

Evaluation of Techniques Employed in the
Study of Alanine Metabolism in Sheep

by

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ABSTRACT

In view of the importance of alanine as a gluconeogenic precursor in ruminants, the objective of the present study was to assess the effectiveness of three techniques in estimating the metabolic parameters surrounding alanine in wethers fed a maintenance diet of alfalfa hay.

A preliminary experiment utilized a blood flow technique to study the net production and/or utilization of both alanine and glucose by the portal drained viscera. Such a method involved evaluating the arterio-venous concentration differences of alanine and glucose, in conjunction with determining the rate of portal vein blood flow.

Radioactively labelled ^{14}C - alanine was administered as a single injection in the second series of experiments to estimate the metabolic parameters of alanine as well as its contribution to glucose synthesis. The L-U- ^{14}C -alanine was given intravenously through previously implanted jugular catheters and the fall in the specific activity of plasma alanine with time was determined. The line of best fit for the decay curve of the specific activity of plasma alanine was constructed by means of a computer using a multi-term exponential function which enables the estimation of such parameters as the pool size, space, total entry rate, irreversible loss and recycling of alanine. The per cent conversion of alanine to glucose was determined by the corresponding peak of glucose specific activity following the single injection of ^{14}C - alanine.

The turnover of alanine was also studied using a continuous infusion of L-U- ^{14}C - alanine without a priming injection. The specific activity of plasma alanine reached a plateau five hours after the beginning of the infusion. It was from these plateau levels that the rate of irreversible loss of alanine as well as its percent conversion to glucose was estimated.

The results indicated that the single injection technique was able to partition the total entry rate of alanine into irreversible loss and recycling and thus prove more informative than a continuous infusion method. The present study also suggested that under certain physiological stress conditions in ruminants, where recycling becomes prominent, a continuous infusion approach may overestimate the actual rate of irreversible loss of alanine.

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INTRODUCTION

In contrast to monogastric animals ruminants, fed primarily a roughage diet absorb negligible amounts of glucose through the gastro-intestinal tract. The carbohydrates are fermented by rumen microorganisms to volatile fatty acids, of which acetic, butyric, and propionic acids predominate (Annison and Armstrong, 1970, Bergman et al., 1965). Thus the ruminant animal depends upon gluconeogenesis or the endogenous synthesis of glucose from non-carbohydrate sources, as a means of satisfying its energy demands.

Ruminants possess a glucose requirement which is just maintained under normal dietary and environmental conditions. However this critical energy balance can be readily disrupted under stress conditions. Such traumas are manifested by a reduction in blood glucose concentration (hypoglycemia) and an excessive production of ketone bodies, whose concentration in the blood rise (ketosis). In the dairy cow this condition is referred to as acetonemia and occurs during periods of heavy lactation, whereas in sheep hypoglycemia and ketosis arise during late pregnancy and is called pregnancy toxemia or twin lamb disease. While these disturbances of dairy cows and sheep are not identical, both disorders have a number of common characteristics, which include a negative energy balance, a reduction of glucose in the blood and liver, and an increased fat metabolism. The heavy drain of glucose in the ruminant also becomes acute under conditions of starvation.

Of the three major end products of rumen microbial

fermentation it has been shown that between 27 and 54 per cent of the glucose may be produced from compounds arising from propionate, either before or upon absorption into the blood draining the rumen (Bergman, 1973). Further work by Annison et al. (1963a) confirmed the glucogenic role of propionate and, by employing ^{14}C -labelled butyrate and acetate, indicated that these endproducts of carbohydrate fermentation contribute little if any to the production of glucose.

Glucogenic properties have been assigned to such precursors as the glycerol moiety of triglycerides as well as lactate and pyruvate. The former becomes a major contributor under conditions of underfeeding or starvation when the free fatty acids are mobilized from the adipose tissue. The glycerol which is released to the blood is transported to the liver where it readily gives rise to glucose (Bergman, 1963). The role lactate, and to a lesser degree pyruvate, play in gluconeogenesis in ruminants has undergone considerable controversy. The situation results because of the variable and unknown amounts which are absorbed into the gastro-intestinal tract and the degree of metabolism which occurs through the rumen wall. A detailed review of the present understanding of this potential glucogenic precursor is presented in the following section.

