated over the corresponding 24 hour interval, is used as a measure of albumin catabolism (Berson et al, 1953; Berson and Yalow, 1954). After an initial period of 24 to 48 hours, during which iodide activity accumulates in the body water, urine activities are assumed to equal iodide activities released by catabolism during the collection interval. This is a lengthy procedure of at least one week, and is dependent on normal renal function and accurate urine collections. It is clear that any method over a short period of time or performed in the presence of compromised renal function must provide a measure of the non-protein-bound activity released into the body water. This has been suggested by McFarlane (1963c) and exploited by Donato et al (1967) and Vitek et al (1967). Recently, McFarlane and Koj (1970) have reinvestigated the possibility of short-term measurement of catabolic rates using mean plasma albumin activities, iodide excreted in the urine and iodide accumulated in the body water over the period of measurement. They concluded that "until the pool from which protein molecules are withdrawn to be catabolised has been defined more clearly and more is known about the nature of the catabolic process itself, it is unlikely that meaningful measurement of the fractional catabolic rates can be made

within 36 hours of injecting an iodine labelled protein".

The author has used iodine-labelled albumin for measuring albumin catabolic rates both in man and in the isolated rat liver perfusion.

A. Man .

The more accurate technique initially described by Berson and Yalow (1954) was used by the author. The F.C.R. of albumin was calculated from the urinary iodide excreted per day, after 48 hours, expressed as a fraction of the mean protein-bound activities in the plasma over the same interval.

B. Liver perfusion study.

In the isolated perfused liver the system is simplified (see Figure 11). The fractional catabolic rate is derived from the iodide released per hour, expressed as a percentage of the mean total protein-bound radioactivity during that hour.

The assumptions include :-

- (i) Those common to the intact animal or man. For example
 - (a) iodide released is dependent on peptide bond rupture, (b) iodide is not reutilised for further protein synthesis.
- (ii) The system is a closed one. (a) No "body pool" of iodide exists to allow for iodide distribution. The only potential component of the "body pool" is the liver which can be ignored for practical purposes. (b) Bile is a potential avenue for iodide or iodinated albumin excretion. However, albumin excretion in bile is known to be negligible while bile iodide excretion was shown by Cohen and Gordon (1958) to constitute less than 3% of the total non-protein radioactivity in the perfusing blood. This was verified by the author prior to commencing this work (unpublished) and quantitatively can be ignored.

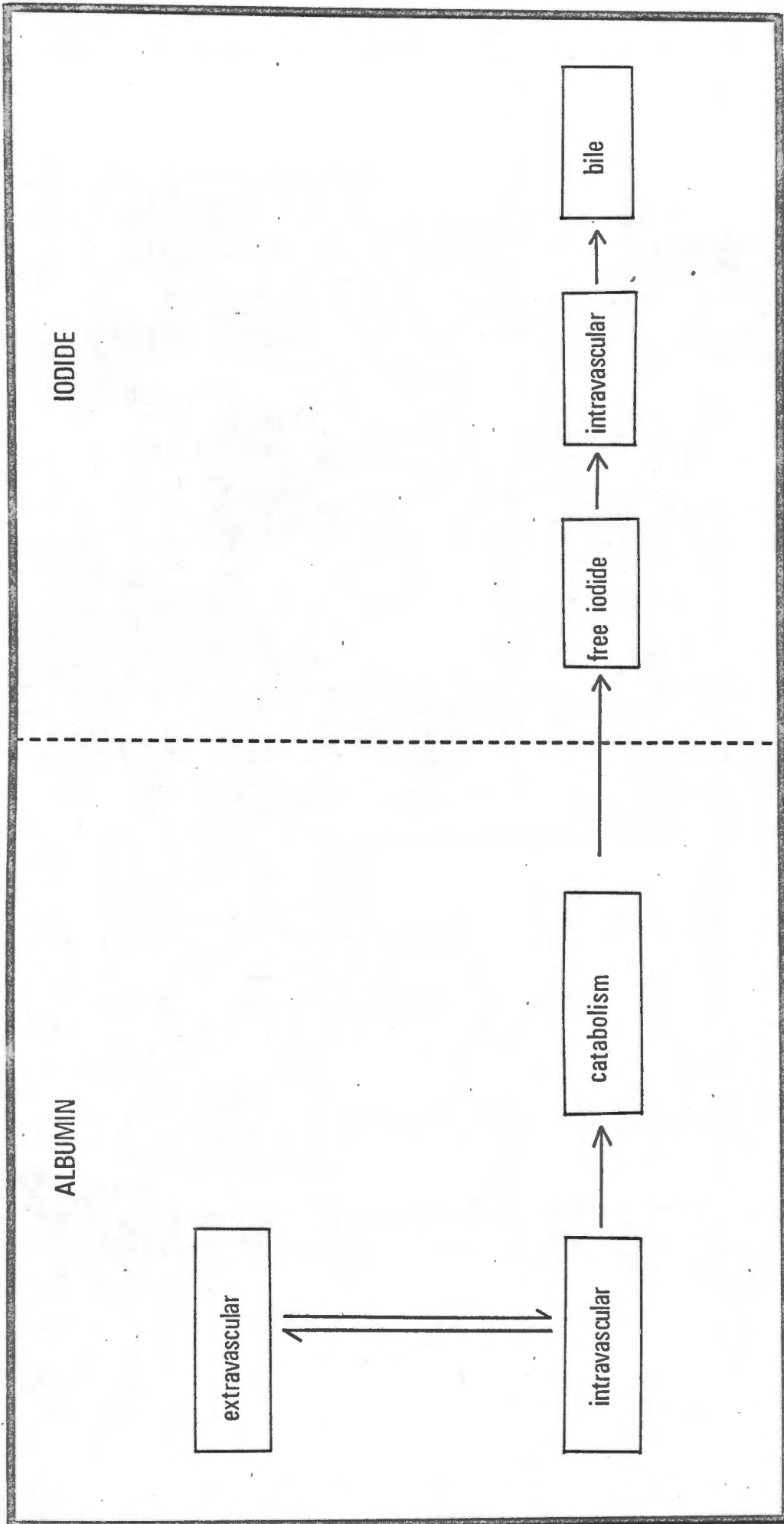


Figure 11: THE POTENTIAL PATHWAYS OF IODOALBUMIN IN THE PERFUSION SYSTEM

(iii) That (^{131}I) albumin is stable in the absence of a liver. Gordon (1957) demonstrated that radioactivity is not released from undenatured (^{131}I) albumin pumped through the perfusion circuit in the absence of a liver.

(iv) That the injected preparation of (^{131}I) albumin contains negligible amounts of free (^{131}I) iodide. Reference to Figure 11 illustrates that any free labelled iodide injected into the system will result in confusion of the final F.C.R. as these rates are extremely low in this system. Non-protein radioactivity was shown to constitute less than 0.2% of total radioactivity injected.

RIBOSOMAL PROFILES.

In 1963 it was demonstrated by electron microscopy and sucrose gradient analysis that ribosomes and mRNA are arranged in aggregates called polysomes (see Review on Amino Acid Pools). A ribosomal profile represents the size distribution of the ribosomes. The ribosomes are closely packed together and the size of the polysome aggregates is therefore a measure of the length of the messenger strand. Wettstein et al (1963) have shown that from the centre of one ribosome to the next on a polysome, the messenger extends for 19 nucleotides or 30 codons. Therefore a protein with 560 amino acids such as albumin will be coded for by a messenger carrying 19 ribosomes. The aggregate size-to-messenger relationship has been confirmed by several workers (Borun et al, 1967; Heywood et al, 1967; Kiho, 1968). Furthermore, following the suggestion of Birbeck and Mercer (1961), the bulk of evidence favours the functional separation of ribosomes into free and membrane-bound components. Redman et al (1968), Takagi and Ogata (1968), and Hicks et al (1969) have confirmed that albumin is synthesized exclusively on the membrane-bound ribosomal population. Also, from the work of Gaetani et al (1969), it appears that the two populations can behave differently under different conditions. Monosome accumulation is a fairly sensitive indicator of polysome breakdown and therefore decreased protein synthesis. Ribosomal profile analysis can therefore yield valuable information concerning either overall protein synthesis (ratio of monosomes to polysomes) or specific protein synthesis (different populations of ribosomes or different size proteins within these ribosomes.)

Many modifications of the original method of preparing C ribosomes (Wettstein et al, 1963) have been described. For considerations mentioned above, the procedure of Blobel and Potter (1967) was used by the author. The five basic steps of the method are discussed below :-

- (1) The use of RNase inhibitor.
- (2) Differential centrifugation.
- (3) Zonal centrifugation.
- (4) Release of membrane-bound ribosomes.
- (5) Profile preparation and analysis.

(1) The use of RNase inhibitor.

In the cell there is a delicate balance between RNA, RNase and RNase inhibitor. Blobel and Potter (1966) and Lawford et al (1966) have shown that any prolonged procedure results in breakdown of RNA as a consequence of RNase activity. Blobel and Potter (1967) recommend adding liver cell sap which contains RNase inhibitor to the homogenising medium to prevent this action. This liver cell sap is prepared from the post mitochondrial supernatant by centrifugation at 105,000 g for 4 hours. The RNase inhibitor contained in the cell sap is stable for at least one month at -20° (Shortman, 1962). Therefore all steps of the method are carried out in the presence of RNase inhibitor.

(2) Differential centrifugation.

This step involves the separation of the total ribosomal population from the cell wall, nuclei, debris and mitochondria. Unfortunately, a large percentage of the membrane-bound ribosomes sediments and is therefore not included in the post

mitochondrial supernatant. Blobel and Potter (1967) have shown that the post mitochondrial supernatant fraction contains more than 90% of the free ribosomes but only about 20% of the bound ribosomes. However, Lawford et al (1966), by preparing polysomal profiles from the pellet usually discarded, showed that the post mitochondrial membrane-bound ribosomes are probably representative of the total membrane-bound ribosomes.

(3) Zonal centrifugation.

The separation of free from bound ribosomes is based on the lower molecular weight of the lipoprotein-bound fraction. The free ribosomes pass through 2M sucrose and form a pellet at the bottom of the centrifuge tube, whereas the membrane-bound fraction is held at the interface.

(4) Release of membrane-bound ribosomes.

The interface containing the lipoprotein-ribosome combination is recovered. The lipoprotein membrane is removed by homogenisation with a detergent and the bound ribosomes prepared by centrifugation through sucrose.

(5) Ribosomal profile preparation and analysis.

The pellets are spun through linear gradients and passed through a flow-through cuvette in a spectrophotometer, set at 260 m μ linked to an automatic recorder. Essentially, the size distribution of the ribosomes in the gradient is indicated by the distribution of the RNA as there is no evidence to suggest variation in ribosomal RNA content.

The final profiles are probably defective in the

following ways:-

(i) Munro (1970) considers that the technique used in centrifuging the free and later the bound ribosomes through 2M sucrose suffers from the same criticism as the original C-ribosome procedure of Wettstein et al (1963). Munro considers that some monosome particles with sedimentation properties similar to those of ferritin are excluded from the final population. The author is aware that this may be a serious limitation to the technique of Blobel and Potter (1967) used, in that monosome changes may be the most sensitive index of changes in protein synthesis. The preferred technique of excluding ferritin without losing these monosomes by the use of ferritin antiserum was not available to the author (Drysdale and Munro, 1967).

(ii) The cell sap added in the preparation of the final gradient contains ferritin, an iron-containing protein which sediments over the range 20-120S and is maximal at 60-70S. The iron of ferritin absorbs strongly in ultraviolet light and its presence can therefore lead to errors in estimating monosomes and disomes (Jackson et al, 1964).

(iii) The author was unable to obtain the quality of separation achieved by workers such as Noll (1969), although profiles were obtained very similar to those of Blobel and Potter (1967) and others using this method.

(iv) The disome peak in liver cell preparations is preferentially increased under some conditions in which monosomes accumulate. This is considered to be due to physical aggregation of monosomes which have neither peptide chains nor messenger attached to them.

In view of the limitations mentioned, it must be stressed that all specimens were handled in an identical fashion. Furthermore, no attempt was made to quantitate profile peaks. Instead minor differences were ignored and only gross qualitative differences compared. Thus ribosomes were considered to show either "aggregated" or "disaggregated" patterns. Essentially, the author has therefore examined ribosomal profiles considering both overall protein synthesis ("aggregated" or "disaggregated") and specific protein synthesis (the profile of free or membrane-bound ribosomes.)

LIVER PERFUSION.

The intact animal has a remarkable ability to maintain the internal environment in response to external stimuli. Conversely, isolated cell function is frequently too crude to provide useful information. Between the two lies perfusion of the isolated organ. While liver perfusions were attempted in the late 19th Century and early 20th Century, it was largely through the efforts of Miller et al (1951) and Brauer et al (1951) that the isolated rat liver perfusion became established as a major tool for biochemical research.

The perfusion technique has varied with the investigator and the requirements of the experiment. Basically, the technique consists of cannulation of the portal vein for inflow of the perfusion medium, cannulation of the bile duct and transfer of the liver, removed from the carcass, in situ to a temperature controlled cabinet. The two-component system (liver and perfusion medium) is kept at physiological pH, oxygen and carbon dioxide tension and the essential nutrients are supplied. The duration of perfusion has varied with the modifications and requirements of the investigator. Basic technical details include minimising manipulation of the liver during its removal, avoiding lengthy periods of hepatic anoxia and establishing a perfusion pressure comparable to that in the rat.

Functional criteria include basic parameters such as the general appearance of the liver, blood flow and bile secretion. Light and electron-microscopy have been used to establish viability. As in clinical medicine, however, the diverse and often intricate function of a liver precludes

simple or even satisfactory estimates of liver function. Comprehensive estimates should be based ideally on hepatic parenchymal cell, reticulo-endothelial cell and biliary cell function. Thus various parameters of protein, carbohydrate and fat metabolism, phagocytic and degradative capacity and biliary excretory function should be considered.

Miller and colleagues (1951) justified the use of the isolated rat liver for the study of protein synthesis by showing that the perfused organ : (1) discriminated between the d- and l- isomers of lysine with a virtual failure of response to the unnatural isomer; (2) repeated its action quantitatively when a second dose of (^{14}C) labelled substrate was given, responded to the removal and oxidation of amino acids and synthesized plasma proteins; (3) removed amino acids continuously from the perfusing blood and (4) responded quantitatively in a similar fashion to that of the intact animal in the conversion of (^{14}C) amino acids to (^{14}C) CO_2 and liver (^{14}C) proteins.

Furthermore, a simple two-compartment system offers the unique opportunity of varying the nutrient or hormonal status of the liver or blood together or independently without widespread compensatory mechanisms which occur in the intact animal. In addition, isotopic studies, as previously mentioned, are simplified by the use of this relatively closed system and the absence of a large extravascular compartment.

Perfusions were performed by the author according to the method of Miller et al (1951) as modified by Fisher and Kerly (1964). Full details of the methodology are described in a later chapter and in the Appendix.

IODINATION.

The biological behaviour of a protein varies in relation to its sensitivity to iodination, albumin being relatively resistant, a mean of one atom of iodine per molecule being accepted as the safe level of substitution. Cationic iodine is substituted into the benzene ring of tyrosine, and to avoid a significant population of protein molecules having excessive substitution, mean levels are utilised which are well below the level resulting in denaturation. The chemistry of iodination has been discussed in some detail by Hughes (1957) and Helmkamp et al (1967a,1967b) while the problems and techniques involved have been described by McFarlane (1958, 1964).

Cationic iodine may be formed from iodide by the use of oxidising agents such as hydrogen peroxide or chloramine T. However, the alternative methods utilising hypiodous acid (HOI) in aqueous solution are less harmful to plasma protein molecules. The iodine monochloride method (McFarlane, 1958) is the most successful. At an alkaline pH, HOI is formed from iodine monochloride. Addition of radio-iodine results in the formation of HO^{131}I . Rapid introduction of the HO^{131}I solution into the purified albumin solution increases the efficiency of iodination. 60-70% iodination is achieved and the residual free radio-iodide is removed by passage through a chloride ion exchanger or by dialysis against distilled water. Self-irradiation damage (Yalow and Berson, 1957; Cohen, 1959) is reduced by the addition of unlabelled carrier protein (Yalow and Berson, 1957) and by the use of the iodo-albumin almost immediately after preparation. Sterilisation is

achieved by Seitz filtration or the use of "millipore" filter units avoiding the denaturation caused by heat sterilisation (Merchant et al, 1957).

While no abnormal physiochemical properties may be detected, the iodinated albumin may still behave in an attenuated fashion. This may be tested for by (1) introduction of the product into a control for comparison with the experimental model, (2) comparison of its plasma half-life with that of bio-synthetically labelled (^{14}C) albumin, (3) detecting high catabolic rates of albumin in the perfused rat liver or humans, and (4) finding excessive urinary radio-iodide within the first 48 hours after injection.

The author is satisfied that the final product used met the criterion of biological integrity. In the human studies, an acceptably pure commercial preparation of human albumin was used and the subsequent procedures performed, as outlined above. In the perfusion catabolic studies, the rat albumin was extracted by the ammonium sulphate technique (McFarlane, 1963a) and the purity checked by cellulose acetate electrophoresis. After iodination as described above, albumin was biologically screened (McFarlane, 1956) by intra-peritoneal injection into rats and bleeding 48 hours later (Gordon, 1957; Cohen and Gordon, 1958).

P A R T I .

THE EFFECTS OF DIETARY PROTEIN RESTRICTION
ON ALBUMIN SYNTHESIS, ALBUMIN CATABOLISM
AND THE PLASMA AMINOGRAM.

Introduction.

The McFarlane technique for the direct measurement of albumin synthesis has recently been applied to man (Jones et al, 1968; Tavill et al, 1968; Wochner et al, 1968; Jeejeebhoy et al, 1969; Rothschild et al, 1969a; Coles et al, 1970). However, little attention has been directed :-

1. to protein intake in relation to synthesis, and
2. to synthesis and catabolism in the same patient on different dietary intakes.

Hoffenberg et al (1966) and James and Hay (1968), using indirect methods of measurement, concluded that albumin synthesis is decreased in man by protein restriction. These studies are supported by the work of Kirsch et al (1968) who, using the (^{14}C) carbonate technique in the rat, demonstrated a rapid decrease in albumin synthesis following protein restriction.

In protein calorie malnutrition (PCM) in children, low plasma albumin and specific plasma amino acid changes are found (Holt et al, 1963; Saunders et al, 1967). Few studies of the plasma aminogram on controlled dietary protein restriction have been reported. No studies relating albumin synthesis to the plasma aminogram have been published.

In view of the above considerations, it was felt that a study of albumin synthesis, albumin catabolism and the plasma aminogram in the same subject before and during protein restriction would be important :-

1. to determine the magnitude of change, measuring albumin synthesis by a direct technique,
2. to interpret similar studies in patients with cirrhosis,

malabsorption and uraemia where protein depletion is frequently present, and

3. to determine whether any relationship exists between plasma amino acid levels and albumin metabolism.

Materials and Methods.

Patients.

Eight adult male subjects were hospitalised in the metabolic ward of Groote Schuur Hospital. Informed consent was given by each patient. All subjects had normal liver, renal, cardiac and gastro-intestinal function and no evidence of infection.

Protocol.

After a two week screening period in the hospital on a 70 gram protein diet, albumin synthesis and catabolism were measured. The subjects were then placed on a 10 gram iso-caloric protein diet, supplemented with adequate minerals and vitamins. The full diet was consumed by all subjects. After 4-6 weeks of this diet, the synthesis and catabolism studies were repeated. 3 days before each study, iodine uptake by the thyroid gland was blocked by the administration of Lugol's iodine (10 minims 3 times daily) and the bowel was sterilised using neomycine sulphate (1 gram 4 times daily). The Lugol's iodine was maintained for 14 days and the neomycine sulphate for 4 days. On the first day of the synthesis rate study, plasma was taken from each subject after an overnight fast for amino acid analysis. The subjects

voided urine, and then 80 microcuries of (^{125}I) albumin was administered intravenously together with 200 microcuries of sodium (^{14}C) carbonate. Blood samples were withdrawn after 10 minutes, 3 hours and 6 hours and then daily for 10 days. Each day, 24-hour urine volumes were collected. The following day, after another overnight fast, 5 microcuries of (^{14}C) urea were administered and blood samples taken at 3, 3½, 4, 4½, and 5 hours. On both days of synthesis rate measurement, a light, non-protein snack was provided with a liberal fluid intake.