Other than propionate, the second most important contributor to endogenous glucose production is the amino acids. Most of these are glucogenic, with the exception of lysine, leucine and taurine (Krebs, 1964). Recent research with humans

(Felig et al., 1970), rats (Aikawa et al., 1972) and sheep (Wolff and Bergman 1972a) has provided convincing evidence that alanine and glutamine are the principle amino acids extracted by the liver for gluconeogenesis. In addition these amino acids are the main amino acids released from the skeletal muscle. From this finding alanine (Felig et al., 1970) and glutamine (Marliss et al., 1971) cycles have been proposed as important means of linking amino acid metabolism with the control of gluconeogenesis. The nature and effect of these cycles are discussed fully in the following literature review.

In view of the importance of alanine as a gluconeogenic precursor in ruminants the present study assessed the effectiveness of various techniques employed to estimate the metabolic parameters of alanine as well as its contribution to glucose synthesis in sheep fed a maintenance diet. The three techniques employed were a modification of the single injection of ^{14}C -labelled alanine, a continuous infusion of ^{14}C -labelled alanine without a priming dose and a portal blood flow study, which included an arterio-venous concentration assay for both glucose and alanine.

LITERATURE SURVEY

Glucose Metabolism in the Ruminant

Glucose plays an essential role in cell metabolism. It serves as a basic component in the construction of complex macromolecules of the cell, including nucleic acids, proteins and lipids. In addition, glucose is utilized for the production of energy which is necessary for the various endergonic processes within the cell. Thus the caloric needs of the body are almost entirely satisfied by this hexose. Other sources include the catabolism of fatty acids, however this mechanism of energy production becomes prevalent only when the former source is depleted.

The importance of glucose in maintaining an animal in an adequate energy balance is paralleled both in ruminants and monogastrics. However, the metabolism of carbohydrates varies in many respects which reflect the anatomical variations between the two, and the subsequent presence of a far greater microbial population in the ruminant species. The metabolism of carbohydrates in the simple stomached animals involves the degradation of dietary carbohydrates to glucose and other simple sugars, which are then absorbed by the portal circulatory system and utilized by the animal. On the other hand, in ruminants, the carbohydrates are fermented by microorganisms present in the rumen, to volatile fatty acids. Consequently

negligible amounts of glucose are absorbed via the gastrointestinal tract. Thus an endogenous glucose production from noncarbohydrate precursors, or gluconeogenesis is relied upon heavily to meet the animals glucose requirements.

A.) Glucose Requirements

The requirements for glucose are equally important both in ruminants and monogastrics, however the blood glucose concentrations differ considerably. The level in ruminants (40-60 mg/100 ml) is lower than that occurring in new born or adult simple stomached animals (80-100 mg/100 ml) (Bergman, 1973). Glucose is needed by five major areas of the body, the nervous system, turnover and synthesis of fat, muscle, fetuses and the mammary gland. The amount utilized by other cells of ruminants such as erythrocytes has been shown to be negligible (Leng and Annison, 1962).

1. Utilization by the Nervous System

Work by Cahill et al., (1970) on metabolism in postabsortive humans has indicated that the human nervous system utilizes as much as 80 percent of the glucose released into the blood. Such a study has yet to be performed in ruminants, however from the arterial - jugular concentration differences in sheep, considerable glucose must be removed by the nervous system and the brain in particular, of these species (McClymont

and Setchell, 1956); (Setchell, 1961). Thus the cells of the nervous system with the brain being most important, are absolutely dependent upon a regular supply of glucose for its oxidative metabolism. An interesting variation to the amount of glucose utilized during prolonged starvation in the sheep, dog and man has been referred to as a "glucose exclusion". (Owen et al., 1967 Raju et al., 1972 and Weiner et al., 1971). This adaptation is present in all tissues of the body but is most strikingly evident for the brain and consists of a conservation of glucose and a utilization of ketone bodies and fatty acids for energy purposes during periods of starvation.

2. Utilization for Fat Metabolism

Glucose plays a dual role in the turnover and synthesis of both milk and body fat. It is the precursor of glycerol, and glycerophosphate, which are utilized in the esterification of fatty acids to form triglyceride. Vaughn, (1961) has shown that the enzyme glycerol kinase is lacking in both adipose tissue and mammary gland. Thus the glycerol requirements of these tissues must be met by glucose metabolism.

The second role of glucose in fat metabolism is in the provision of adequate supplies of NADPH which is required as a reducing agent in the synthesis of long-chain fatty acids. (Ballard et al., 1969; Krebs, 1966). In addition to the above two, glucose can also serve as a carbon substrate for fatty acid

synthesis. However, in the ruminant animal carbon atoms for fat synthesis are supplied to a greater extent by acetate. Nonruminants rely on glucose rather than acetate for lipogenesis.

3. Utilization by Muscle Tissue

Muscle glycogen synthesis depends on glucose. The total quantity of glucose stored in the muscles remains constant and is greater than that present in the liver or body fluids (Lehninger, 1970). This is due to the much larger tissue mass that is involved. The glycogen stores of muscle serve an essential function by providing an anaerobic energy supply for the muscle during exercise or when oxygen becomes limiting (Bergman, 1973). The majority of the glycogen is converted to lactate and pyruvate which is returned to the blood and then to the liver for resynthesis back into glucose. This glucose, when liberated in the blood, may again return to the muscles to be converted to glycogen. Such a process is referred to as the Cori cycle (Cori, 1931).

4. Utilization by the Fetus

One of the greatest glucose demands in ruminants is during late pregnancy and lactation. Such metabolic disorders as pregnancy toxaemia in sheep and ketosis in cows occur at this time. Since the principal metabolic fuel of the fetus is carbohydrate, it must maintain a constant supply of glucose from the mother. In this regard work by Huggett (1961) firmly established that the placentas of ruminants convert a portion of the maternal glucose to fructose so that the concentrations of both sugars reach even higher levels in the body fluids of the fetus than the mother. In addition, late in fetal life large glycogen reserves are built up in the placenta, and in the fetal liver, lung and skeletal muscle. In a review of fetal physiology, Daves (1968) stated glycogen concentrations to be .8 to 10 percent in liver and about 4 percent in muscles, which are 2 to 8 times the corresponding values for adults of the same species.

5. Utilization during Lactation

Lactation also makes a large demand for glucose upon the animal since milk contains approximately 90 times as much total sugar as does blood (Bergman, 1970). Of the two metabolic disorders involving glucose, pregnancy toxaemia and ketosis, the former is manifested with a far more severe hypoglycemia than the latter since the glucose supply for milk production can

readily be reduced or cut off completely. Such is not the case for a pregnant animal where the glucose demands of the fetus must be sustained.

From this brief survey of the metabolism and requirements of glucose by the ruminant, it appears evident that these animals depend heavily upon gluconeogenesis for their glucose supply.

B.) Glucose Production in the Ruminant

1. Dietary Source

The sites of glucose production in the body of all mammals are the gut (by absorption), the liver and the kidneys. As mentioned earlier ruminant animals differ from the simple stomached species in that little or no glucose is absorbed from the gastro-intestinal tract. However, there still exists controversy regarding the quantity of glucose absorbed in ruminants fed grain or high concentrate diets. One approach to this problem is to detect the amount of starch which escapes fermentation and flows into the small intestines. MacRae and Armstrong (1966) and Topps et al., (1968) employed this approach and indicated that barley and oat diets are readily fermented in the rumen and only little starch appears in the intestine. Further research in this area has postulated that a fine grinding of the grain may result in a faster rate of passage through the rumen and thus enable more glucose to be absorbed

(Sutton and Nicholson, 1968). Another method used to study the glucose absorption under concentrate diets is to measure the glucose concentrations in portal and arterial blood. With this technique a number of workers detected no actual glucose absorption into blood of animals fed a maintenance diet of hay or a 50 percent hay - grain (wheat, corn or oats) mixture (Bergman, et al., 1970; Katz and Bergman, 1969; Roe et al., 1966). In a review article Bergman (1973) suggested that the kind of feed, as well as the amount and frequency of feed eaten, may determine whether or not glucose is absorbed.