Materials and Methods.

The principles of methodology have been discussed. Detailed methodology can be found in the Appendix. A summary of the essential features follows.

Catabolic rates were derived from urinary excretion of radioactive iodine as a function of plasma specific activity. Iodination of human albumin was achieved by the McFarlane iodide monochloride technique to give 0.5 to 1.0 gram - atoms of iodine per molecule of albumin. Free iodine was removed by dialysis against distilled water. Trichloroacetic acid precipitation showed less than 1% free iodine. Carrier albumin was added to reduce radiation damage and the preparation was sterilised by Seitz filtration. The labelled albumin was used within 48 hours of preparation. The radioactivity of the serum and urine samples was measured in a Packard Autogamma Spectrometer. Plasma volume was obtained by isotope dilution at 10 minutes. Albumin was measured in duplicate by the method of Fernandez et al (1966). The intra-vascular albumin pool was estimated

by multiplying the plasma albumin pool by the degradation rate constant (mean of day 3 to day 14).

Albumin synthesis was measured by the technique developed by McFarlane (1963) and Reeve et al, (1963). The formula used (Equation 8) was that proposed by Koj and McFarlane (1968). The specific radioactivity of urea was calculated by extrapolating the slope of the (^{14}C) urea specific radioactivity curve to zero time applying the modifications already described. The hypothetical specific radioactivity of albumin was obtained by extrapolation to zero time, correcting the 6 hour value by a factor derived from the distribution and catabolism of the injected (^{125}I) albumin. The urea synthesis rate was obtained from the slope of the semilog plot of (^{14}C) urea specific activity versus time.

Urea specific activity was measured on deproteinised plasma samples, incubated with urease to produce ($^{14}\text{CO}_2$) which was then released by the addition of acid. The volume of gas produced was measured manometrically on a high vacuum gas train, collected in phenylethylamine-methanol and counted in a PPO-POPOP toluene liquid scintillator in a Beckman Automatic Scintillation Spectrometer. Samples were counted to an error of less than 3%. The specific activity of the guanidine carbon of arginine in albumin was obtained by extraction of albumin from the plasma by the acid ethanol method (Korner and Debro, 1956). After acid hydrolysis at 110° , the sample was passed through a bicarbonate resin column to separate arginine which was then incubated with arginase to produce urea. The sample was then treated as for urea. Urea was measured using an

autoanalyser. Tryptophan was measured by the method of Hess and Udenfriend (1959), while the other amino acids were analysed on a Technicon amino acid analyser.

Results.

Table I shows the absolute albumin synthesis and catabolic rates on the 70 gram and 10 gram isocaloric protein diet. Patient 2 was reported to be disobeying dietary instructions intermittently. Table II summarises the changes in absolute and fractional synthesis and catabolic rates, plasma urea, plasma albumin and the plasma volume. While the plasma volume was unchanged, the plasma urea and the plasma albumin were reduced. Both the fractional and the absolute albumin synthesis and catabolic rates were markedly reduced following protein restriction.

Table III shows the changes in the fasting plasma amino-gram. Alanine is the only amino acid to show any change and is markedly increased on the restricted protein diet.

T A B L E I.

The absolute albumin synthesis and catabolic rates in subjects on a 70G and a 10G iso-caloric protein diet.

Patient	70G Protein Diet		10G Iso-caloric Protein Diet	
	Synthesis Rate *	Catabolic Rate*	Synthesis Rate*	Catabolic Rate*
1	398	250	101	184
2	225	138	257**	82
3	327	163	82	123
4	307	168	79	125
5	161	211	88	133
6	95	171	33	102
7	256	147	83	89
8	193	132	126	53
Mean and standard deviation	245 ₊₉₈	173 ₊₄₀	85 ₊₂₈	111 ₊₃₉

* Expressed in mg/Kg/day

** Reported to be eating 70G protein diet intermittently.

T A B L E II

Plasma albumin, plasma urea, plasma volume, fractional and absolute albumin synthesis and catabolic rates.

	70G Protein Diet:+	10G Iso-caloric Protein diet. +	Differences between two diets.
Plasma albumin	4.11 ± 0.45 g%	3.71 ± 0.35 g%	0.02 < P < 0.05
Plasma urea	15.5 ± 5.3 mg%	7.0 ± 4.2 mg%	0.001 < P < 0.01
Plasma volume	2,330 ± 515 ml	2,247 ± 428 ml	0.7 < P < 0.8
Fractional synthesis rate	14.9 ± 6.2%/day	5.9 ± 2.1%/day	0.001 < P < 0.01
Absolute synthesis rate	245 ± 98mg/Kg/day	85 ± 28 mg/Kg/day	P < 0.001
Fractional catabolic rate	10.9 ± 2.65%/day	6.5 ± 2.7%/day	0.001 < P < 0.01
Absolute catabolic rate	173 ± 98mg/Kg/day	111 ± 39mg/Kg/day	0.001 < P < 0.01

+ Mean and standard deviation.

T A B L E III:

Fasting plasma aminograms on a 70G and a 10G iso-caloric protein diet.

Amino Acid.	70G Protein Diet. +	10G Iso-caloric Protein Diet.+
Glutamic Acid	0.169 ± .125	0.332 ± .368
Glycine	0.304 ± .070	0.349 ± .082
Alanine ++	0.461 ± .096	0.719 ± .144
Valine	0.227 ± .022	0.215 ± .038
Methionine	0.025 ± .008	0.031 ± .010
Isoleucine	0.079 ± .018	0.080 ± .015
Leucine	0.128 ± .034	0.130 ± .036
Tyrosine	0.051 ± .008	0.051 ± .015
Phenylalanine	0.056 ± .014	0.064 ± .048
Ornithine	0.082 ± .016	0.074 ± .033
Lysine	0.192 ± .060	0.181 ± .038
Histidine	0.106 ± .014	0.094 ± .040
Arginine	0.098 ± .042	0.109 ± .028
Tryptophan	0.024 ± .003	0.025 ± .006

+ Mean (micromoles/ml) and standard deviation.

++ 0.001 < P < 0.01 All other amino acids showed no difference.

Discussion.

A. Albumin synthesis and catabolism.

The adaptation of man to a low protein intake has been extensively investigated over the past decade. An overall picture is gradually emerging. Picou and Taylor-Roberts (1969), measuring total protein synthesis and catabolism and nitrogen turnover in infants by constant intragastric infusion of (^{15}N) glycine, concluded that nitrogen turnover, protein synthesis and protein catabolism were all significantly higher in malnourished compared to recovered infants. These results, together with data obtained in the rat by continuous intravenous infusion of (^{14}C) lysine (Waterlow and Stephen, 1968), led Waterlow to conclude that "the organism adjusts to a low protein intake not by a reduction of overall nitrogen turnover but by an alteration or diversion of metabolic pathways, such that a smaller proportion of available nitrogen is excreted and a larger proportion (though not a larger absolute amount) is used for protein synthesis" (Waterlow, 1968). Moreover, different proteins are affected to different degrees. Thus Picou et al (1966) were able to show severe depletion of total and non-collagen protein with conservation of collagen protein in infantile protein malnutrition. Waterlow and Stephen (1968), using a continuous infusion of (^{14}C) lysine, showed that protein deprivation caused large reductions in the rate of muscle protein synthesis, small changes in the rate of serum protein synthesis and no change in liver protein synthesis.

Albumin metabolism, particularly catabolism and distribution, has been fairly extensively studied. It has been clearly established that the body attempts to maintain the

circulating albumin mass at a constant level within fairly narrow limits. In children, Picou and Waterlow (1962) and Cohen and Hansen (1962) showed a reduction in the catabolic rate of albumin during protein depletion. Similar changes were shown by James and Hay (1968) and Hoffenberg et al (1966), who showed a marked fall in albumin catabolism in adults on a very low protein intake. Gitlin and Borges (1953), Humphrey and McFarlane (1954) and Gitlin (1955) claimed that catabolism of plasma protein is mainly a first-order process. This homeostatic mechanism would tend to maintain a constant plasma protein pool size. A change in pool size would be associated with a proportional change in the absolute amount of protein catabolised, and the pool would be restored to its original size provided that protein synthesis continued at the same rate. However, the work of Reeve and Roberts (1959), Hoffenberg et al, (1966) and others suggests that this may be an oversimplification, as changes in the fractional and absolute rates of albumin catabolism may occur without corresponding changes in serum albumin concentration or total intravascular albumin mass.

Numerous workers (Hoffenberg et al, 1966; Cohen and Hansen, 1962; Sellers et al, 1966) have shown that on protein restriction the body attempts to maintain the constancy of the intravascular albumin mass by the transfer of albumin from the extra- to the intra-vascular compartment. This shift is rapidly reversed on refeeding protein (James and Hay, 1968).

Until recently, albumin synthesis measurements were technically inaccurate. There are few studies of albumin

synthesis during protein depletion. Using the "synthesis and transfer rate" index described by Matthews (1961), Freeman and Gordon (1964) and Hoffenberg et al (1966) found that albumin synthesis was reduced in rats, rabbits and human subjects on a low protein diet. James and Hay (1968) studied the fate of injected iodinated albumin using a method of computer analysis in children on varying protein intakes. Their results suggest that the rate of synthesis is more dependent on the immediate dietary protein intake than on the serum albumin concentration or the intra-vascular albumin mass, and that the reduced rate of synthesis occurs earlier than the compensatory adjustment in catabolism. Kirsch et al (1968), using the direct method of McFarlane (1963), drew similar conclusions in rats. Recently, Coles et al (1970) measured albumin synthesis by the method of McFarlane in 2 patients with severely compromised renal function on 20 gram protein diets. The present study represents the first comprehensive attempt to measure albumin synthesis and catabolism independently on different dietary protein intakes in the same subjects free from complicating disease states.

The McFarlane technique has been validated by the agreement obtained between synthesis and catabolic rates when these are estimated independently under steady state conditions, in which the 2 are presumed to be equal. Tavill et al (1968), Jeejeebhoy et al (1969), Rosenoer et al (1967), McFarlane (1963a), and Kirsch et al (1968) obtained fairly good agreement between the albumin synthesis rate measured by the (^{14}C) carbonate technique and the catabolic rate measured with iodinated albumin in a steady state. It is

relevant that after two weeks on a 70 gram protein diet, there is poor correlation between synthesis and catabolic rates measured independently in the same subject in this series. It is possible that at this stage the subjects were still adapting to a protein load greater than normal.

A major attribute of the (^{14}C) carbonate technique is the minimal recycling of the label. In the case of the labelled guanidine carbon of arginine, recycling is minimised by the presence of arginase in the liver. Since hepatic arginase is reduced (Schimke, 1962) on a low protein diet, it is conceivable that labelled arginine released by catabolism is then re-utilised and could thus give fallacious albumin synthesis rates. However, catabolism is decreased in these circumstances and moreover, any re-utilisation would tend only to underestimate the reduction in the albumin synthesis rate.

This study therefore demonstrates a marked reduction in albumin synthesis and catabolic rates on protein restriction. The disproportionate fall in synthesis relative to catabolism results in a drop in the serum albumin concentration in spite of the compensating shift of albumin from the extra-vascular to the intra-vascular pool.

It is interesting to compare the results of this study with those of Hoffenberg et al (1966) who measured albumin catabolism and "synthesis and transfer" in subjects under the same conditions as reported in this study. (Table IV). The results obtained by the different methods are similar but indicate that "synthesis and transfer" tends to overestimate the albumin synthesis rate on protein restriction. While this work was being completed, Coles et al (1970) reported reduced albumin catabolism and synthesis in uraemic patients. They

T A B L E IV

Comparison between the results of Hoffenberg et al (1966)
and those of present study.

	NORMAL DIET		10G PROTEIN DIET	
	Present study	Hoffenberg et al	Present study	Hoffenberg et al
Fractional synthesis rate *	14.9	8.9 ^{xxx}	5.9	5.8 ^{xxx}
Fractional catabolic rate *	10.9	8.8	6.5	6.5
Absolute synthesis rate **	245	150 ^{xxx}	85	103 ^{xxx}
Absolute catabolic rate **	173	151	111.0	103

* % of intravascular pool/day

** mg/Kg/day

xxx "synthesis and transfer".

obtained albumin synthesis rates of 120 and 100 milligrams per kilogram per day in 2 uraemic patients on 20 gram protein diets. They concluded that changes in albumin metabolism in uraemia were probably secondary to the dietary changes rather than to the renal disease itself. This study, using a direct technique for measuring albumin synthesis, therefore confirms the work of previous investigators and serves to emphasise the importance of measuring albumin catabolism and particularly albumin synthesis under controlled dietary conditions.

It is therefore quite clear that the dietary protein supply directly influences albumin synthesis. Mariani et al (1963) and Gaetani et al (1964) have shown that there is an increase in the activity of amino acid activating enzyme systems in the liver on a low protein diet. In these circumstances, there is a decrease in the amino acid activating enzymes of muscle (Stephen, 1968) and a decreased activity of urea cycle enzymes (Schimke, 1962). Therefore an amino acid entering the body under conditions of reduced protein supply will have a greater chance of being incorporated into protein and a smaller chance of being deaminated or incorporated into muscle protein than would occur on a normal protein intake. Following this initial adaptation, albumin synthesis would be progressively slowed, presumably due to lack of further substrate, defective machinery or altered controlling mechanisms. At what level is the protein load exerting control? Apart from early post-natal life, proteins are absorbed only as amino acids. Therefore are the amino acids acting as substrate, trigger mechanisms for protein synthesis or do they influence synthesis by their release of hormones (see review of amino acid pools) or by some as yet undetermined manner? Furthermore, are

some amino acids invested with special controlling properties or, at a higher degree of sophistication, are certain amino acids influencing the synthesis of only certain proteins?

The relationship between amino acid levels and augmented protein synthesis has been discussed in general in the review on amino acid pools. There can be little doubt that hormones figure predominantly in, that not only do they influence tissue and plasma amino acid levels and are themselves influenced by amino acid levels, but also their influence on protein synthesis is well described (see review on amino acid pools.) It has been shown that excess thyroid hormone (Rothschild et al, 1957) and excess steroid hormones (Rothschild et al, 1958; Grossman et al, 1960; Sterling, 1960) increase both the absolute and fractional synthesis and catabolic rates of albumin. Moreover, Kernoff (1970) has shown growth hormone to play a role in increasing both albumin synthesis and albumin catabolism in the hypophysectomised rat. The role of amino acids in albumin synthesis is described in the following section. Here hormonal influences have been excluded by using the isolated perfused rat liver.

One would anticipate that a consideration of the functions of albumin would provide insight into controlling mechanisms other than by amino acids. The functions which have been ascribed to albumin include :-

1. acting as an amino acid pool in a form unable to be lost from the body via the kidneys;
2. acting as a transport system; and
3. maintaining the colloid osmotic pressure within the vascular system.

It is interesting to note that the 12 grams of amino acids released by albumin catabolism per day together with the catabolic products of other plasma proteins would go a long way toward providing the daily requirement of 50-60 grams of amino acids. However, if albumin were an amino acid store, then protein deficiency would be expected to result in increased and not decreased metabolism.

Albumin certainly participates in the transport of numerous substances by virtue of its extensive surface area (Bennhold, 1965) and its particular avidity for anions (Scatchard et al, 1944; Karush, 1950). Normally, albumin provides the carrier for bilirubin, porphyrins, uric acid, acetylcholine, fatty acids, copper, calcium and zinc. When present in excess, vitamin A, vitamin C, thyroxine, triiodothyronine, cortisol, progesterone, aldosterone, oestrone and histamine are transported by albumin. In addition, numerous drugs are transported such as barbiturates, digoxin, aspirin, penicillin, sulphonamides, dyes, P.A.S., tolbutamide and others. However, in all cases the albumin molecule is a non-specific vehicle and its role can be replaced by other proteins.

Albumin exerts 75% of the total colloid osmotic pressure within the intra-vascular compartment by virtue of its high concentration and relatively small size. However, once again this function appears to be non-specific.

In spite of the above proposed functions, patients with analbuminaemia appear to be little inconvenienced by their lack of albumin. Feedback control mechanisms examined from a functional point of view have been limited. Nonetheless, a

colloid osmotic regulatory system exerting control over albumin synthesis has been proposed (Bjorneboe, 1946; Rothschild et al, 1962). Rothschild et al (1965) considered that their data was consistent with the hypothesis that albumin synthesis was regulated by the albumin concentration within the hepatic interstitial space. However, albumin infusions in man (Tavill et al, 1968) and in the rabbit (Rothschild et al, 1968) were unable to retard albumin synthesis consistently. However, dextran and hypergammaglobulinaemia were able to depress albumin synthesis in rabbits (Rothschild et al, 1961, 1962, 1968). Moreover, dextran and excess albumin were shown to inhibit albumin synthesis in the isolated perfused rabbit liver system (Rothschild et al, 1967, 1969b). Oratz et al (1970), from subcellular work, considered that dextran both decreases the fraction of membrane-bound ribosomes and prevents the ribosome from binding to mRNA. It is uncertain whether this is related to changes in colloid osmotic pressure induced by dextran or to other changes such as amino acid or tissue hormone levels.

From this discussion it would appear that only amino acid supply, hormones and the colloid osmotic pressure have been shown to influence albumin synthesis.

B. Plasma aminogram.

In a review of the plasma aminograms of children receiving low protein intakes in many countries, Holt et al (1963) concluded:

1. that there is a decrease in the concentration of the plasma essential amino acids, particularly of the branched chain amino acids, and least of phenylalanine and lysine, and
2. that the nonessential amino acids tyrosine, arginine,

citrulline and alpha-amino N-butyric acid decrease. The other non-essential amino acids glycine, alanine, serine, proline, histidine and aspartic acid are either unchanged or even rise above normal values.

Studies in normal adults on low protein or protein-free diets have shown varying patterns. Tuttle et al (1957) and Swendseid et al (1968) showed a reduction in valine and alpha-amino N-butyric acid on a low protein diet, but a rise in methionine, glutamine, asparagine, glycine and alanine. However, Adibi (1968) found a marked elevation of plasma alanine concentration and, to a lesser extent, an elevation of the glycine concentration in normal subjects after 2 weeks on an iso-caloric protein-free diet. This is similar to the findings reported in this thesis of a raised plasma alanine after 4 to 6 weeks on a 10 gram iso-caloric protein diet. When one considers that alanine, together with glycine, glutamic acid and aspartic acid constitute 80% of the free amino acids in the body, it is hardly surprising that any changes would be detected first in this group. In fact, Felig et al (1969) consider that alanine serves as a vehicle of transport for nitrogen arising from amino acid catabolism in the periphery. The differences between the studies reported above are probably due to the variations in individuals, protein intake, caloric intake, meal spacing and duration of the studies. Interpretation of animal studies is even more difficult as protein restriction is accompanied by anorexia and decreased caloric intake. Pair-fed controls are unsatisfactory as these animals consume the reduced intake so rapidly that they are virtually fasting by the time of the experiment. Forced feeding is limited by the metabolic changes

which occur (Sidransky and Farber, 1958; Sidransky and Verney, 1964). Since plasma amino acid concentrations may have no bearing on hepatic intracellular amino acid levels, the relationship of the low synthesis rates to the elevated plasma alanine levels may even be fortuitous or the result of 2 processes acting at different sites.

P A R T I I .