2. Renal Gluconeogenesis

The kidneys have been suspected of playing a role in the production of glucose since no appreciable amounts are absorbed from the gastro-intestinal tract and the liver has been estimated to contribute 85 percent of the total glucose turnover in nonpregnant fed sheep (Bergman et al., 1970). Research by Kaufman and Bergman (1971) reported that glucose production by the two kidneys averaged 0.4 - 0.8 g/hr and was higher in pregnant than in nonpregnant sheep. Renal gluconeogenesis appears to account for 8 - 10 percent of the total body glucose turnover in normal fed sheep and for approximately 15 percent during fasting. To further emphasize the significance of renal gluconeogenesis in ruminants, these workers pointed out that the sum of the glucose production rates of both liver and kidneys averaged 98 percent, which accounts for virtually all of the body's total glucose production.

3. Hepatic Gluconeogenesis

The liver is the major contributor of glucose for the animals energy demands, with an estimate of 85 per cent of the total glucose turnover arising from the hepatic source in ruminants under a steady state condition (Bergman et al., 1970). Thus the sources of hepatic glucose production consist of four major precursors, which include propionate, glycerol, and lactate and amino acids.

A. From Propionate

The short chain fatty acids arising from the fermentation of dietary carbohydrates in the rumen supply approximately 70 per cent of the animal's caloric requirements (Bergman et al., 1965). Of these only propionate can contribute significantly to glucose synthesis (Annison et al., 1963; Bergman and Wolff, 1971; Black et al., 1966 and 1972; Leng and Annison, 1963). The net contribution of propionate to the synthesis of glucose has been subject to some controversy. Uncertainty still exists as to whether the rate of propionate production in the ruminant is sufficient to meet the demands for gluconeogenesis.

Early work in measuring, quantitatively, the amount propionate absorbed from the rumen has been complicated by the lack of complete control in timing the experiments with respect to feeding and also by the fact that some propionate is metabolized by the rumen epithelium during absorption

(Pennington, 1954). Thus the gluconeogenic role of propionate remained obscure. The first advance in this area was made by Annison et al., (1957), who, by measuring the portal vein - arterial blood concentration differences of the volatile fatty acids, concluded that nearly all of the propionate was removed by the liver. Bergman et al., (1966), extended the study by using a constant infusion of labelled propionate into a rumen vein of nonpregnant, nonlactating ewes and found that the amount of propionate absorbed depends greatly upon the quantity and quality of the feed being digested. The results indicated that normal sheep absorbed about 24 mM propionate per hour. These figures estimate that approximately 50 percent of the propionate entering the portal bed is converted to glucose which accounts for 27 per cent of the glucose entry rates.

The higher absorption rate figures found under direct infusion of ^{14}C - propionate into the rumen are justified since up to 70 percent of propionate produced in the rumen is first converted to lactate in the rumen epithelium during absorption (Leng et al., 1967). This particular study demonstrated that the conversion of propionate into glucose may undergo two pathways, either direct or indirect. The following reactions are a mere outline of these pathways:

rumen (Leng et al., 1967) or in portal blood (Bergman et al., 1966). The rate of propionate metabolised by rumen epithelium is not clear. Contrary to Leng's findings, Weigand et al., (1972) obtained blood from thoracic aorta and portal vein of Holstein calves and estimated that only 1.0-4.6 per cent of rumen derived propionate is converted to lactate by the rumen epithelium. Regardless of these differences, it can be stated that the amount of lactate actually appearing in portal blood is small under conditions of normal rumen fermentation (Roe et al., 1966). However high grain diets will increase both lactate (Dunlop and Hammond, 1965) and propionate (Lindsay, 1959) production and absorption.

Of the remaining primary volatile fatty acids, acetate has been established to play a dominant role in ruminant lipogenesis since ATP-citrate lyase is deficient in ruminants and glucose can supply only limited amounts of acetyl - CoA for fatty acid synthesis (Ballard et al., 1969). Butyrate, on the other hand, has been ascribed a glucogenic role by a number of workers for several years. For example, Potter (1952) demonstrated that butyrate was more effective than propionate in increasing blood sugar levels and relieving hypoglycemic convulsions in lambs and sheep treated with insulin. Kleiber et al., (1954) showed that, unlike acetate, butyrate was markedly glucogenic in lactating cows and that the ^{14}C from butyrate was utilized more for synthesis of lactose than for synthesis of milk fat. These and other studies on the utilization of butyrate in ruminants fail to demonstrate a glucogenic pathway in either lactating cows (Black et al., 1966) or in sheep (Leng and Annison, 1963).