THE EFFECTS OF AMINO ACIDS ON ALBUMIN SYNTHESIS IN
THE ISOLATED PERFUSED RAT LIVER.

Introduction.

While recent workers have confirmed that protein restriction reduces albumin synthesis both in man (Coles, 1970; present thesis, Part I) and in the rat (Kirsch et al, 1968), the mechanism involved remains to be established. The changes in the plasma aminogram in PCM (Holt et al, 1963; Saunders et al, 1967) suggested that certain amino acids may influence protein synthesis selectively. Indeed, Pronczuk et al (1968) have shown that tryptophan deficiency results in diminished incorporation of amino acids into rat liver protein, with associated ribosomal disaggregation. Similar results have been obtained in mice (Sidransky et al, 1968). While tryptophan appears to have this specific function in vivo, the work of Hanking and Roberts (1964, 1965) suggests that individual amino acids can stimulate protein synthesis and increase ribosomal activity in vitro, irrespective of the size of the amino acid pool. In contrast, Baliga et al (1968) have shown that the absence of any amino acid (with the exception of isoleucine) results in ribosomal disaggregation.

The author has studied the influence of amino acids on albumin synthesis in the isolated perfused rat liver. This technique permitted the study of individual amino acids using both fed and fasted liver donors without changes in hormonal levels.

While this work was in progress, Rothschild et al (1969c) reported that tryptophan, and, to a lesser extent, isoleucine were able to stimulate albumin synthesis in a fasted rabbit liver. Important differences between the present study and

that of Rothschild et al (1969c) suggest that more than one mechanism is operative.

Materials and Methods.

The principles of the methods have been discussed. The detailed methodology is given in the Appendix. Essential features are summarised.

Perfusion apparatus and technique.

Liver donors were 320-350 g male Wistar rats housed under controlled conditions of temperature, humidity and lighting.

Two groups of donors were used :-

1. rats fed a 20% diet ad lib, and
2. rats fasted for 24 hours.

Both groups had free access to water.

The perfusate consisted of 60 ml blood, 2 ml 4.2% sodium bicarbonate, 40 mg of heparin (heparin injection B.P. Boots Drug Co. Ltd., Nottingham, England) and 25 ml plasmalyte B (Baxter-Saphar Laboratories Limited, Johannesburg, South Africa) containing, in mEq/L, sodium 130, chloride 109, potassium 4, magnesium 3, sodium bicarbonate 28. The final packed cell volume of the perfusate was 24-28%. Blood was obtained by cardiac puncture under diethyl ether anaesthesia from 2 groups of rats: (1) rats fed ad lib, and (2) rats on a zero protein diet for 48 hours. This feed consisted of maize starch with mineral and vitamin supplements (2.2% vitamin diet fortification mixture, National Biochemical Company, Ohio).

Perfusions were performed according to the method of Miller et al (1951) as modified by Fisher and Kerly (1964).

Under diethyl ether anaesthesia, the bile duct and portal vein were cannulated, the liver flushed with plasmalyte B and then transferred to the perfusion cabinet. Outflow from the hepatic veins drained directly into a reservoir. A 95% oxygen and 5% CO₂ mixture was passed into a glass "thin film" oxygenator at a flow rate of 750 ml/minute. The cabinet was humidified, the temperature controlled at 38°C and the portal pressure at 18 cm of water.

250 microcuries of sodium (¹⁴C) carbonate were added to the reservoir after 30 minutes of perfusion. The perfusion was continued for a further 120 minutes to allow for complete release of labelled albumin into the perfusate. pH, PO₂ and PCO₂ were maintained at 7.35 - 7.42, 150-175 millimetres of mercury and 35-40 millimetres of mercury respectively. Perfusate urea and glucose were measured on a technicon autoanalyser with appropriate standards.

Albumin synthesis rate.

This was measured by the technique of McFarlane and of Reeve et al as previously described. The formula used is given in Equation 10.

Plasma albumin was measured by the method of Fernandez et al (1966). Only L isomers of amino acids (British Drug House, Poole, England) were added to the perfusate, each to concentrations of 10 micromoles/ml, except in some experiments in which tryptophan and the branched chain amino acids were added to a concentration of 0.05 micromoles/ml. Arginine, asparagine, isoleucine, lysine, methionine, phenylalanine, proline,

threonine, tryptophan and valine were added together to a concentration of 10 times their normal peripheral blood levels. Glucagon (U.S.P. Eli Lilly and Co., Indianapolis, U.S.A.) when used, was added initially in doses of 1 mg followed by 0.5 mg/hour. Amino acids were measured on a technicon amino acid autoanalyser.

Results.

The amino acid concentrations of the perfusates used are shown in Table V. 48 hours of protein restriction reduced the concentrations of isoleucine, leucine, valine, tyrosine, phenylalanine, histidine, ornithine, methionine and glycine. Lysine was the only essential amino acid remaining unchanged.

Table VI shows the changes in albumin and urea synthesis rates expressed in mg/hour/300 g rat using well-nourished liver donors. When perfusate I was used, a synthesis rate of 1.75 ± 0.18 was obtained. The use of perfusate II dropped the synthesis rate to 1.11 ± 0.31 ($0.01 < P < 0.02$). Perfusate II with each amino acid added singly to a concentration of 10 micromoles/ml significantly increased albumin synthesis rates (tryptophan $0.01 < P < 0.02$; isoleucine $0.001 < P < 0.01$; lysine $0.001 < P < 0.01$; glycine $0.02 < P < 0.05$; alpha-amino isobutyric acid $0.02 < P < 0.05$ and lysine, methionine and threonine together $0.02 < P < 0.05$). Neither the branched chain amino acids nor tryptophan, added to a concentration of 0.05 micromoles/ml to perfusate II, had any effect on albumin synthesis ($0.3 < P < 0.4$ and $0.05 < P < 0.10$).

T A B L E V

Initial amino acid concentrations of
the perfusates.

Amino Acid.	Perfusate I. ⁺	Perfusate II ⁺⁺	Significance. ++++
	μ moles/ml ⁺⁺⁺	μ moles/ml ⁺⁺⁺	
Isoleucine	0.056 \pm 0.005 (7)	0.038 \pm 0.009 (5)	.001 < P < .01
Leucine	0.107 \pm 0.012 (7)	0.062 \pm 0.021 (5)	.001 < P < .01
Valine	0.110 \pm 0.011 (7)	0.051 \pm 0.020 (5)	P < .001
Histidine	0.047 \pm 0.014 (6)	0.028 \pm 0.008 (5)	.02 < P < .05
Phenylalanine	0.045 \pm 0.005 (7)	0.030 \pm 0.011 (5)	.01 < P < .02
Tyrosine	0.058 \pm 0.005 (7)	0.028 \pm 0.008 (5)	P < .001
Lysine	0.160 \pm 0.023 (7)	0.140 \pm 0.011 (5)	N.S.
Alanine	0.407 \pm 0.109 (6)	0.518 \pm 0.039 (5)	N.S.
Citrulline	0.049 \pm 0.013 (3)	0.040 \pm 0.017 (2)	N.S.
Ornithine	0.123 \pm 0.027 (7)	0.074 \pm 0.020 (5)	.001 < P < .01
Taurine	0.120 \pm 0.005 (5)	0.120 \pm 0.007 (3)	N.S.
Methionine	0.032 \pm 0.005 (7)	0.020 \pm 0.001 (5)	P < .001
Glycine	0.286 \pm 0.049 (6)	0.133 \pm 0.042 (4)	P < .001
Tryptophan	0.018 \pm 0.004 (8)	0.014 \pm 0.003 (8)	.02 < P < .05

⁺Perfusate I: consisted of 60 ml blood from rats on a normal diet, 25 ml Plasmalyte B, 4 ml heparin (40 mg), 2 ml. 4.2% sodium bicarbonate.

⁺⁺Perfusate II: as above with the 60 ml. blood taken from rats on a protein-free diet for 48 hours.

⁺⁺⁺Each result represents the mean and Standard Deviation with the number of observations in parentheses.

⁺⁺⁺⁺p Value indicating significance of change between Perfusate I and Perfusate II. N.S. = Not significant.

T A B L E VI

Albumin and urea synthesis rates (mg/hr/300G rat) in
isolated rat liver perfusions using well nourished
rats as liver donors.

Perfusate.	Albumin Synthesis Rate ++++	Urea Synthesis Rate ++++
Perfusate I	1.75 ± 0.18 (8)	5.81 ± 1.93 (8)
Perfusate II	1.11 ± 0.31 (7)	6.03 ± 1.28 (7)
Perfusate II + Tryptophan ⁺	1.50 ± 0.23 (8)	6.93 ± 1.16 (8)
Perfusate II + Isoleucine ⁺	1.82 ± 0.34 (5)	6.18 ± 0.98 (5)
Perfusate II +(Lysine + Methionine ⁺ Threonine ⁺	1.58 ± 0.36 (5)	15.58 ± 2.69 (5)
Perfusate II + Lysine ⁺	2.04 ± 0.61 (5)	12.90 ± 1.32 (5)
Perfusate II + Glycine ⁺	1.45 ± 0.24 (5)	11.02 ± 2.10 (5)
Perfusate II + Alpha- amino isobutyric acid ⁺	1.47 ± 0.16 (5)	6.02 ± 0.81 (5)
Perfusate II + Tryptophan ⁺⁺	1.45 ± 0.34 (8)	6.59 ± 0.55 (8)
Perfusate II + Branched chain amino acids ⁺⁺	1.23 ± 0.12 (5)	5.92 ± 0.66 (5)
Perfusate I + 11 amino acids ⁺⁺⁺	2.38 ± 0.43 (5)	14.30 ± 1.37 (5)

⁺ Amino acid added to a concentration of 10 μ moles/ml.

⁺⁺ Amino acid added to a concentration of 0.05 μmoles/ml.

⁺⁺⁺ Amino acids arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added together to a concentration of 10 x their normal peripheral blood levels.

⁺⁺⁺⁺ Each result represents the mean and Standard Deviation with the number of observations in parenthesis.

respectively). Arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added together to perfusate II, each to 10 times their normal peripheral blood concentrations, enhanced synthesis rates to a value of 2.38 ($0.02 < P < 0.05$). Urea synthesis rates were the same whether perfusate I alone or perfusate II alone was used. The addition of lysine, glycine, the 11 amino acids given together (see before) or a combination of methionine, lysine and threonine significantly increased urea synthesis. However, the addition of tryptophan, isoleucine and alpha-amino isobutyric acid had no effect on urea production despite an increase in albumin synthesis rates.

Table VII shows the changes in albumin and urea synthesis rates expressed in mg/hour/300 g rat using liver donors from rates fasted for 24 hours. Perfusion of the fasted liver resulted in a fall in the albumin synthesis rates without affecting urea synthesis rates. The combination of the 11 amino acids mentioned above increased albumin synthesis rates markedly. Irrespective of the other amino acids added or the perfusate used, there was no change in the albumin synthesis rates. Glucagon with tryptophan failed to influence albumin synthesis. However, lysine, alone or in combination, glucagon with tryptophan and the 11 amino acids increased the urea synthesis rates.

Glucose production was dependent on the nutritional state of the liver and was uninfluenced by the addition of amino acids or a change in the perfusate. Glucose production increased from 60 mg% in both groups to 110 mg% and 215 mg% using liver donors of fasted or fed rats respectively.

In all perfusions the liver was uniformly perfused. Bile production was 0.5-1.0 ml/hr and the perfusion rate varied from 1.5-2.0 ml/g liver/min.

T A B L E VII

Albumin and urea synthesis rates (mg/hr/300G rat) in
isolated rat liver perfusions using 24 hour fasted
rats as liver donors.

Perfusate.	Albumin Synthesis Rate ++++	Urea Synthesis Rate ++++
Perfusate I	0.50 ± 0.24 (5)	6.28 ± 1.28 (5)
Perfusate II	0.65 ± 0.43 (5)	6.34 ± 0.97 (5)
Perfusate II + Tryptophan ⁺	0.46 ± 0.14 (5)	7.36 ± 1.83 (5)
Perfusate II + Isoleucine ⁺	0.59 ± 0.08 (3)	6.63 ± 0.50 (3)
Perfusate II + Lysine +	0.48 ± 0.26 (5)	14.44 ± 1.31 (5)
Perfusate II +(Tryptophan ⁺ (Lysine ⁺	0.70 ± 0.22 (5)	14.14 ± 2.09 (5)
Perfusate I + Tryptophan ⁺	0.61 ± 0.10 (3)	7.17 ± 1.42 (3)
Perfusate I + 11 Amino Acids ⁺⁺	1.65 ± 0.33 (4)	15.90 ± 1.71 (4)
Perfusate I +(Glucagon ⁺⁺⁺ (Tryptophan	0.59 ± 0.03 (3)	10.53 ± 2.70 (3)

+ Amino acid added to a concentration of 10 µmoles/ml.

++ Amino acids arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added together to a concentration of 10 x their normal peripheral blood+ levels.

+++ Tryptophan added to a concentration of 10 µmoles/ml. 1 mg glucagon added initially, followed by 0.5 mg/hr.

++++ Each result represents the mean and Standard Deviation with the number of observations in parentheses.

Discussion.

A striking feature of albumin synthesis rate measurements in isolated rat liver perfusions has been the marked variation and the reduced rates compared with the in vivo values obtained by different investigators (see Table VIII).

Both Katz et al (1967) and Hoffenberg et al (1971) have demonstrated a fair correlation between the immunochemical and (^{14}C) carbonate techniques. The remarkably raised rate obtained by Katz et al (1967) is unexplained. However, the bulk of evidence would suggest a rate of about 40-60% of in vivo values, irrespective of techniques used, in spite of the fact that the liver is the only site of albumin synthesis (Miller and Bale, 1954; Kukral et al, 1961).

The mean value for albumin synthesis rates in the normal rat in vivo, as measured by the author using the (^{14}C) carbonate technique, was 9 mg/hour/300 g body weight (unpublished). In contrast, when perfusate II was used the value was 1.11 mg/hour/300 g. However, perfusate I, with its minimally increased levels of isoleucine, leucine, valine, histidine, phenylalanine, tyrosine, ornithine, methionine, glycine and tryptophan, was able to increase synthesis rates to 1.75 mg/hour/300 g rat. Clearly, the varying synthesis rates obtained by different workers could reflect many factors including the perfusate amino acid concentration, dilution of blood and the nutritional and hormonal state of blood or of liver donors.

Reference has already been made to the augmented protein synthesis achieved with increased amino acid supply (see review on amino acid pools.) The increased amino acid supply provided by perfusate I led to increased albumin synthesis rates and

T A B L E VIII.

Albumin synthesis rates obtained by different workers using the isolated perfused rat liver.

A. IMMUNOLOGICAL TECHNIQUES

Marsh and Drabkin (1960)	6 mg/hr/300 g body wt.
Gordon and Humphrey (1960)	9-12 mg/hr/300 g body wt.
John and Miller (1966)	6 mg/hr/300 g body wt.
Hoffenberg et al (1971)	4 mg/hr/300 g body wt.

B. (¹⁴C) CARBONATE TECHNIQUE

Gordon and Mutschler (1964)	60% of in vivo values.
Katz et al (1967)	30 mg/hr/300 g body wt.
Kirsch et al (1969)	1.64 mg/hr/300 g body wt.
Rosenoer et al (1970)	2.7 mg/hr/300 g body wt.
Hoffenberg et al (1971)	5 mg/hr/300 g body wt.

would tend to support this. However, other changes in the blood, such as altered hormonal levels, could have occurred leading to this change. Nonetheless, John and Miller (1969), in an extensive study, demonstrated the major role of amino acids in accelerating albumin synthesis and the modifying influence of insulin, cortisone and growth hormone.

Levitan and Webb (1969) and Jefferson and Korner (1969) have shown progressive ribosomal disaggregation during perfusion. Jefferson and Korner (1969) demonstrated that the 11 amino acids arginine, asparagine, isoleucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added together to a concentration of 10 times their normal peripheral blood levels were able to prevent ribosomal disaggregation. The author has confirmed and extended these observations to the endoplasmic reticulum-bound ribosomes (see later chapter). Under these conditions, albumin synthesis rates increased to 2.38 milligrams/hour/300 g body weight.

The question has arisen whether protein synthesis is limited by specific amino acid deficiencies. Reference has already been made to the correlation of a low serum albumin and specific blood amino acid changes in PCM. Moreover, Elwyn et al (1968), in their study of the quantitative role of the gut and the liver in amino acid metabolism found that net hepatic protein synthesis was associated with the uptake of the branched chain amino acids. The output of other amino acids was unrelated to hepatic protein synthesis. In the present study, the author, using diluted blood, was unable to confirm the findings of Kirsch et al (1969), using whole blood, that the branched chain amino acids in physiological amounts were able

to increase albumin synthesis.

The study of the role of specific amino acids in protein synthesis has been focussed to a large extent on ribosomal profiles. Jefferson and Korner (1969) demonstrated that omission of any one of the above 11 amino acids will result in diminished protein synthesis. Baliga et al (1968) could obtain ribosomal aggregation in vitro only if all 20 amino acids (except isoleucine) were present. Eliasson et al (1967) have shown that Chang liver cells in tissue culture undergo ribosomal disaggregation when arginine or glutamine is removed from the medium. In vivo, in the rat (Pronczuk, 1968) and in the mouse (Sidransky et al, 1968), tryptophan occupies this unique role. It appeared that the limiting amino acid was determined by the ease with which its pool could be depleted to a critical level. This concept is supported by the low intracellular levels of tryptophan in the liver of the rabbit (Rothschild et al, 1969c) and the finding of Pronczuk et al (1969) that, on feeding an imbalanced amino acid mixture under conditions when amino acid recycling is minimal, other amino acids influence the ribosomal profile. While the present study was in progress, Rothschild et al (1969c) reported that tryptophan and, to a lesser extent, isoleucine increased albumin synthesis when added to the blood perfusing an isolated liver taken from a fasted rabbit. This would suggest that, normally, the liver is dependent for short term supply on protein catabolism for the bulk of amino acids, with the diet regulating synthesis by providing tryptophan. Fasting is known to result in elevated glucagon levels (Aguilar-Parada et al, 1969) and the latter has been shown to

accelerate liver protein catabolism (Miller, 1960). Moreover, Gan and Jeffay (1967) have demonstrated the accelerated recycling of liver amino acids on fasting. If a supply of tryptophan is not available, the increased supply of amino acid is wasteful and it appears that this spurious adaptation then gradually declines.

The author, like Rothschild et al (1969c) obtained a marked slowing of albumin synthesis using the fasted liver. However, unlike Rothschild et al, single amino acids, including tryptophan and isoleucine in excess, were ineffective in stimulating albumin synthesis. An attempt to increase protein catabolism by glucagon infusion and then adding tryptophan failed to increase albumin synthesis. The fasted liver, however, responded to the addition of the previously mentioned 11 amino acids added to 10 times their peripheral blood concentrations. Probably the fasted rat liver, with its faster turnover than the rabbit liver, is too protein depleted by the time of perfusion to provide the other amino acids required.

The remarkable and unexpected finding by the author was the increased albumin synthesis resulting from the addition of single amino acids to the blood obtained from protein depleted rats, perfusing livers derived from fasted rats. This would tend to support the finding of Hanking and Roberts (1964, 1965) that single amino acids are capable of stimulating protein synthesis in vitro. Furthermore, the finding of Rothschild et al (1969c) that both isoleucine and tryptophan individually increased albumin synthesis, suggests that a mechanism other than that dependent on critical amino acid pool depletion is operative. It is of interest that alpha-amino isobutyric acid,

a non-metabolisable model amino acid also has this property, suggesting that the mechanism is not one involving energy expenditure nor direct participation in amino acid metabolism. In addition, lysine is also effective, supporting the concept that transamination is not involved.

However, caution is required in interpreting this data. Rothschild et al (1969c) were unable to influence albumin synthesis by the addition of tryptophan to blood perfusing livers derived from fed rats. No experiments were conducted by the author adding single amino acids to the perfusion system with both liver and blood derived from well-nourished animals. It is possible that it is only in the setting of altered hormonal levels or other factors in the blood derived from 48 hours protein-depleted animals that single amino acids in excess influence albumin synthesis.

P A R T I I I

THE EFFECTS OF AMINO ACIDS AND HORMONES ON THE
FRACTIONAL CATABOLIC RATE OF ALBUMIN.

Introduction.

While albumin degradation has been fairly extensively studied during the past decade, little is known of the site(s) or factors operative in this process.

Campbell et al (1956) and McFarlane (1957) showed that, subsequent to the injection of iodoalbumin, iodide excreted daily in the urine represents a constant fraction of the mean protein-bound activity circulating in the plasma at the same time, and not of the total retained radioactivity. This data implied that albumin catabolism occurs in cells in close proximity to the intra-vascular pool. Recently, McFarlane and Koj (1970) favoured the view "that catabolism occurs in a pool of significant protein content which is either a subunit of the extra-vascular pool or is independent and sandwiched between the plasma and extra-vascular pools".

At an organ level, the kidney has been estimated to degrade about 10-15% (Katz et al, 1960), the gut less than 20% (Waldmann, 1966) and the liver 10-15% (Cohen and Gordon, 1958; Gordon, 1962) of the albumin catabolised by the whole animal.

While the absolute degradation of albumin varies generally with the pool size, the fractional rate remains relatively constant (first order kinetics). Therefore, any change in pool size due to abnormal losses or altered synthesis will result in changes in absolute albumin degradation. However, in certain circumstances, such as protein deprivation, the fractional degradation rates have been shown to alter (Hoffenberg et al, 1966; Kirsch et al, 1968; Part I of this thesis). The mediator of this important change in the

fractional rate is largely unknown. The author has investigated this aspect with emphasis on :-

(1) the role of amino acids in the fractional catabolic rate of albumin (FCRA). This has 2 objectives : (a) to validate the assumption, made earlier in this thesis during measurement of albumin synthesis in the isolated perfused rat liver, that amino acids do not influence albumin catabolism, and (b) to verify that albumin degradation is unrelated to amino acid supply but dependent on the albumin pool size (Kirsch et al, 1968).

(2) the measurement of albumin degradation under conditions of maximum protein synthesis, and therefore presumably under conditions of accelerated proteolytic enzyme activity, and

(3) the influence of growth hormone, hydrocortisone, insulin, glucagon and triiodothyronine on the FCRA.

Materials and Methods.

The principles of liver perfusion, extraction, iodination and screening of albumin and the measurement of catabolic rates in the isolated perfused system have been described while the detailed methodology appears in the Appendix.

Both liver and blood donors were obtained from well-nourished Wistar rats of 320-350 g, housed under controlled conditions of humidity and lighting and with free access to food and water. The iodinated albumin was used shortly after preparation. After perfusion for 30 minutes, the labelled albumin was added and blood sampled $\frac{1}{2}$ hourly for a further 3 hours. The fractional catabolic rate was

derived from the iodide released per 30 minutes, expressed as a percentage of the mean total protein-bound radioactivity during that time. Amino acids, when added, were arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added together to a concentration of 10 times their peripheral blood levels. Hormones, when added, were given at the beginning of the perfusion and thereafter infused continuously. Initially, 2 units of soluble insulin B.P. (Boots Pure Drug Co. Ltd., Nottingham, England), 2 mg of hydrocortisone (Glaxo-Allenbury's, SA(Pty) Ltd), 2 mg of bovine growth hormone (N.I.H.), $\frac{1}{2}$ mg of glucagon(U.S.P. Eli Lilly & Co., Indianapolis, USA) and 20 mg of triiodothyronine (Glaxo Lab. Ltd., Greenford, Middlesex, England) were added in individual perfusions. Following the initial addition, the respective hormones were infused continuously at the rate of $\frac{1}{2}$ the initial dose per hour.

Results.

Table IX shows the results of the 28 liver perfusions. Hydrocortisone, growth hormone, amino acids, triiodothyronine and glucagon failed to influence the FCRA. However, insulin appeared to slow the fractional catabolic rate marginally.

The albumin concentration in all perfusions varied from 1.0 g/100 ml to 1.3 g/100 ml.

Discussion

Catabolism of albumin is thought to occur throughout the body. Nonetheless, the liver provides a useful, although limited, model for studying the control of albumin degradation.

T A B L E IX.

The fractional catabolic rate of albumin in isolated rat liver perfusions using well nourished rats as liver and blood donors.

Perfusate	Fractional catabolic rate of albumin ⁺
(1) Perfusate alone	0.075 ± 0.023 (7)
(2) Added hydrocortisone	0.069 ± 0.031 (5)
(3) Added growth hormone	0.085, 0.058 (2)
(4) Added insulin ⁺⁺	0.044 ± 0.017 (5)
(5) Added amino acids	0.069 ± 0.020 (3)
(6) Added triiodothyronine	0.078 0.060 (2)
(7) Added glucagon	0.051 ± 0.004 (4)

⁺ Mean, Standard deviation and number of observations in parentheses.

⁺⁺ Significance (1) versus (4) $0.02 < P < 0.05$
 (1) versus (7) $0.05 < P < 0.1$
 (1) versus (2), (3), (5) and (6) $P > 0.1$

Catabolic studies in the isolated rat liver perfusion have emanated chiefly from investigators from Mill Hill. Thus Gordon (1957), Cohen and Gordon (1958) and Gordon (1962) showed that the perfused rat liver catabolises about 10-15% of the homologous albumin broken down in the whole animal. Freeman et al (1958) demonstrated that the catabolism of (^{14}C) heat-denatured chicken serum proteins was decreased while that of native serum (^{131}I) albumin was increased fivefold in perfusions using livers from carbon-loaded rats. More recently, Hoffenberg et al (1970) have published interesting data relating the FCRA to the intra-vascular albumin pool. They found that when livers from protein depleted rats were perfused with normal blood the FCRA was slightly reduced. When these livers were perfused with blood derived from protein deprived rats, minimal catabolism could be detected. Catabolism was restored by the addition of unlabelled albumin. These results suggest that both the nutritional state of the liver and particularly the intra-vascular pool of albumin regulate the FCRA. It should be noted, however, that the albumin concentration at which the FCRA was markedly reduced in their work was still higher than the albumin concentration reported in the present study, at which normal FCRA values were obtained. The normal FCRA results obtained by the author are similar to those obtained by Gordon (1962) and Hoffenberg (1970). Only screened albumin was used, and it is unlikely that the present results are high due to denatured material. Is it the albumin itself, is the operative factor something in the blood from protein deprived animals or is it a combination of both?

Could the non-screened unlabelled albumin load added by Hoffenberg et al (1970) have acted as a carrier of some agent or even functioned as a "carbon load" (Freeman et al, 1958)? Certainly, more experiments are required to finalise these points.

It seemed a plausible hypothesis to the author that the 10-15% value for the liver contribution to total body albumin catabolism was an underestimation dictated by the functional state of the isolated liver. The enzymes or proteolytic mechanisms involved in protein degradation are largely unknown (see review of amino acid pools.) Furthermore, Levitan and Webb (1969), Jefferson and Korner (1969) and the author of this thesis (part IV) have shown that ribosomal disaggregation occurs during perfusion without specific amino acid supplements. However, the perfusion studies with qualitative and quantitative amino acid supplements shown to aggregate both the free and the endoplasmic reticulum-bound ribosomes and also to augment albumin synthesis, failed to influence the FCRA. This justifies the assumption made in Part II and the implication of Kirsch et al (1968) that amino acids have no direct influence on albumin catabolism. Furthermore, certainly for short periods, altered protein synthesis is not rate limiting to albumin catabolism.

Reference to the lysosomal system has been made in the "Review of Amino Acid Pools". Plasma proteins are thought to be engulfed by a process of pinocytosis and then transferred in small vesicles to secondary lysosomes. While this process has been clearly implicated with respect to denatured albumin, native albumin degradation is ill-defined. Glucagon induces

autophagocytosis with participation of lysosomal enzymes (Ashford and Porter, 1962; Deter and De Duve, 1967; Guder et al, 1970). Moreover, glucagon increases protein degradation in the isolated perfused rat liver (Green and Miller, 1960; Miller, 1961). The reciprocal role of insulin and glucagon are well established and it was therefore not surprising that Mortimore and Mondon (1970) have recently shown that insulin can almost completely suppress proteolysis in the isolated rat liver. It is therefore very tempting to conclude that the lysosomal system and its control are the cornerstone of protein, including plasma protein, degradation.

In vivo, thyroid hormone (Rothschild et al, 1957), steroid hormones (Rothschild et al, 1958; Grossman et al, 1960; Sterling, 1960) and growth hormone (Kernoff, 1970) have been shown to increase the fractional rate of albumin catabolism. No effect of these 3 hormones could be shown by the author on the FCRA in the isolated perfused rat liver. However, insulin retarded albumin degradation. These preliminary results raise a number of points :-

(1) Is a system other than the lysosomes involved in catabolism of albumin? Certainly, the in vivo results obtained with steroids, the perfusion results with glucagon, the existence of other proteolytic enzymes apart from those of the lysosomes and the inability to demonstrate the participation of lysosomes in the degradation of undenatured albumin suggest that this is a strong possibility.

(2) Is the addition of hormones to an isolated system justifiable? Problems in interpretation arise from dose-response

relationships, and from the fact that some hormones have been shown to require endogenous modification for biological activity.

(3) To what extent does the liver perfusion dictate the pattern of biological activity by virtue of its isolation? This important question arises in any study utilising the isolated perfused system. There can be little doubt that most biological processes are the result of numerous inter-relating control mechanisms, whether the latter be hormones, amino acids, or other factors. Therefore the results obtained will, to some extent, depend on the half-life of those factors in the perfusion. Hormones provide a prime example of this. The system used by the author rapidly degrades insulin and glucagon but growth hormone, proinsulin and parathyroid hormone are relatively resistant (Kelman et al, 1971). The net effect would be impossible to predict. It is possible that while glucagon and insulin degradation are balanced, the release of insulin from proinsulin would continue to provide an overall "insulin effect". To attempt to extrapolate perfusion data to absolute data in vivo is dangerous and this certainly applies to the 10-15% estimate of albumin catabolism by the liver.

P A R T I V

THE EFFECTS OF PERFUSION, AMINO ACIDS AND HORMONES ON
THE RIBOSOMAL PROFILE OF THE ISOLATED RAT LIVER

Introduction.

The isolated perfused rat liver is widely used for metabolic studies. Criteria of adequate function include the appearance of the liver, blood and bile flow, glucose and urea production, histology, bromsulphthalein excretion and incorporation of amino acids into trichloroacetic acid-precipitable protein. However, analysis of ribosomal profiles has revealed that, despite the perfusion meeting all of the above criteria, there is a progressive disaggregation of ribosomes (Levitan and Webb, 1969). The same authors have suggested that the change is due to the degradation or utilisation of an essential substrate or hormone. Jefferson and Korner (1969) found that the presence of arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine at 10 times the normal peripheral blood concentration in the perfusate prevented this disaggregation. In contrast, Rothschild et al (1969c) were able to aggregate the total ribosomes of the isolated perfused liver of a fasted rabbit by adding either tryptophan or isoleucine only to the perfusate.

It should be noted that Gaetani et al (1969) have shown that free and endoplasmic reticulum-bound ribosomes behave differently under certain conditions. They found that on a protein-free diet, the membrane-bound and free ribosomes of the rat liver disaggregate rapidly. Thereafter, there is a progressive aggregation of the free ribosomes with maintenance of the disaggregation of the endoplasmic reticulum-bound ribosomes.

Accordingly, the author has investigated the effect of amino acids both singly and in combination, not on the total

ribosomal profile, but on both free and endoplasmic reticulum-bound ribosomal profiles in the isolated perfused rat liver. In addition, because of the widespread influence of hormones on protein metabolism, the effects of glucose, insulin, cortisone, pancreozymin, secretin, gastrin, growth hormone, glucagon and testosterone have been studied.

Materials and methods.

Perfusion apparatus and techniques.

Liver donors were 320-350 g male Wistar rats housed under controlled conditions of temperature, humidity and lighting.

2 groups of donors were used :-

- (1) rats fed ad lib a 20% protein diet, and
- (2) rats fasted for 24 hours.

Both groups had free access to water. Blood was obtained by cardiac puncture under diethyl ether anaesthesia from well-nourished rats. 42 perfusions were performed according to the method of Miller et al (1951) as modified by Fisher and Kerly (1964). The technique is described in detail in the Appendix.

Amino acids, when added, were L-isomers. They were dissolved in the perfusate at the following levels :
tryptophan, isoleucine, lysine, alpha-amino isobutyric acid, each at 10 micromoles/ml and a combination of arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine each at 3 or 10 times their normal peripheral plasma concentration, depending on the experimental design. The hormones were added separately at a concentration of 1 unit of pancreozymin,

(Boots Pure Drug Co.Ltd., Nottingham, England), $1\frac{1}{2}$ units of secretin (Boots Pure Drug Co. Ltd., Nottingham, England), 5 mg of gastrin (Peptavlon. Imperial Chemical Industries Ltd., Cheshire, England), 2 units of soluble insulin, B.P. (Boots Pure Drug Co. Ltd., Nottingham, England), 2 mg of hydrocortisone (Glaxo-Allenbury S.A., Pty.Ltd.), 2 mg of testosterone propionate (Petersen Ltd., Johannesburg) 2 mg of bovine growth hormone and $\frac{1}{2}$ mg of glucagon (U.S.P. Elli Lilly & Co., Indianapolis, USA) at the start of the perfusion. Thereafter, $\frac{1}{2}$ the initial concentration per hour was infused.

Free and bound ribosomal profiles.

The method of Blobel and Potter (1967) was used to isolate free and bound ribosomes. The principles of the methodology have been described. Full details are given in the Appendix. After 30 or 150 minutes perfusion, depending upon experimental design, the liver was perfused rapidly with 50 ml of chilled 0.154 molar saline and the ribosomes isolated as described in the Appendix. Non-perfused, anaesthetised rat livers were used as controls.

Results

Results have been expressed in a qualitative rather than a quantitative fashion. The "A1" and "A2" pattern represent a non-perfused, anaesthetised free and bound profile respectively (Figure 12). The "B1" and "B2" profiles are free and bound profiles respectively after 30 minutes perfusion with no supplementation of the perfusate. The limitations of the methodology have been discussed previously. All groups were handled in the same way and at least two perfusions

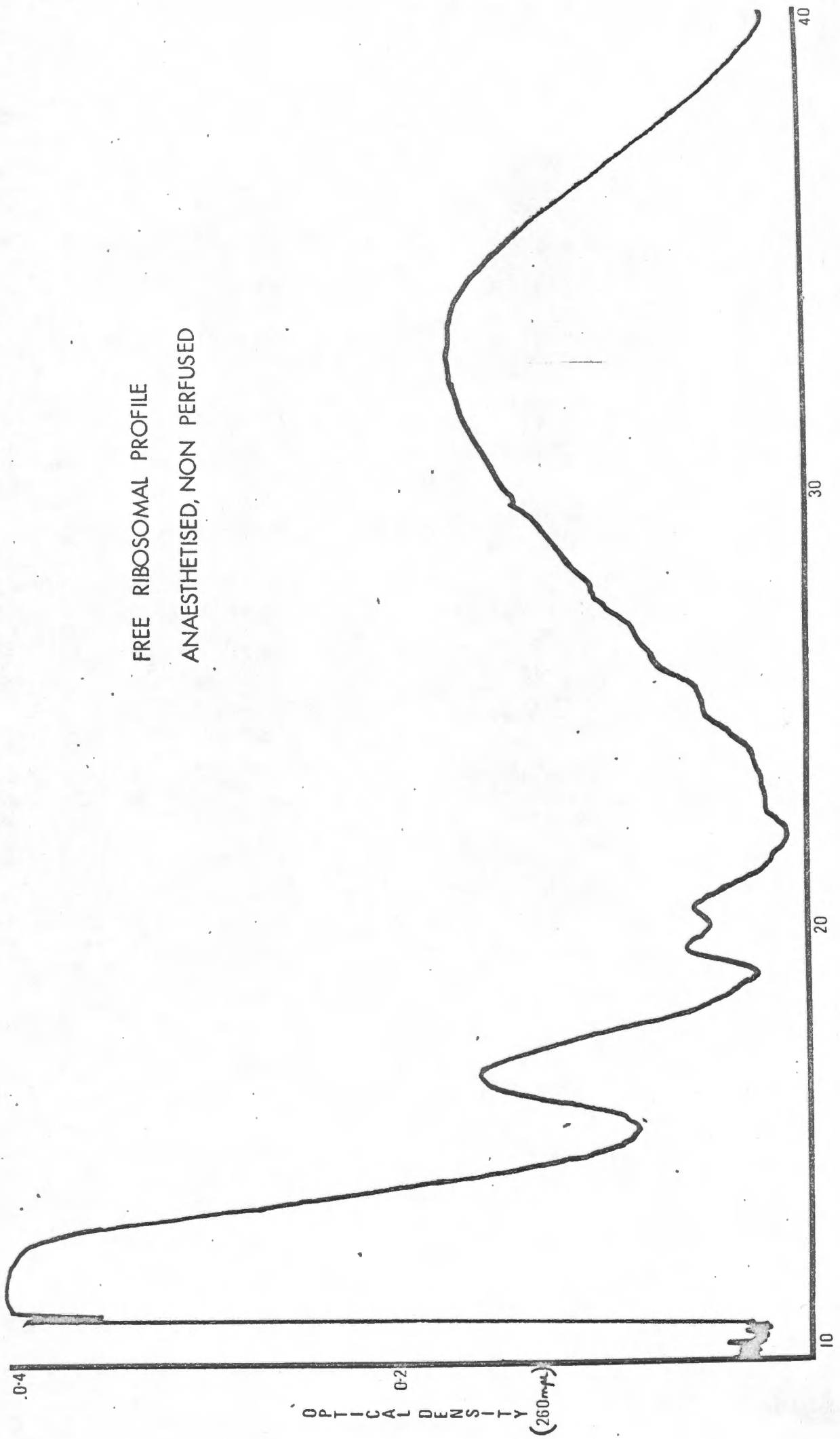


Figure 12a: "A1" PROFILE

SUCROSE IN GRADIENT (%)