The metabolic role of butyrate in ruminants was investigated by Black et al., (1966). These workers gave single injections of pyruvate 2- ^{14}C and propionate 2- ^{14}C into the jugular vein of lactating cows and utilized the intra-molecular labelling patterns of glutamic acid to assess the pathway for metabolism of these two glucogenic compounds. The results from this study demonstrated that when butyrate was injected together with pyruvate 2- ^{14}C it caused preferential utilization of pyruvate to form oxaloacetate and consequently less pyruvate was decarboxylated to form acetyl - COA.

Therefore they have suggested there are two effects which contribute to the glucogenic effect of butyrate in the cow, the first is the sparing effect of butyrate on pyruvate oxidation, and the second is the greater activity of pyruvate carboxylase and hence an increased rate of gluconeogenesis. This work is contrary to the results of Cook (1970), who investigated the effect of route of administration on transfer of ^{14}C volatile fatty acids to liver glycogen and various blood substrates by administering (1- ^{14}C) acetate, (1- ^{14}C) propionate and (1- ^{14}C) butyrate via the jugular vein, portal vein or added directly into the rumen of sheep and goats. The work demonstrated that a tracer dose of (^{14}C) butyrate injected into the jugular vein labeled blood glucose more than (^{14}C) acetate because of the higher specific activity of blood butyrate. When unlabelled butyrate was infused along with a tracer dose of (1- ^{14}C) butyrate the specific activity of blood glucose was similar to that observed when acetate was administered. Thus the glucogenic

effect of butyrate that has been observed in the past was a misconception and may be explained by the fact that a tracer dose of (1- ^{14}C) butyrate injected into the jugular vein is not diluted with unlabelled butyrate since butyrate is not normally present in peripheral blood. Cook (1970) provided an alternative explanation for the stimulation of ^{14}C transfer from (^{14}C) pyruvate to glucose by butyrate observed by Black *et al.*, (1966) in that the level of butyrate administered (0.60 μM per kg body weight) provided a substrate for the tissues. Therefore, butyrate has a sparing effect on the oxidation of (^{14}C) pyruvate to $^{14}\text{CO}_2$ and H_2O .

Despite the controversy regarding the glucogenic role of butyrate, the study of Cook (1970) revealed that results on the metabolism of ^{14}C labelled volatile fatty acids depend on whether the acids are administered via the jugular vein, portal vein or added directly to the rumen.

B. From Glycerol

Glycerol is considered to be a potent glucogenic compound and is released from the adipose tissue along with free fatty acids during starvation, ketosis or other periods of body fat mobilization (Steinberg and Vaughn, 1965). Bergman, (1968) has shown that while the turnover rate of glycerol is low in fed sheep, it increases markedly during fasting and especially during hypoglycemic ketosis. In the case of rats and humans,

approximately 80-90 per cent of the glycerol is removed from the blood by the liver and the kidneys apparently remove most of the remainder (Borchgrevink, and Havel, 1963; Larsen, 1963; and Lundquist et al., 1965). The free glycerol thus removed enters the gluconeogenic pathway at the triose phosphate stage as illustrated in Figure 1, which summarizes the major metabolic pathways in the liver and kidneys of ruminants (Bergman, 1973). These findings along with the established high glycerokinase activity in the liver and kidneys suggest the major importance of glycerol for gluconeogenesis. In addition, glycerol metabolism seems similar to that of propionate metabolism in that most of the former is also removed by the liver (Annison et al., 1957). Though, glycerol is known to be glucogenic precise estimates of its actual glucogenicity from quantitative standpoint seem to be lacking. The first work to assess this problem quantitatively came from Cahill et al., (1970) who calculated, on the basis of the gross energy metabolism and respiratory quotient, the mobilization of glycerol in the fasted human. They theorized that this glycerol could account for the formation of approximately 20 g glucose/day for the average man. Studies on the glucogenicity of glycerol in ruminants by Bergman et al., (1968) using a continuous infusion of ^{14}C labelled glycerol, demonstrated that in fasted, ketotic and hypoglycemic sheep, the glycerol turnover or rate of release from adipose tissue was high and as a maximum, could account for nearly 40 per cent of the animal's glucose production. However, on an average pregnant ketotic sheep derived about 28 per cent of the glucose from glycerol. The results also illustrated that about