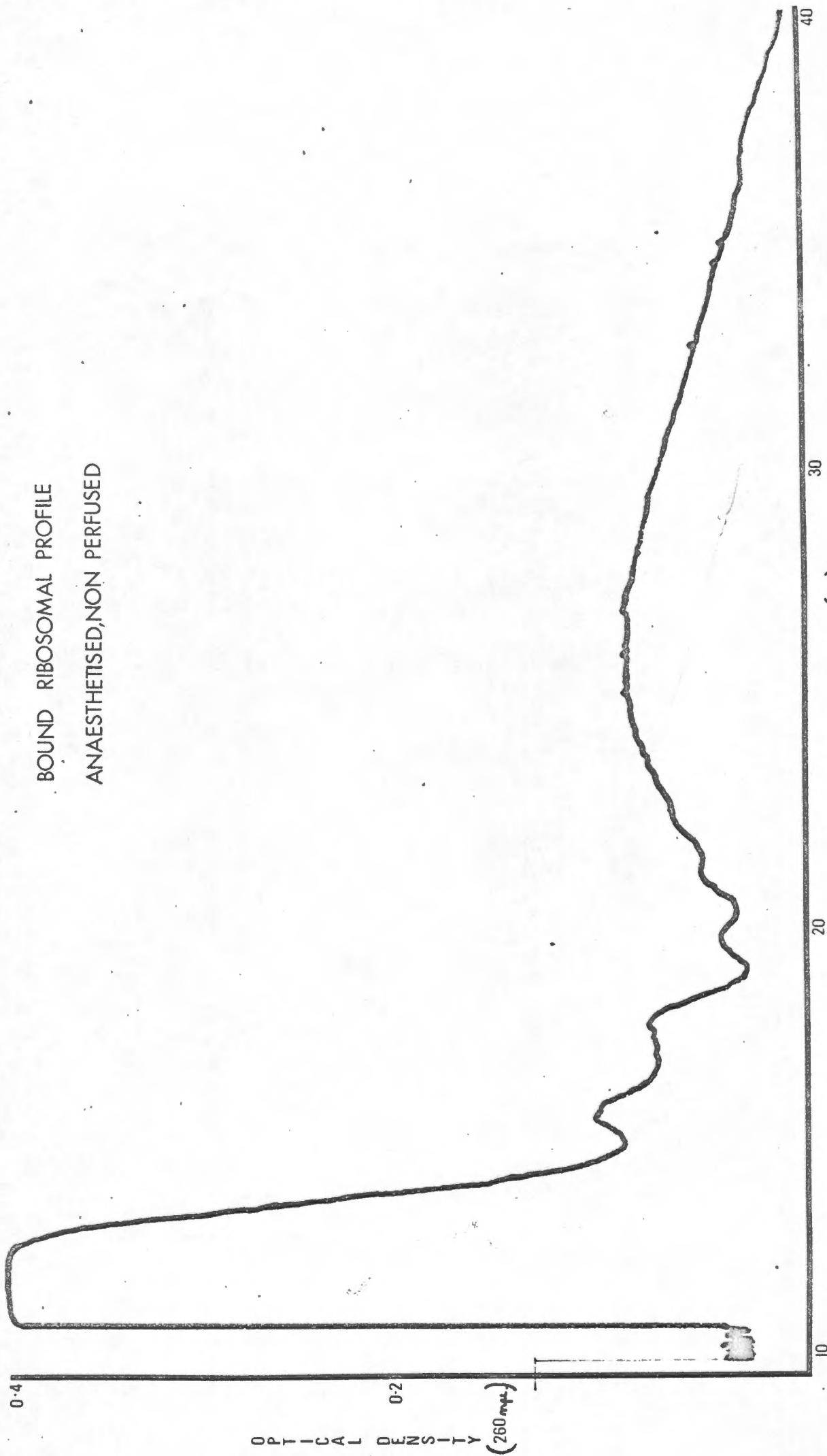


Figure 12b: "A2" PROFILE

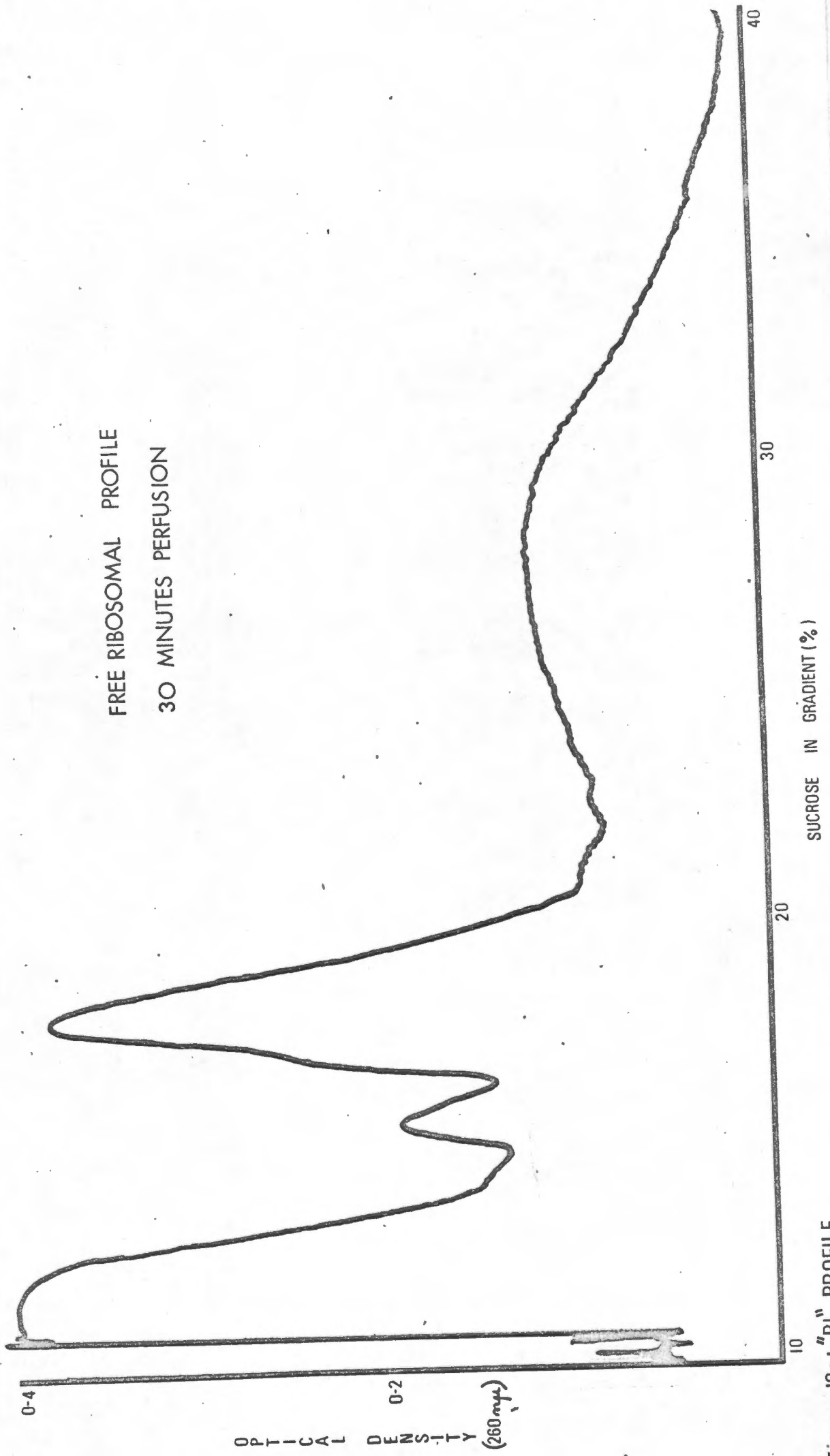
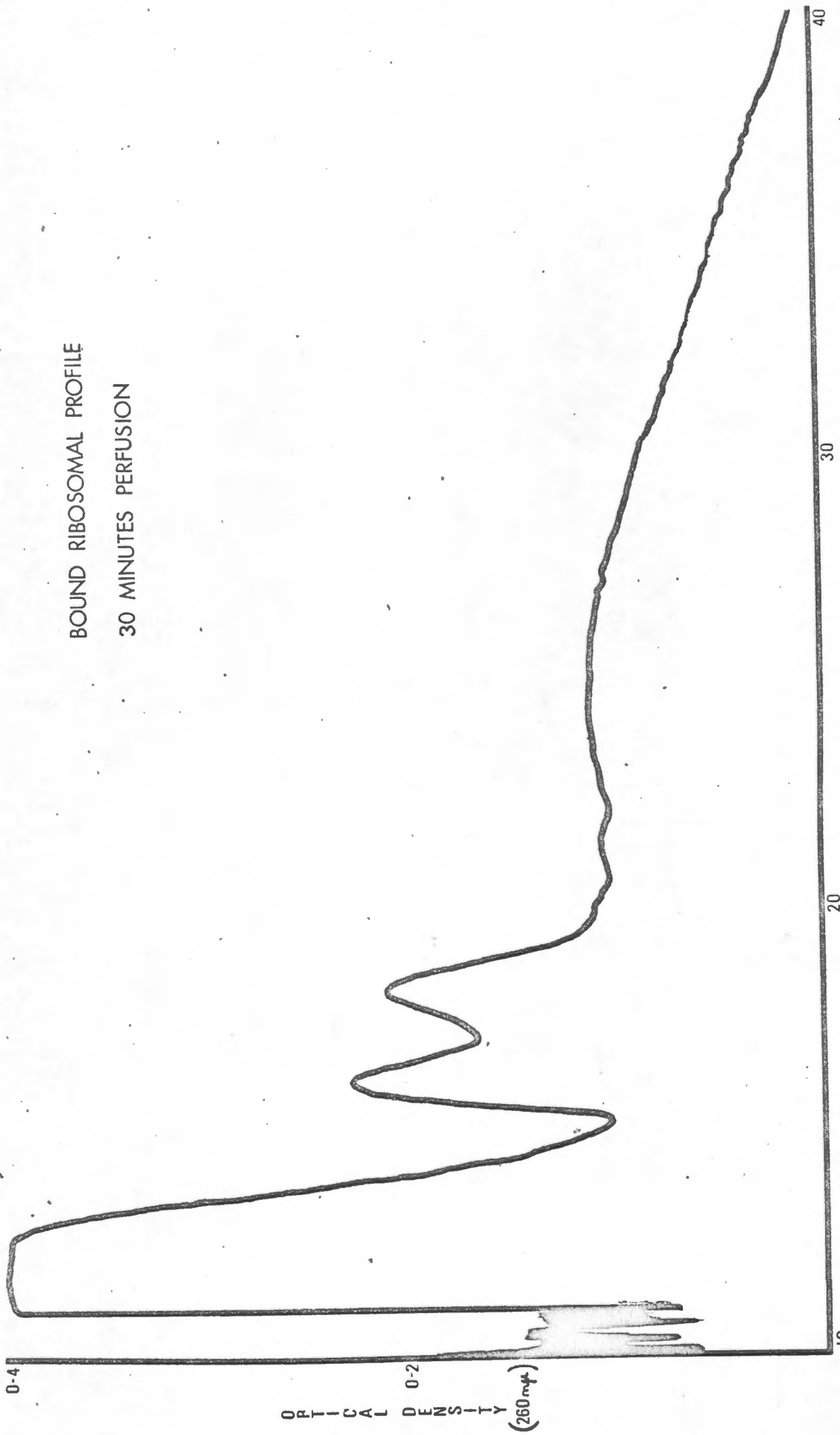


Figure 12c: "B1" PROFILE



BOUND RIBOSOMAL PROFILE
30 MINUTES PERFUSION

OPTICAL DENSITY
(260 mμ)

SUCROSE IN GRADIENT (%)

Figure 12d: "B2" PROFILE

were performed for each group.

In all profiles the free and bound components behaved in the same fashion. 30 minutes of perfusion resulted in the B pattern which was accentuated by perfusing for a further 2 hours. Amino acids added singly were unable to influence the B pattern, whether the liver donor was fasted or fed. The addition of 3 times the peripheral concentration of the 11 amino acids used by Jefferson and Korner (1969) to the perfusate had no effect. Ten times the concentration of these 11 resulted in the A pattern, both in the free and bound profiles.

B profiles were obtained when glucose, glucose and insulin, testosterone, growth hormone, gastrin, pancreozymin, secretin, glucagon or insulin were added to the perfusate.

Discussion.

From this work and that of Jefferson and Korner (1969) and Levitan and Webb (1969), it is apparent that the ribosomes require examination before results from isolated perfused organs can be extrapolated to the whole animal.

Rothschild et al (1969c) found that tryptophan, in keeping with its unique role in vivo (Pronczuk et al, 1968; Sidransky et al, 1968), aggregated the ribosomes in perfused fasted rabbit livers. However, the author has been unable to influence the ribosomal profile with single amino acids using livers from either fasted or fed rats. Moreover, an attempt to increase amino acid supply with glucagon infusion and subsequent addition of tryptophan, as previously described in Part II, failed to alter the profile. In contrast, the

findings of the author support the work of Jefferson and Korner (1969) in that arginine, asparagine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and valine added to 10 times their normal peripheral blood concentration aggregated the ribosomes. The concept of differential function of free and bound ribosomes has been discussed in the "Review of Amino Acid Pools" and the results of the present author suggest that both intracellular and export protein are affected. It is difficult to account for the discrepancy between the work in the rabbit and the rat. It is possible that the difference is related to a difference in the dietary habits or in the protein turnover rate of the 2 animals.

The fact that portal blood concentrations of amino acids are usually less than 10 times their peripheral concentration even post prandially suggests that other factors are involved. In addition, Wittman et al (1969) showed that a protein-free diet fed to protein deprived rats was able to aggregate the ribosomes. This response was possibly mediated by insulin and glucose together, as they were found to achieve the same effect when added instead of the protein-free diet. However, in the author's system, glucose and insulin, either together or singly, and pancreozymin, secretin, growth hormone, cortisone, testosterone, glucagon and gastrin were ineffective. The final common pathway may yet be a critical level of amino acid supply with hormones regulating amino acid availability by influencing amino acid transport, compartmentation, degradative or synthetic pathways or protein catabolism.

S U M M A R Y

In recent decades, this Medical School has made important contributions to the study of clinical malnutrition, largely through the work of Professors J.F. Brock and J.D.L. Hansen. With the emergence of Dr. R. Hoffenberg as an established investigator in the field of albumin metabolism, the laboratory achieved a new dimension. Both Dr. Hoffenberg, and later Dr. R.E. Kirsch, made notable contributions to the understanding of albumin synthesis, catabolism and distribution. Recently Dr. L. Kernoff has expanded the field to include the role of growth hormone in albumin metabolism. Professor S.J. Saunders' expertise in amino acids and interest in albumin metabolism provided an active and remarkably topical area for research by the author.

The concept of depressed albumin synthesis rates during protein depletion is well established. However, the author considers the study in humans to be the first comprehensive attempt to measure albumin synthesis directly and independently of catabolism during controlled conditions of protein intake. This study serves to confirm the work of Hoffenberg and others, who inferred from their data that albumin synthesis is reduced during protein restriction. Furthermore, the demonstration that plasma alanine is the only amino acid the concentration of which is influenced by dietary protein deprivation, is a useful contribution in a field with few established facts. Moreover, few studies have included the measurement of tryptophan. The relationship of the low synthesis rate to the elevated plasma

alanine levels is felt by the author to be either fortuitous or the result of 2 processes acting at different sites. However, the possibility of a cause-and-effect relationship cannot be excluded. Emphasis has been placed on the complex interplay of factors regulating the hepatic intracellular amino acid pools and the fact that plasma amino acid levels may bear no relation to tissue levels or to protein synthesis at other sites.

Clearly, the accelerated albumin synthesis rates on protein loading are related to amino acid supply. However, the mechanism has not been established. It was unclear whether the amino acids were only acting as substrate or whether their influence was indirect. Furthermore, were they equally effective ?

Perfusion of the isolated rat liver was employed by the author to attempt to clarify this issue. The defects of the perfusion system used have been emphasised, but nonetheless, it remains a powerful tool in the research laboratory. From these perfusion studies the following points relating amino acids to albumin synthesis were established :-

- (1) It was confirmed that fasting markedly reduces albumin synthesis.
- (2) The plasma aminogram is altered by depriving rats of protein for 48 hours.
- (3) The use of the above plasma as the perfusate, instead of normal plasma, reduces albumin synthesis rates significantly.

- (4) The use of the above perfusate with the addition of single amino acids increases albumin synthesis rates.
- (5) Single amino acids have no effect on albumin synthesis when the liver is derived from fasted rats. Yet the addition of 11 amino acids added to a concentration of 10 times their normal peripheral blood concentration restores albumin synthesis to control levels.

While the study was in progress, the work of Rothschild et al. (1969c) in rabbits was published. Reference has been made to the important differences between the studies. These include :-

- (1) The inability of the author to influence albumin synthesis after adding single amino acids to fasted livers. The increased synthesis observed by the addition of the 11 amino acids demonstrated the potential activity of the system. However, even in the latter circumstances, no overshoot beyond control levels occurred. The postulate that the relatively stable mRNA of albumin no longer has to compete with more labile mRNA of other proteins for available amino acid supply, did not appear to be operative in this study.
- (2) Single amino acids including non-essential and non-metabolisable amino acids increased albumin synthesis rates in livers from well nourished rats.

The work on ribosomes confirmed and extended the work of other investigators. The author has demonstrated

that

- (1) both free and endoplasmic reticulum-bound ribosomes disaggregate progressively during perfusion;
- (2) single amino acids fail to influence the ribosomal profile;
- (3) the 11 amino acids together used by Jefferson and Korner (1969) aggregated both populations of ribosomes;
- (4) infusion of growth hormone, cortisone, insulin, secretin, gastrin, glucagon and a combination of glucose and insulin failed to influence the profiles;
- (5) unlike Rothschild et al (1969c) in the rabbit, the author was unable to influence the ribosomal profile of fasted rats by the addition of tryptophan or isoleucine. However, ribosomal aggregation, like accelerated albumin synthesis, still occurred when the 11 amino acids were added.

Bearing in mind the quantitative limitations of the technique used by the author, it is unwise to place too much emphasis on the association of unchanged ribosomal profiles with increased albumin synthesis rates after the addition of single amino acids.

The concept of short-term regulation of protein synthesis by dietary tryptophan has not been supported by the perfusions using fasted livers, possibly as a result of insufficient supply of other amino acids. As mentioned above, tryptophan together with 10 other amino acids increased albumin synthesis and aggregated the ribosomes. The alternative method of increasing the supply of other amino acids by

infusing glucagon and adding tryptophan was unsuccessful.

The short study of the influence of amino acids, glucagon, insulin, growth hormone and hydrocortisone on the fractional catabolic rate of albumin represents the only study investigating this control by factors other than carbon loading or albumin pool size known to the author.

A few interesting observations were noted :-

- (1) Amino acids do not influence the fractional rate of catabolism.
- (2) Accelerated protein synthesis, and thereby possibly augmented proteolytic enzyme activity, failed to influence the fractional catabolic rate of albumin.
- (3) None of the hormones except insulin had any influence on the catabolic rate of albumin. However, insulin effected a marginal slowing of catabolism.
- (4) Unlike Hoffenberg et al (1970), normal fractional catabolic rates were obtained in the presence of low perfusate albumin concentrations. The implications of this important finding were discussed.

In summary, it would appear that this thesis consolidates and extends existing literature and opens new avenues for exploration.

A P P E N D I X .

A. FRACTIONATION OF RAT ALBUMIN

- (1) To 1 volume of plasma add 2 volumes of 0.9% (W/V) sodium chloride.
- (2) Add 3 volumes of ammonium sulphate solution, saturated at 37°C.
- (3) Allow to stand for 30 minutes. Centrifuge to remove globulin precipitate.
- (4) Bring supernatant to pH 4.6 with 10% (V/V) acetic acid.
- (5) Allow to stand for 10 minutes. Centrifuge.
- (6) Dissolve albumin precipitate in distilled water.
- (7) Dialyse overnight against distilled water at 4°C to remove residual salt.
- (8) Check (a) concentration of albumin by biuret method.
(b) purity of fractionation by cellulose acetate electrophoresis.

B. IODINATION.Reagents

- (1) Approximately 15 mg human albumin (Sigma Chemical Company, St. Louis, USA) in Part I and 15 mg rat albumin (prepared as described above) in Part III, both contained in 0.5 ml volume is used for labelling.
- (2) Iodine monochloride (stock solution).
 - (i) Dissolve 150 mg sodium iodide in 8.0 ml 6N HCl.
 - (ii) Dissolve 108 mg sodium iodate monohydrate in 2.0 ml distilled water.
 - (iii) Forcibly inject (ii) into (i) to avoid precipitation of iodine.
 - (iv) Dilute to 40.0 ml with distilled water and shake with 5.0 ml carbon tetrachloride.

The presence of a red discolouration in the organic solvent indicates the presence of free iodine. The carbon tetrachloride is removed with a pipette, and the extraction process is repeated until the carbon tetrachloride remains unchanged in colour.

- (v) Bubble moist air through ICl solution for one hour to remove any residual carbon tetrachloride.
 - (vi) Make up to 45.0 ml with distilled water. This solution is stored at 4°C.
 - (vii) Before use, dilute 1 volume of stock solution with 9 volumes of 2M NaCl.
- (3) Glycine buffer (stock solution)
- (i) Dissolve 7.5 g glycine in 75.0 ml distilled water.
 - (ii) Add 25.0 ml 1N NaCl.
- (4) Alkaline glycine buffer (pH 9.0-9.5).
Add 0.2 ml 1N (W/V) NaOH to 1.8 ml of the stock solution.

Method.

- (a) Prepare alkaline glycine buffer as above.
- (b) Prepare 1.0 ml of a solution of ICl containing 0.1 ml ICl with 0.9 ml 2M NaCl.
- (c) Place required amount of radioiodine solution in 0.3 ml of the ICl prepared in (b). (Tube A).
- (d) Adjust pH of albumin solution to 8.5 with alkaline glycine buffer (Tube B).
- (e) Similarly, bring pH of Tube A to 8.5.

- (f) Without delay vigorously and only once inject contents of Tube A into Tube B.
- (g) Remove the free iodine by dialysis against distilled water.
- (h) Check the free radioiodine content on an aliquot of the final preparation by trichloroacetic acid precipitation to ensure less than 1% free radioiodine for human studies and less than 0.02% for perfusion studies.
- (i) Check that the label is confined only to the albumin fraction by cellulose acetate electrophoresis.
- (j) Sterilise by Seitz filtration prior to use in human studies.

C. CATABOLIC RATES IN LIVER PERFUSIONS

- (1) Prepare the rat albumin by ammonium sulphate precipitation until pure preparation is obtained as checked by cellulose acetate electrophoresis.
- (2) Iodinate as described.
- (3) Remove the denatured molecules by screening the iodinated albumin: inject the preparation into the peritoneal cavity of a rat. Bleed the rat 48 hours later.
- (4) Dialyse the above plasma against distilled water to remove non-protein radioactivity.
- (5) Thirty minutes after the start of the perfusion add a volume of the plasma to the reservoir to achieve a dose of 10-20 microcuries for ^{131}I or 15-40 microcuries for ^{125}I .
- (6) Withdraw samples at 30 minute intervals for 3 hours.

- (7) Prepare a protein-free supernatant :

To 1.5 ml of plasma add 1.5 ml of 0.33 mM-NaI and 3.0 ml of 20% (W/V) trichloroacetic acid. Centrifuge. Filter the supernatant through small cotton wool pledgets.

- (8) Measure the radioactivity on 0.1 ml samples of whole plasma and 2 ml samples of the non-protein supernatant. Count the samples for two 20 minute periods in a Packard Autogamma Spectrometer.
- (9) On linear graph paper plot the fraction of iodide released per 30 minutes, expressed as a fraction of the mean total protein-bound radioactivity during that period, against time.
- (10) Derive the best fitting line using the least mean squares method.
- (11) Derive the fractional catabolic rate of albumin from the slope of the line.

D. CATABOLIC RATES IN HUMANS.

- (1) Prepare the sterilised, labelled human albumin as described.
- (2) Draw up 80 microcuries of (^{125}I) albumin in a disposable syringe.
- (3) Weigh the full syringe.
- (4) Inject the dose intravenously without withdrawing blood.
- (5) Weigh the empty syringe.
- (6) Take a blood sample with a heparinised tube 10 minutes after injection.
- (7) Take daily blood samples into heparinised tubes and collect 24 hour urine samples from day 2 to day 14.
- (8) Prepare a 1 in 200 solution of the dose to use as a standard.

- (9) Pipette 1 ml of daily plasma sample, 2 ml of daily urine sample and 1 ml of the standard and count on a Packard Autogamma Spectrometer.

Calculation.

- (1) Calculate the dose injected (D).

Step (5) - Step (3) = Volume of dose administered (ml)

$$\frac{\text{Total dose (counts/minute)}}{\text{(counts/minute)}} = \frac{\text{volume of dose (ml)} \times \text{standard (counts/min/ml)}}{\text{(ml)} \quad \text{(counts/min/ml)}}$$

- (2) Calculate the percentage of the dose remaining in the plasma on each day regarding the 10 minute sample (P) as 100%. Plot these values on linear graph paper.
- (3) From the graph read off the daily average of the percentage of the dose in the plasma (Q).
- (4) Calculate the fraction of the total dose excreted in the urine during each 24 hour period ($U = \frac{u}{D}$. Where U = fraction of total dose and u = amount of radioactivity excreted per day.)
- (5) The fractional catabolic rate of albumin (FCRA) =

$$\frac{\frac{U_1}{Q_1} + \frac{U_2}{Q_2} + \frac{U_3}{Q_3} \dots \dots \frac{U_n}{Q_n}}{n}$$

where n = number of days of measurement excluding the first 2 days.

- (6) The plasma volume (K) = $\frac{D}{P}$

- (7) Plasma albumin pool (PAP in milligrams) =

albumin concentration (mg/ml) x plasma volume (ml).

- (8) The absolute albumin catabolic rate (ACR in mg/Kg/day) =

$$\frac{\text{FCRA} \times \text{PAP}}{100 \times \text{patients wt. (Kg)}}$$

Modification for repeat experiment in same patient.

(1) Sample plasma before injection of second dose. Let X be residual plasma radioactivity.

(2) Let P be 10 minute plasma radioactivity.

Then $P - X =$ true 10 minute radioactivity (P_t)

$$\text{Plasma volume (K)} = \frac{\text{Dose (D)}}{\text{10 minute sample}} = \frac{(D)}{P_t}$$

therefore "equivalent dose" (injected + residual activity)

$$= K \times P_t$$

(3) In calculation (4) above use corrected equivalent dose.

E. DETERMINATION OF PLASMA ALBUMIN CONCENTRATION.

Reagents.

(1) 0.9% (W/V) NaCl.

(2) HCl - Ethanol.

Prepare a mixture of 95% absolute ethanol and 5% methanol. To 600 ml of this ethanol-methanol mixture add 1.0 ml of concentrated HCl. Store at 4°C.

(3) 0.2M. Sodium acetate-ethanol.

Dissolve 2.7218 mg sodium acetate in 5.0 ml methanol. Add 95 ml absolute ethanol. Store at 4°C.

(4) 3% (W/V) sodium hydroxide.

(5) Biuret reagent.

Dissolve (i) 17.3 g copper sulphate pentahydrate in 100 ml hot distilled water.

(ii) 173 g sodium citrate and 100 g anhydrous sodium carbonate in 800 ml distilled water while heating.

When cool, pour (ii) into (i) with rapid stirring.

Dilute to 1 litre with distilled water.

(6) Protein Standard.

30 mg/ml aqueous solution of crystalline human albumin.

Method :

- (a) To 0.2 ml of plasma add 0.8 ml of 0.9% (W/V) sodium chloride.
- (b) Add 9.0 ml HCl-ethanol. Stopper and mix by inversion.
- (c) Incubate for 30 minutes at 37°C.
- (d) Centrifuge.
- (e) Transfer 5.0 ml of the supernatant to another centrifuge tube.
- (f) Add 0.5 ml of 0.2M acetate-ethanol. Stopper and mix by inversion.
- (g) Allow to stand at room temperature for 10 minutes.
- (h) Centrifuge. Pour off supernatant. Invert tubes on filter paper to ensure that all supernatant fluid is removed.
- (i) Dissolve precipitate in 5.0 ml 3% sodium hydroxide, add 1.0 ml biuret reagent, and mix.
- (j) Read intensity of colour produced after 15 minutes using a 54 filter on a Klett calorimeter.

A reagent blank consists of 5.0 ml 3% sodium hydroxide plus 1.0 ml biuret reagent. The known protein standard is made by adding 4.9 ml of 3% sodium hydroxide and 1.0 ml biuret reagent to 0.1 ml of a 3g% human albumin solution.

F. MEASUREMENT OF ALBUMIN SYNTHESIS.

Reagents

- (1) 10% (W/V) and 5% (W/V) trichloroacetic acid.
- (2) Absolute ethanol.
- (3) CO₂-free 1N sodium hydroxide.
 - (i) Prepare CO₂-free water by boiling 200 ml distilled water to half its volume.
 - (ii) To 10.0 ml of 10N sodium hydroxide add 90 ml CO₂-free water. Stopper tightly and prepare a fresh solution for each batch of samples processed.
- (4) 4 M citric acid.

Make 84 g citric acid up to 100 ml volume with distilled water.
- (5) Phosphate buffer (pH 6.7).

Dissolve 41.75 g sodium pyrophosphate and 5.7 ml orthophosphoric acid in 500 ml distilled water.
- (6) Urease solution.

A 1 mg/ml solution of urease (Nutritional Biochemical Corporation, Cleveland, Ohio, USA) in phosphate buffer. Store at 4°C and renew monthly.
- (7) 10% (W/V) sodium tungstate.
- (8) Tungstic acid.

Make up 10.0 ml 10% (W/V) sodium tungstate and 3.8 ml 1N HCl to 100 ml with distilled water.
- (9) Citric-tungstic acid solution.

A 1:1 mixture of 4 M citric acid and tungstic acid.
- (10) 0.66 N sulphuric acid.
- (11) Resins : (Deacidite FF resin is obtained commercially in the chloride form - 100-200 mesh; 3.5% cross linkage.)

A. Hydroxide form

- (i) Wash the chloride form with distilled water until eluate is clear.
- (ii) Wash with 4 litres of 4 N NaOH.
- (iii) Wash with 4 litres of 2 N NaOH.
- (iv) Determine if eluate is free of chloride ions by testing with silver nitrate at acid pH.
- (v) If necessary repeat washings with 4 N and 2 N NaOH until eluate is chloride free.
- (vi) Wash with distilled water to pH 7.0.

B. Carbonate form.

- (i) Wash the hydroxide form with several litres of 0.5 N sodium bicarbonate.
- (ii) Wash with distilled water to pH 7.0.

C. Mixed form

A 1:1 mixture of chloride and hydroxide form.

(12) Activated arginase solution.

Prepare manganese/maleic buffer.

- (i) Dissolve 8.90 g sodium maleate in 200 ml distilled water.
- (ii) Bring pH to 9.7-9.8 with 1 N NaOH.
- (iii) Add 11.5 g manganese sulphate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$) and make up to 500 ml with distilled water.
- (iv) Bring to pH 7.0 with 1 N HCl.

Arginase solution is prepared as a 1 mg/ml solution of arginase (Nutritional Biochemical Corporation) in manganese/maleic buffer. Add required amount of arginase to the appropriate volume of buffer, and incubate for 3 hours at 37°C. The solution is stored at 4°C and

renewed every 2 months.

(13) Phenylethylamine-methanol.

- (i) Redistil commercially obtained phenylethylamine until yellow colour is removed.
- (ii) Mix with an equal volume of methanol.
- (iii) Store in the dark.

(14) Scintillation mixture.

Dissolve 7.5 g 2,5-diphenyloxazole (PPO) and 0.75 g 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene (POPOP) in 2.5 litres anhydrous toluene.

Method

A. Preparation of samples for measurement of albumin guanidine carbon specific activity.

(1) Extraction of albumin from plasma.

(i) Human study.

To 5 ml of plasma add an equal value of 10% trichloroacetic acid.

Perfusion study.

To 1 ml of plasma add 1 ml of bovine albumin (100 mg/ml British Drug House Chemicals Limited, Poole, England) and add 2 ml of 10% trichloroacetic acid.

- (ii) Mix well, centrifuge and discard supernatant.
- (iii) Wash precipitate with an equal volume of cold 5% trichloroacetic acid. Resuspend. Centrifuge and discard supernatant.
- (iv) Add 3 volumes of absolute ethanol to precipitate. Mix thoroughly and centrifuge to remove globulin precipitate.

- (v) Dialyse albumin-rich supernatant against distilled water overnight at 4°C.
 - (vi) Concentrate sample to approximately 2 ml. Confirm homogeneity of fraction by cellulose acetate electrophoresis. Measure the volume and concentration by the biuret method to give the total amount of albumin present.
- (2) Acid hydrolysis of albumin.
- (i) Given that 100 ml of 6 N HCl is added to 250 mg protein, determine amount of 6 N HCl required for sample (1 vi).
 - (ii) Add volumes of water and concentrated HCl to sample so that the final solution is 6 N with respect to HCl.
 - (iii) Transfer to hydrolysis tubes and hydrolyse for 16 hours at 110-115°C.
 - (iv) Transfer the hydrolysate to a round-bottom flask and evaporate to dryness.
 - (v) Wash dried sample in flask with 3-5 ml distilled water and evaporate to dryness again.
- (3) Neutralisation of hydrolysate, and incubation with arginase.
- (i) Dissolve amino acid residue in 2-3 ml distilled water.
 - (ii) Bring to pH 7.0 by the addition of Deacidite FF resin in the carbonate form.
 - (iii) Pass through a glass column containing approximately 5 mm of the carbonate resin.
 - (iv) Wash the flask with about 10 ml distilled water and add washings to the column.

(v) Collect eluate in a round-bottom flask.

If less than 100 mg of albumin was hydrolysed :

- (a) add 2 ml activated arginase solution.
- (b) bring to pH 9.0 with 1 N NaOH.
- (c) Incubate for 16 hours at 38°C.

If more than 100 mg of albumin was hydrolysed:

- (a) Prepare a column (9 by 1 cm) of mixed chloride and hydroxide resins. (1:1). Wash with distilled water to pH 7.0.
- (b) Concentrate eluate (3 v) to approximately 2 ml and place on column.
- (c) Wash column with distilled water, collecting 4 ml aliquots of the eluate. Collect 6 such aliquots.
- (d) Spot one drop of each aliquot on filter paper and dry in a hot oven (\pm 110°C). Spray with ninhydrin and return to oven until dry. The presence of arginine is indicated by a purple spot.
- (e) Place the aliquots containing arginine into a round-bottom flask, add 3 ml activated arginase solution, adjust to pH 9.0 and incubate as above.

After incubation

(vi) Bring sample to pH 2.0 with 4 M citric acid.

(vii) Evaporate to dryness.

(viii) Dissolve this urea in 1 ml of distilled water and 0.5 ml phosphate buffer. Bring to pH 7.0 with CO₂-free 1 N NaOH.

Sample is now ready for the gas-train.

B. Preparation of samples for the measurement of urea carbon SA

(i) I. Human study

- (a) Normal diet : to 1 ml of plasma add 2 ml stable urea (1 mg/ml) and 6 parts of water. Precipitate proteins by adding $\frac{1}{2}$ part of 10% sodium tungstate and $\frac{1}{2}$ part of 0.66 sulphuric acid.
- (b) Low protein diet : to 5 ml of plasma add 2 ml stable urea (1 mg/ml) and 6 parts of water. Precipitate by adding $\frac{1}{2}$ part of 10% sodium hydroxide and $\frac{1}{2}$ part of 0.66 sulphuric acid.

II. Perfusion study.

To 0.5 ml plasma add 2.0 ml of stable urea (1 mg/ml). Precipitate proteins by adding 1.0 ml of 10% sodium tungstate, and 1.0 ml 0.66 N sulphuric acid.

- (ii) Mix well by inversion and leave to stand for 5 minutes. Centrifuge.
- (iii) Place protein-free supernatant in round-bottom flask and evaporate to dryness.
- (iv) Redissolve in 1.0 ml distilled water and 0.5 ml phosphate buffer.
- (v) Bring to pH 7.0 with CO_2 -free 1 N NaOH. Sample is now ready for the gas-train.

C. Measurement of $^{14}\text{CO}_2$ Specific Activity (See Figure A:1)

- (i) Place sample in centre bulb of reaction vessel (a). Place 1 ml urease in one side-arm and 1 ml citric-tungstic acid solution in the other side-arm.
- (ii) Briefly immerse vessel in a solid CO_2 -ethanol mixture

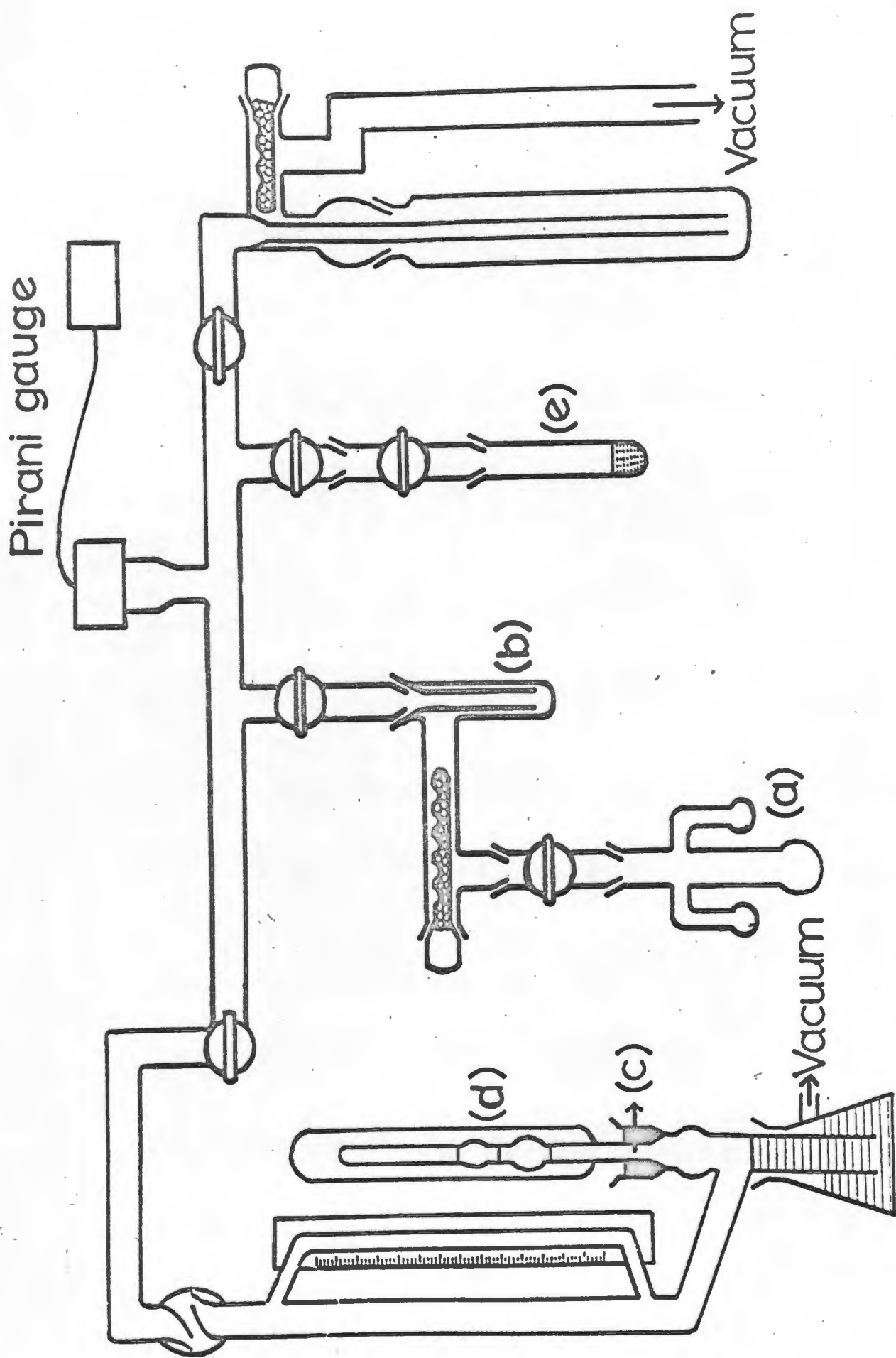


Figure A-1: THE HIGH VACUUM GAS TRAIN

and evacuate gases present in solution while gently thawing. Seal vessel by closing tap.