30 per cent of the glycerol was oxidized to CO and no marked differences seemed to occur during the fed, fasted or hypoglycemic states. Thus glycerol becomes an important glucogenic precursor and nearly replaces propionate only during periods of undernutrition, starvation or other periods of body fat metabolism. Bergman et al., (1968) summarize their findings by stating that in the well fed ruminant the contribution of glycerol to gluconeogenesis is small and probably accounts for less than 5 percent of the total glucose produced.

C. From Lactate and Pyruvate

Lactate and to a lesser degree pyruvate have been attributed with gluconeogenic properties, primarily in monogastric animals such as rats (Exton, 1972), dogs (Issekutz et al., 1972) and humans (Katz and Dunn, 1967). In ruminants however, evaluation of lactate metabolism is difficult due to the variable and unknown amounts which are absorbed from the digestive tract. This situation results because lactate can be produced by rumen fermentation or from propionate metabolism in the rumen wall during absorption (Leng et al., 1967; Wergand et al., 1972).

Lactate is synthesized from the anaerobic metabolism of glucose and is carried to other areas of the body for complete aerobic oxidation. The major route for lactate disposal is the synthesis of glucose in liver and kidneys. Thus the lactate is

utilized for the resynthesis of glucose and constitutes what is known as the Cori cycle (Cori, 1931). The operation of this cycle does not result in a net increase in glucose formation for the body since lactate originates from glucose. It does, however, transfer energy between the liver and other tissues. The energy to drive such a system is ultimately derived from oxidation of fatty acids and other non-gluconogenic compounds.

The studies previously mentioned monogastrics estimated the Cori cycle contributing between 10 and 33 per cent of the total glucose turnover with higher rates occurring during underfeeding or starvation. Considering the problems associated with determining lactate metabolism in ruminants, a number of researchers have suggested that this cycle constitutes no more than 4 to 10 per cent of the glucose turnover in fed sheep (Bergman et al., 1970); Annison et al., 1963).

D. From Amino Acids

Almost all amino acids are glucogenic to varying degrees, with the exception of lysine, leucine and taurine (Krebs, 1964). The majority of amino acids are stored in muscle and along with dietary amino acids, are converted to glucose in both liver and kidney cortex with the liver accounting for approximately 85 per cent of the net glucose production from this source in fed sheep (Bergman et al., 1970; Kaufman and Bergman, 1971). The pathway of conversion of the various amino acids to glucose is

illustrated in Figure 14 (Appendix). When the amino acids are utilized for glucose production by these sites, most of the nitrogen is quickly metabolized to urea and excreted into the urine. In ruminants, however, some of the urea can enter the digestive tract and the nitrogen reincorporated into additional amino acids (Nolan and Leng 1972).

Similar to the previous precursors, gluconeogenesis from amino acids will vary widely depending upon the nutritional and physiological state of the animal. Attempts to estimate the proportion of the total glucose production arising from amino acids have been limited in number and the interpretation of results is associated with considerable difficulty. For example, calculations of the quantity of total amino acids passing through the abomasum of sheep per day have been performed (Clarke et al., 1966; Hogan and Weston, 1967). By assuming that 55 grams of glucose can be synthesized from 100 grams of protein, Leng (1970) has estimated that this could supply up to 70 per cent of the glucose production in nonpregnant sheep. This approach is indirect and probably grossly overestimates the contribution of dietary amino acids to gluconeogenesis since the actual absorption of amino acids into the blood may be considerably less than its disappearance from the rumen due to metabolism by gut tissues. Attempts have also been made to assess the contribution of amino acids to glucose from measurements of urinary nitrogen excretion (Bergman et al., 1966) or urea production rates (Nolan and Leng, 1970). These estimates are also of limited value since considerable amounts of ammonia are absorbed from the rumen to be converted to urea

in the liver and urea itself is recycled through secretions into the digestive tract (Bergman, 1973).