- (iii) Tip in urease from side-arm and allow to incubate for one hour at room temperature.
- (iv) Tip acid mixture into reaction vessel and shake gently to release CO_2 .
- (v) Place reaction vessel on gas-train and immerse in solid CO_2 -ethanol.
- (vi) Evacuate gas line.
- (vii) Immerse cold finger (b) in liquid nitrogen and allow CO_2 to pass from reaction vessel to cold finger.
- (viii) Evacuate any contaminant gases present while the CO_2 sample is trapped under liquid nitrogen in the cold finger.
- (ix) Close tap above cold finger. Thaw cold finger in warm water and then immerse in solid CO_2 -ethanol, releasing the CO_2 sample but trapping any contaminant gases.
- (x) Fill cuff (c) surrounding manometer arm with liquid nitrogen and allow CO_2 sample to pass from cold finger to manometer arm (previously, evacuated) by opening the intervening taps and closing the pump tap.
- (xi) Raise mercury column to trap CO_2 sample in the manometer arm and thaw by adding warm water to the cuff.
- (xii) Adjust mercury column to the reference mark on the modified MacLeod chamber (d) to obtain the displacement of mercury by CO_2 .

- (xiii) Note the scale reading indicated by the height of of the mercury column in the manometer side-arm.
 - (xiv) Evacuate the rest of the gas-train including the tube (e) containing 2 ml of phenylethylamine-methanol mixture frozen under liquid nitrogen.
 - (xv) Close the pump tap, lower the mercury and transfer the CO₂ to the prepared tube (xiv).
 - (xvi) Remove the tube and after thawing transfer the solution to a counting vial containing 8 ml of the PPO-POPOP scintillation mixture.
- Count each sample for two 20 minute periods in a Beckman Liquid Scintillation Counter.

D. Calculation of albumin synthesis rate.

Human study.

- (1) Urea carbon specific activity measurements.
 - (i) Convert volumes of CO₂ into mg C by means of a standard graph.
 - (ii) Calculate SA (expressed as counts per minute/mg C) for each of urea samples.
 - (iii) Correct these SA measurements for the stable urea added.⁺
 - (iv) Plot corrected SA measurement of day 2 on semilog graph paper against time.
 - (v) By the least mean squares method plot the best fitting line through these points.
 - (vi) Apply the slope to the 6 hour value obtained from day 1 of the experiment.

- (vii) Extrapolate the line back to the Y axis and determine urea SA at t_0 .
- (viii) From the slope determine the fractional SR of urea per hour.

NOTE: (1) When repeated studies are made in the same patient 4-6 weeks later the residual labelled urea is negligible.

* (2) Equal recovery of the stable and labelled urea occurs.

(2) Albumin guanidine carbon specific activity measurement.

- (i) Calculate albumin SA as in (i) and (ii) above.
- (ii) Correct the SA measurement for the added bovine albumin**
- (iii) Determine albumin SA at t_0 by multiplying the observed SA at t_6 by the factor derived from the disappearance of the iodinated albumin sample over the 6 hour experimental period.

Then :

Fractional SR of albumin
(% IVP/day)

$$= \frac{\text{Fractional SR urea} \times 24 \times \text{albumin SA at } t_0}{\text{Urea SA at } t_0}$$

Absolute SR albumin
(mg/kg/24 hours)

$$= \frac{\text{FSR albumin} \times \text{intra-vascular albumin pool (mg)}}{\text{Weight in Kg} \times 100}$$

NOTE : (1) When repeated studies are made in the same patient 4-6 weeks later, allowance must be made for the residual (^{14}C) albumin. Subtract this residual activity from the corrected SA (2 ii) before proceeding with (2 iii).

** (2) Equal recovery of the stable and labelled albumin occurs.

Perfusion study(1) Urea carbon radioactivity

(i), (ii) and (iii) as before.

(iv) Calculate the urea pool in mg C.

Urea pool in mg C

$$= \frac{\text{Perfusate volume (ml)} \times \text{final urea concentration (mg\%)}}{500}$$

where $\frac{12}{60}$

= factor for converting mg urea to mg C in urea.

(v) Calculate the total radioactivity incorporated into urea.

Total radioactivity incorporated into urea
(counts/min)

$$= \frac{\text{urea pool (mg C)} \times \text{urea SA (C/min/mgC)}}{1}$$

(2) Arginine carbon radioactivity.

(i) and (ii) as before.

(iii) Calculate the arginine pool in mg C.

Arginine pool
(in mg guanidine C)

$$= \frac{(100-PCV) \times \text{perfusate volume} \times \text{perfusate albumin concentration} \times 5.95 \times 12}{17,400 \times 100}$$

Where (1) $\frac{(100-PCV) \times \text{perfusate volume (ml)}}{100} = \text{plasma volume (ml)}$

(2) PCV = Packed Cell Volume.

(3) $\frac{5.95 \times 12}{174 \times 100} =$ factor for converting mg albumin to mg guanidine carbon of arginine in albumin.

(4) Albumin concentration expressed in mg/ml.

- (iv) Calculate the total radioactivity incorporated into arginine.

Total radioactivity incorporated into arginine
(counts/min)

$$= \text{arginine pool (mg C)} \times \text{arginine SA (c/min/mg/C)}$$

- (v) Calculate urea synthesis rate.

Urea synthesis rate (mg/hr)

$$= \frac{\text{perfusate volume} \times (\text{post} - \text{pre urea concentration in mg/ml})}{2.5}$$

- (vi) Calculate the arginine synthesis rate.

Arginine synthesis rate (mg/hr)

$$= \frac{\text{radioactivity incorporated into arginine} \times \text{urea synthesis rate}}{\text{radioactivity incorporated into urea}}$$

- (vii) Calculate the albumin synthesis rate.

Albumin synthesis rate (mg/hr)

$$= \frac{\text{arginine synthesis rate} \times 100}{5.95}$$

Where $\frac{5.95}{100}$ = amount of arginine in albumin.

- (viii) Correct the albumin synthesis rate for body weight.

Corrected albumin synthesis rate
(mg/hr/300 g)

$$= \frac{\text{albumin synthesis rate} \times R}{300}$$

Where R = Rat body weight.

G. MEASUREMENT OF PLASMA FREE AMINO ACID CONCENTRATIONS.

Tryptophan

Reagents.

- (1) 10% T.C.A. (iced)
- (2) 18% formaldehyde (1:1 with distilled water).

- (3) 5% H_2O_2 from 100 volumes of 30% (1:5 with water).
- (4) L. tryptophan.

Note: glassware used should be meticulously decontaminated.

Method.

- (a) Pipette 0.6 ml heparinised plasma into a 15 ml glass centrifuge tube.
- (b) Add 0.2 ml 18% formaldehyde and mix
- (c) Stopper and place in water bath at $100^{\circ}C$ for 20 minutes.
- (d) Remove from water bath and add 0.2 ml 5% H_2O_2 (freshly diluted) while the tube is still hot.
- (e) Stopper. Replace in H_2O_2 bath for a further 20 minutes.
- (f) Cool to room temperature in cold water.
- (g) Run appropriate standards of L-tryptophan and sample blanks concurrently.
- (h) Read on a Beckman fluorimeter.

Other amino acids :

Apparatus:

- (1) Technicon amino acid auto-analyser.
- (2) Column 127 x 0.62 cm.
- (3) Chromobeads type B (Technicon) resin.

Reagents :

(1) Ninhydrin

- (i) Dissolve 45 g ninhydrin and 3.375 g hydrindantin in 4.5 L of methylcellusolve in a dark bottle. Mix for 15 minutes.
- (ii) Add 900 ml of sodium acetate buffer (sodium acetate 656 g, glacial acetic acid 200 ml, water to 2 L) and 3,600 ml of water to the ninhydrin solution.
- (iii) Nitrogen should be bubbled through the mixture during

mixing and for at least 30 minutes after the addition of the water.

(2) pH 2.875 Buffer.

- (i) Add 14.71 g sodium citrate (0.05M, 0.150 N with respect to sodium) 25 ml of 2 N. (standardised) NaOH and 5.0 ml of thiodiglycol (Pierce Chemical Company) to 900 ml of water.
- (ii) Adjust pH to 2.875 with 6 N HCl and add 10 ml of Brij 35 solution (Atlas Chemical Industries Incorp).
- (iii) Make up to 1 L with deionised water.

(3) pH 3.800 Buffer

As above but adjust the final pH to 3.800.

(4) pH 4.700 Buffer

- (i) Dissolve 471.30 g of sodium citrate in 5.4L of water.
 - (ii) Adjust pH to 4.7 with 6 N HCl. Add 60 ml Brij solution and make up to 6L with deionised water.
- Store all buffers at 4°C and check their pH immediately before use.

Method:

- (a) Deproteinise plasma by the addition of salicylsulphonic acid crystals. Centrifuge.
- (b) Pump 0.2 NaOH through the column for 30 minutes followed by sodium citrate buffer pH 2.875 for a further 90 minutes.
- (c) Apply 0.5 ml of internal standard (Norleucine 0.1 micro-moles/ml) and wash down sides of column with 2 ml of pH 2.875 buffer. Pump norleucine and buffer into the

column with nitrogen at 100 pounds per square inch (p.s.i.).

- (d) Apply 0.2 - 0.5 ml of supernatant from (a) in the same way.
- (e) Fill column to the brim with buffer and connect it to the autograd.
- (f) Prior to the above fill the 9 chambered autograd with buffers as follows :

Chamber 1	75 ml	pH 2.875 buffer
Chamber 2	75 ml	pH 2.875 buffer
Chamber 3	75 ml	pH 2.875 buffer
Chamber 4	75 ml	pH 2.875 buffer
Chamber 5	40 ml	pH 2.875 buffer
	35 ml	pH 3.800 buffer
Chamber 6	6 ml	pH 2.875 buffer
	9 ml	pH 3.800 buffer
	60 ml	pH 4.700 buffer
Chamber 7	75 ml	pH 4.700 buffer
Chamber 8	75 ml	pH 4.700 buffer
Chamber 9	75 ml	pH 4.700 buffer

- (g) Turn on autograd pump and stirrer and then pump the buffers through the column at 30 ml/hour at 200 p.s.i.
- (h) Set temperature of column at 37°C for the first 90 minutes. Then reset to 60°C for the remaining 19 hours of the run.
- (i) Colour development occurs with ninhydrin under nitrogen, the amino acids passing through an oil bath at 95°C before being cooled and entering the flow cuvettes of the calorimeter.
- (j) Set calorimeter to read at 440 mμ and 570 mμ wavelengths.

- (k) Calculate results utilizing the area under each peak with the norleucine peak as a standard.

H. RIBOSOMAL PROFILES.

Reagents and apparatus.

(1) TKM

Dissolve 6.0257 g, Tris, 1.864 g KCl and 1.01665 g $MgCl_2 \cdot 6H_2O$ in 1 litre of water. Bring to pH 7.5 with 1 N.HCl.

- (2) For preparation of special sucrose solutions pass a solution of 2.3 M-sucrose in TKM through a millipore filter (1.2 u) and keep frozen at $-20C$.

(a) 2.0 M-sucrose- S_3 medium = 10 ml stock solution and
1.5 ml S_3

(b) 1.38 M-sucrose- S_3 medium = 10 ml stock solution and
8 ml S_3 .

- (3) Prepare linear sucrose gradients (10-40% W/V in TKM),

(a) Place 15 ml 10% sucrose in TKM in A (Figure A:2).

(b) Open tap B to allow the sucrose-TKM to reach C.

Close tap B.

(c) Fill C with 15 ml of 40% sucrose in TKM.

(d) Start the motor D.

(e) Open taps E and B together.

(f) Chill the linear gradient as soon as it is prepared.

(A) Preparation of RNase inhibitor.

(i) Decapitate fasted rats.

(ii) Remove the livers and chilled them in ice-cold 0.25 M sucrose in TKM.

Note: (1) Use only RNase-free sucrose.

(2) Perform all subsequent steps at $0-4^{\circ}C$.

(iii) Mince the livers with scissors and add 2 volumes of 0.25 M sucrose in TKM.

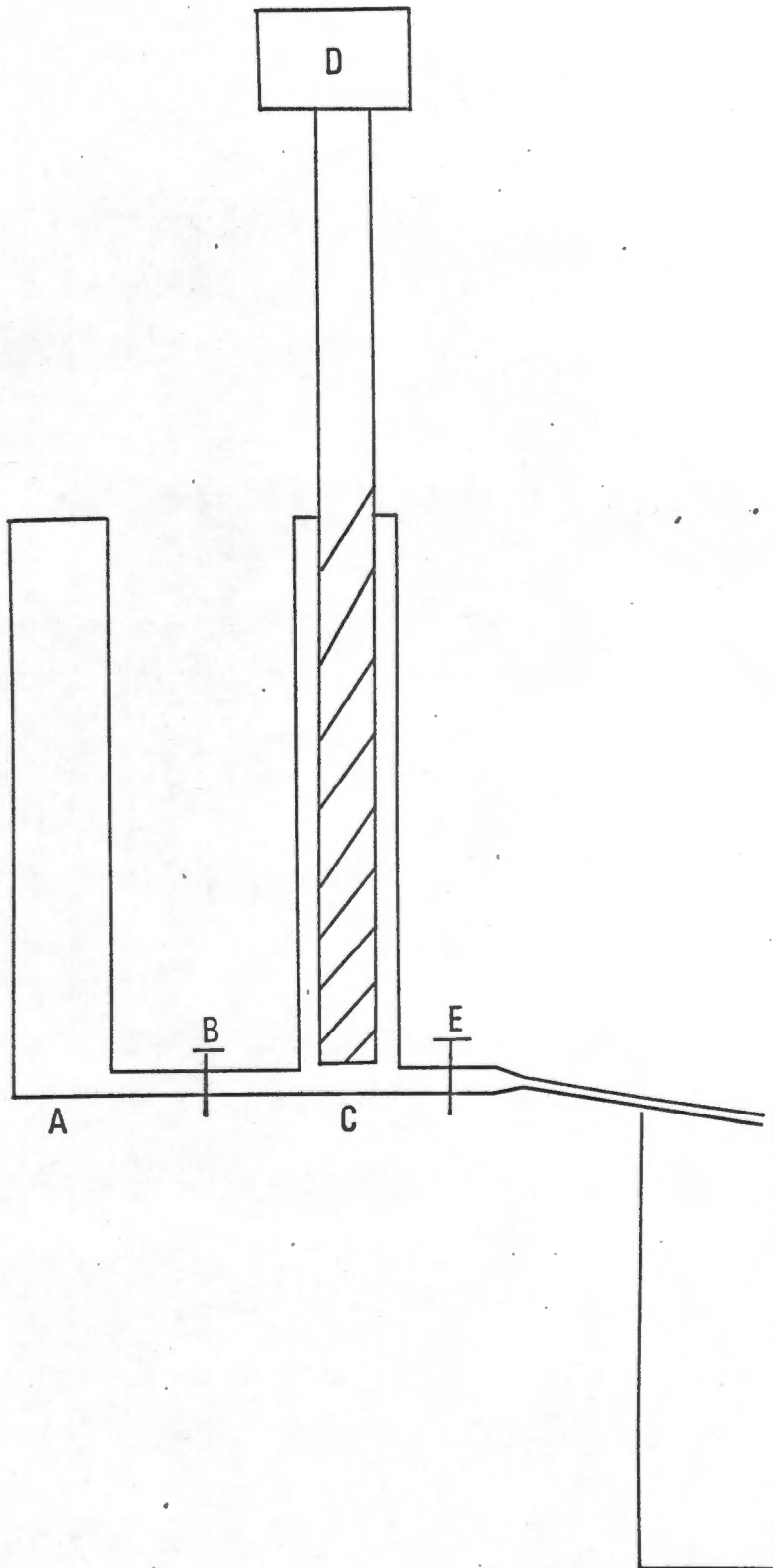
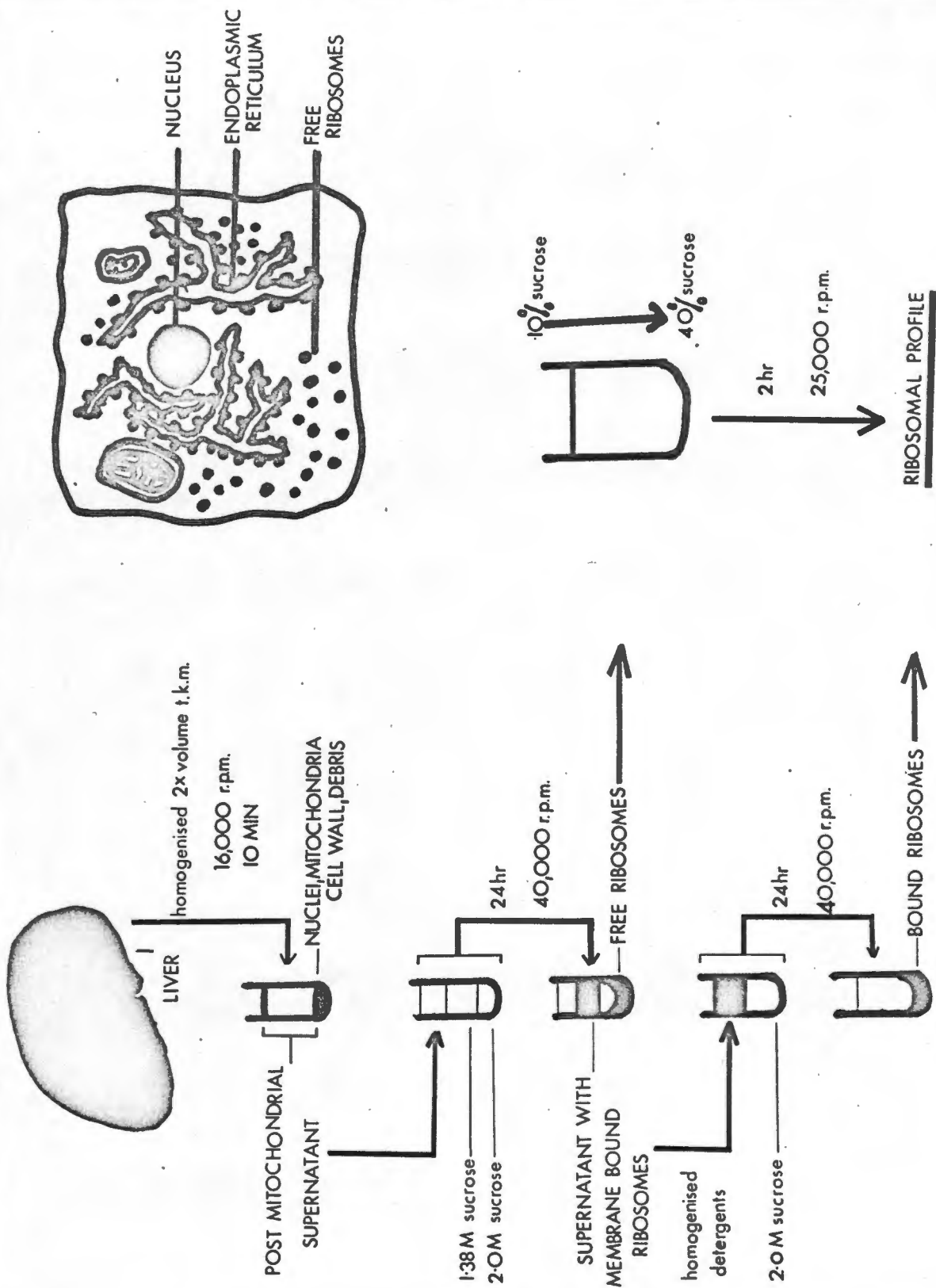


Figure A:2: APPARATUS FOR PREPARATION OF LINEAR SUCROSE GRADIENTS

Figure A:3: THE PREPARATION OF RIBOSOMAL PROFILES



ALL PROCEDURES PERFORMED AT 0°-4°C IN THE PRESENCE OF R.N. ase INHIBITOR

- (iv) Homogenise the livers with 10 strokes in a Potter-Elvehjem homogeniser with a Teflon pestle.
 - (v) Prepare a postmitochondrial supernatant (S_2) by centrifuging the homogenate in the SS 34 rotor in the refrigerated Sorvall centrifuge for 10 minutes at 16,000 rev/min.
 - (vi) Spin S_2 in a Spinco 40 rotor for 4 hours at 40,000 rev/min to obtain a supernatant fraction S_3 containing RNase inhibitor.
 - (vii) Store S_3 for a maximum of 30 days.
- (B) Preparation of pellets of free ribosomes. (Figure A:3)
- (i) Control profiles: remove the livers from the control rats anaesthetised with diethyl ether.
Perfusion profiles: stop the perfusion apparatus.
Perfuse the liver via the portal vein with 50 ml of 0.9% (W/V) sodium chloride).
 - (ii) Chill the livers in a sucrose- S_3 medium (9 parts 0.25 M-sucrose in TKM and 1 part S_3).
 - (iii) Mince the livers with scissors and add the sucrose- S_3 medium to make up to 2 volumes.
 - (iv) Prepare S_2 as previously described (A (iv) and (v)).
 - (v) Layer 4 ml S_2 over a two-layer discontinued sucrose gradient with 3 ml 2.0 M-sucrose- S_3 medium at the bottom and 3 ml 1.38 M-sucrose- S_3 medium at the top of a Polyallomer tube.
 - (vi) Centrifuge for 24 hours at 40,000 rev/min in a Spinco 40 rotor.
 - (vii) Store the pellets of free ribosomes at -20°C .*
- (C) Preparation of pellets of bound ribosomes.
- (i) With a syringe remove and discard the 4 ml above the 1.38 M-layer in B(vi).
 - (ii) With a syringe remove the 2.0 M-layer containing the rough

endoplasmic reticulum.

- (iii) Holding a glass rod in the tube rehomogenise this layer gently with a detergent mixture of Triton X-100 (20% W/V) and sodium deoxycholate (5% W/V) to give a final concentration of 4% Triton X - 100 and 1% sodium deoxycholate.
- (iv) Layer this over 3 ml of 2.0 M-sucrose-S₃ medium.
- (v) Spin for 24 hours at 40,000 rev/min in a Spinco 40 rotor.
- (vi) Store the pellets of ribosomes, originally bound to the endoplasmic reticulum, at -20°C. *

*NOTE: The profiles gradually undergo changes with storage. However, it was found that the qualitative change after one week is negligible.

(D) Examination of profiles.

- (i) Resuspend the ribosomal pellet in 2 ml water and 0.2 ml S₃ by mixing with a glass rod in the tube.
- (ii) Layer the equivalent of 40 optical density units of the ribosome S₃ solution over the linear gradient.
- (iii) Spin the gradient for 2 hours in a Spinco SW 25 rotor at 25,000 rev/min.
- (iv) Using the apparatus shown in Figure A:4
 - (a) Fill the continuous infusion pump A and the tubing B with 50% sucrose.
 - (b) Fit C over the gradient tube to achieve a tight seal. Puncture the bottom of the tube by applying C firmly to D.
 - (c) Connect tubing E to the flow cuvette.
 - (d) Displace the gradient upwards with the 50% sucrose.

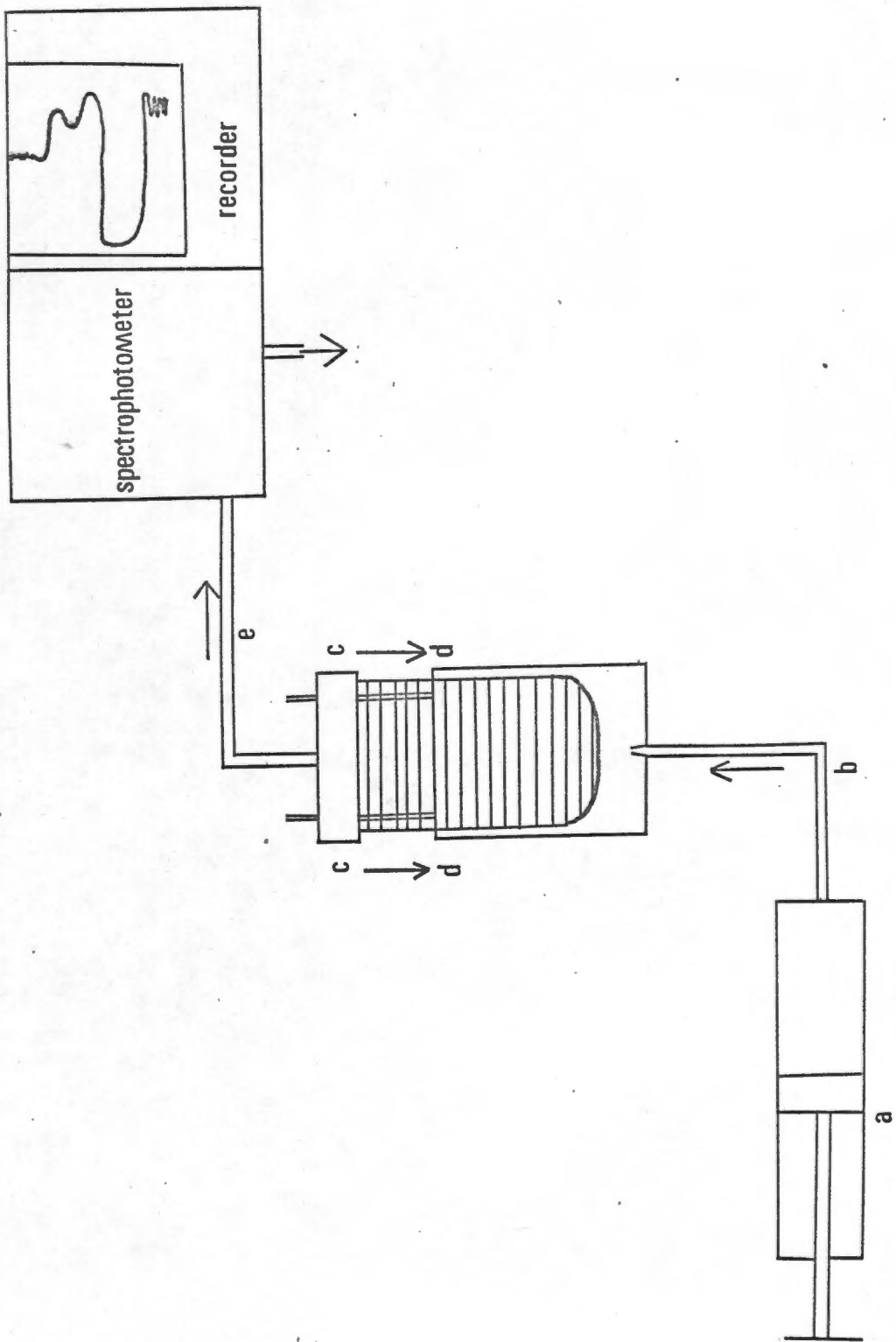


Figure A:4: SYSTEM FOR ANALYSIS OF RIBOSOMAL PROFILES

- (e) Record the profile with an automatically recording D.B. Beckman Spectrophotometer set at an optical density of 260 u.

I. LIVER PERFUSION.

- (1) Set up the apparatus as shown in Figure A: 5.
- (2) Under light diethyl ether anaesthesia bleed rats by cardiac puncture using heparinised syringes.
- (3) Make up the perfusate volume to 87 ml with
 - (i) 56 ml whole rat blood.
 - (ii) 4 ml Heparin (10 mg/ml - Heparin B.P. Boots Pure Drug Co. Ltd., Nottingham, England).
 - (iii) 25 ml Plasmalyte B (Baxter-Saphar Laboratories Ltd., Johannesburg, South Africa) containing mEq/L sodium 130, chloride 109, potassium 4, manganese 3, sodium bicarbonate 28.
 - (iv) 2 ml of 4.2% sodium bicarbonate.
- (4) Pour 77 ml of the perfusate into the reservoir and start the apparatus ensuring that the fan, heater, pump, oxygenator, humidifier and magnetic stirrer are working satisfactorily.
- (5) Anaesthetise the rat for dissection in a bell jar supplied with diethyl ether by an Abingdon Vaporiser. Transfer the rat to the dissecting board continuing the anaesthetic.
- (6) Proceed with dissection :
 - (i) Make a midline abdominal incision. Extend the incision laterally on both sides near the lower abdomen. Reflect flaps of abdominal wall laterally.
 - (ii) Free the stomach from the liver using blunt dissection.

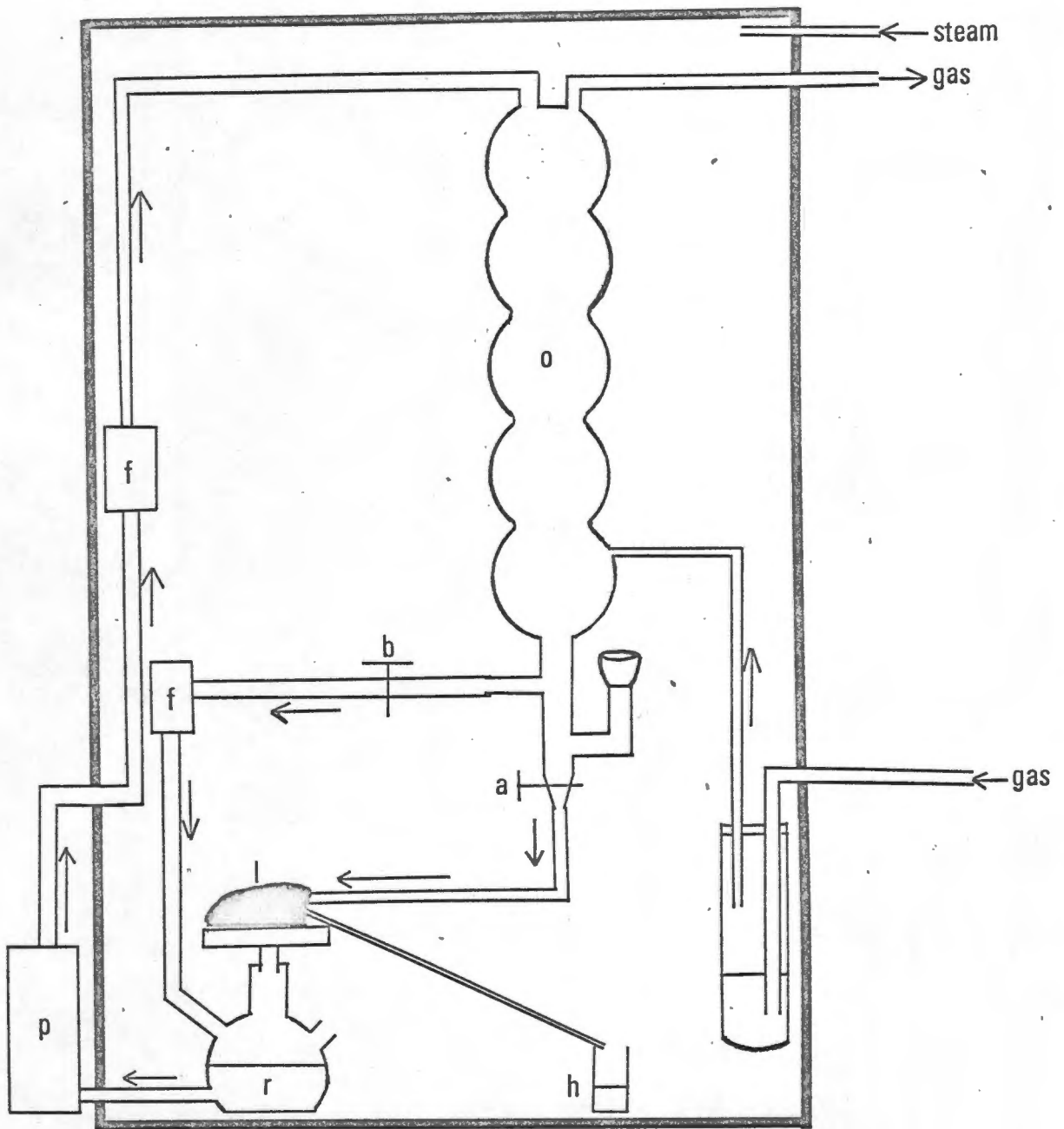


Figure A:5: THE PERFUSION SYSTEM

- l - liver
- r - reservoir with magnetic stirrer
- p - pump
- o - oxygenator
- f - filter
- a - tap
- b - clamp
- h - bile

- (iii) Locate and cut through the oesophagus in close proximity to the diaphragm, allowing the proximal portion to retract into the thorax.
 - (iv) Inject 5 mg heparin into the spleen.
 - (v) Place a ligature loosely around the bile duct.
 - (vi) Incise the bile duct and after cannulation tighten the ligature.
 - (vii) Tie off the pyloric vein at its junction with the portal vein.
 - (viii) Place a ligature loosely around the portal vein close to the hilum of the liver.
 - (ix) Clamp the proximal portion of the portal vein.
 - (x) Incise the portal vein just proximal to the ligature.
 - (xi) Cannulate the portal vein. Tie the ligature firmly.
 - (xii) Cut the hepatic veins flush with the liver.
 - (xiii) Perfuse the liver slowly with warm plasmalyte B at a low pressure.
 - (xiv) Rapidly remove the liver manipulating surrounding tissues rather than the liver itself.
 - (xv) Place the liver on gauze resting on the glass plate.
- (7) Transfer the glass plate to the reservoir.
- (8) Connect the portal vein to the oxygenator and open tap (A).
- (9) Adjust the overflow clamp (B) to keep the portal pressure at 18 cm water.
- (10) Measure the rate of bile production, blood flow and the pH of the perfusate. Adjust the pH with 4.2% sodium bicarbonate if required.

NOTE: The "ischaemic time" (step 6 (ix) - step 8) is approximately 2 minutes.

J. STATISTICAL METHODS

(1) Mean. $\bar{x} = \frac{\sum x}{n}$

Standard deviation of the mean. $S = \frac{\sum (x - \bar{x})^2}{n - 1}$

(2) Student's t-test.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$n_1 + n_2 - 2$ degrees of freedom.

(3) Linear regression analysis (Method of Least Mean Squares)

The least square line approximating the set of points

$(x_1, y_1), (x_2, y_2), \dots, (x_n, y_n)$, is given by the equation

$y = mx + c$ where

$$m = \frac{N \sum xy - (\sum x)(\sum y)}{N \sum x^2 - (\sum x)^2} \quad \text{and} \quad c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N \sum x^2 - (\sum x)^2}$$

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THE ROLE OF AMINO ACIDS IN ALBUMIN SYNTHESIS
AND CATABOLISM.

A Thesis
presented to the University
of Cape Town for the degree
of Doctor of Medicine by
Leslie Kelman, M.B., Ch.B.

1 9 7 1 .

S U M M A R Y

In recent decades, this Medical School has made important contributions to the study of clinical malnutrition, largely through the work of Professors J.F. Brock and J.D.L. Hansen. With the emergence of Dr. R. Hoffenberg as an established investigator in the field of albumin metabolism, the laboratory achieved a new dimension. Both Dr. Hoffenberg, and later Dr. R.E. Kirsch, made notable contributions to the understanding of albumin synthesis, catabolism and distribution. Recently Dr. L. Kernoff has expanded the field to include the role of growth hormone in albumin metabolism. Professor S.J. Saunders' expertise in amino acids and interest in albumin metabolism provided an active and remarkably topical area for research by the author.

The concept of depressed albumin synthesis rates during protein depletion is well established. However, the author considers the study in humans to be the first comprehensive attempt to measure albumin synthesis directly and independently of catabolism during controlled conditions of protein intake. This study serves to confirm the work of Hoffenberg and others, who inferred from their data that albumin synthesis is reduced during protein restriction. Furthermore, the demonstration that plasma alanine is the only amino acid the concentration of which is influenced by dietary protein deprivation, is a useful contribution in a field with few established facts. Moreover, few studies have included the measurement of tryptophan. The relationship of the low synthesis rate to the elevated plasma

alanine levels is felt by the author to be either fortuitous or the result of 2 processes acting at different sites. However, the possibility of a cause-and-effect relationship cannot be excluded. Emphasis has been placed on the complex interplay of factors regulating the hepatic intracellular amino acid pools and the fact that plasma amino acid levels may bear no relation to tissue levels or to protein synthesis at other sites.

Clearly, the accelerated albumin synthesis rates on protein loading are related to amino acid supply. However, the mechanism has not been established. It was unclear whether the amino acids were only acting as substrate or whether their influence was indirect. Furthermore, were they equally effective ?

Perfusion of the isolated rat liver was employed by the author to attempt to clarify this issue. The defects of the perfusion system used have been emphasised, but nonetheless, it remains a powerful tool in the research laboratory. From these perfusion studies the following points relating amino acids to albumin synthesis were established :-

- (1) It was confirmed that fasting markedly reduces albumin synthesis.
- (2) The plasma aminogram is altered by depriving rats of protein for 48 hours.
- (3) The use of the above plasma as the perfusate, instead of normal plasma, reduces albumin synthesis rates significantly.

- (4) The use of the above perfusate with the addition of single amino acids increases albumin synthesis rates.
- (5) Single amino acids have no effect on albumin synthesis when the liver is derived from fasted rats. Yet the addition of 11 amino acids added to a concentration of 10 times their normal peripheral blood concentration restores albumin synthesis to control levels.

While the study was in progress, the work of Rothschild et al (1969c) in rabbits was published. Reference has been made to the important differences between the studies. These include :-

- (1) The inability of the author to influence albumin synthesis after adding single amino acids to fasted livers. The increased synthesis observed by the addition of the 11 amino acids demonstrated the potential activity of the system. However, even in the latter circumstances, no overshoot beyond control levels occurred. The postulate that the relatively stable mRNA of albumin no longer has to compete with more labile mRNA of other proteins for available amino acid supply, did not appear to be operative in this study.
- (2) Single amino acids including non-essential and non-metabolisable amino acids increased albumin synthesis rates in livers from well nourished rats.

The work on ribosomes confirmed and extended the work of other investigators. The author has demonstrated

that

- (1) both free and endoplasmic reticulum-bound ribosomes disaggregate progressively during perfusion;
- (2) single amino acids fail to influence the ribosomal profile;
- (3) the 11 amino acids together used by Jefferson and Korner (1969) aggregated both populations of ribosomes;
- (4) infusion of growth hormone, cortisone, insulin, secretin, gastrin, glucagon and a combination of glucose and insulin failed to influence the profiles;
- (5) unlike Rothschild et al (1969c) in the rabbit, the author was unable to influence the ribosomal profile of fasted rats by the addition of tryptophan or isoleucine. However, ribosomal aggregation, like accelerated albumin synthesis, still occurred when the 11 amino acids were added.

Bearing in mind the quantitative limitations of the technique used by the author, it is unwise to place too much emphasis on the association of unchanged ribosomal profiles with increased albumin synthesis rates after the addition of single amino acids.

The concept of short-term regulation of protein synthesis by dietary tryptophan has not been supported by the perfusions using fasted livers, possibly as a result of insufficient supply of other amino acids. As mentioned above, tryptophan together with 10 other amino acids increased albumin synthesis and aggregated the ribosomes. The alternative method of increasing the supply of other amino acids by

infusing glucagon and adding tryptophan was unsuccessful.

The short study of the influence of amino acids, glucagon, insulin, growth hormone and hydrocortisone on the fractional catabolic rate of albumin represents the only study investigating this control by factors other than carbon loading or albumin pool size known to the author.

A few interesting observations were noted :-

- (1) Amino acids do not influence the fractional rate of catabolism.
- (2) Accelerated protein synthesis, and thereby possibly augmented proteolytic enzyme activity, failed to influence the fractional catabolic rate of albumin.
- (3) None of the hormones except insulin had any influence on the catabolic rate of albumin. However, insulin effected a marginal slowing of catabolism.
- (4) Unlike Hoffenberg et al (1970), normal fractional catabolic rates were obtained in the presence of low perfusate albumin concentrations. The implications of this important finding were discussed.

In summary, it would appear that this thesis consolidates and extends existing literature and opens new avenues for exploration.