Recently the glucogenicity of amino acids in ruminants has been determined by using ^{14}C -labelled amino acids. A mixture of U- ^{14}C amino acids isolated from chlorella protein was used by Reilly and Ford (1971) to estimate that 28.2 per cent of the total glucose turnover was derived from amino acids in sheep. Their work also stressed the importance of arterial rather than jugular blood sampling. In a previous study in which the jugular vein was used for both infusion of labelled amino acids and collection of blood, these same workers estimated amino acids contributed between 12.8 and 14.7 per cent towards gluconeogenesis. Black et al., (1968) employed single intravenous injections of several different U- ^{14}C -labelled amino acids, into lactating cows and goats, to evaluate their role as glucose precursors. The technique studied the rate and extent of incorporation of ^{14}C from the amino acid into plasma glucose with time. They estimated that 30 to 50 per cent of the glucose turnover may arise from amino acids of which alanine and glutamate each represent 6 to 8 per cent. By infusing a mixture of ^{14}C amino acids derived from algal protein into the jugular vein of cows Hunter and Millson, (1964) reported that 12 per cent of milk lactose was derived from protein in lactating ruminants (Hunter and Millsen, 1964). Techniques such as these, which use a mixture of ^{14}C amino acids, tend to underestimate the true glycogenic contribution of amino acids due to a randomization or crossing over of the label between the tricarboxylic acid cycle and the gluconeogenic pathway (Krebs et

al., 1966). Thus due to these difficulties it becomes necessary to measure the ^{14}C -labelled amino acids separately rather than collectively and, in addition, the measurements should be made on portal rather than on jugular blood. These factors were pointed out by Reilly and Ford (1971) since the glucogenicity of individual amino acids (e.g. alanine) can be confounded by an actual anti-glucogenic effect of another (e.g. leucine).

More recent studies have employed two approaches to assess the contribution of amino acids to glucose in sheep fed alfalfa hay. The first method consisted of measuring the quantities of amino acids added to the plasma by the portal-drained viscera (mainly tissues of the gastrointestinal tract) and also the quantities metabolized by the liver of sheep fed a near maintenance diet (Wolff et al., 1972). This was accomplished by collecting blood samples from catheters, previously implanted in the aorta and the portal and hepatic veins, for measurement of plasma amino acid concentrations. Figure 15 (Appendix) illustrates the placement of catheters for infusion and blood collection. In conjunction with these measurement, the blood flow in the portal and hepatic veins was determined simultaneously by the method of Katz and Bergman (1969). The net metabolism of each amino acid was calculated by multiplying the veno-arterial concentration difference by the flow of plasma. In each case net output or production was assigned a positive value and net uptake or utilization a negative value. The positive values obtained for the portal vein-arterial concentration differences indicate a net output by the portal drained viscera with alanine being the amino acid produced in the greatest

quantities. The mean output of 2.3 mM/hr accounted for 19 per cent of the total net appearance of -amino nitrogen in portal plasma. The net hepatic metabolism of the various amino acids was determined by measuring the hepatic vein-portal concentration differences and in most cases almost all of the gut output was removed by the liver. Glycine, alanine and glutamine accounted for about one-half of the total -amino nitrogen removed by the liver (18.4 mM/hr). When the hepatic uptake exceeded the gut output, in the case for these three amino acids, Wolff and Bergman (1972) postulated the occurrence of a net movement from peripheral tissues to the splanchnic viscera. This concept is further indicated by the negative values for the net metabolism by the total splanchnic viscera (liver plus viscera), which were calculated by the difference in concentration between the hepatic vein and arterial source.

The validity of this study rests on the assumption that the net appearance of amino acids in the portal blood reflects the amino acid pattern of the protein digested in the intestine. The net output of amino acids from gut tissues is the result of both absorption of dietary amino acids by the gastro intestinal tract as well as visceral tissue mobilization. With this in mind other workers (Elwyn, 1970) have proposed that, so long as the quantity of endogenous protein in the gut tissues does not change over the period of time that measurements are made (i.e. steady state conditions), then net gut output will measure mainly absorption of amino acids from dietary protein. Thus, considering the conditions of continuous feeding as used in these experiments, the total protein status of the gut tissues

is assumed to have remained constant so that absorption of endogenous protein would have compensated for amino acid removal from the arterial supply. The results from this study indicated large hepatic uptakes of alanine (3.2 mM/hr) and glutamine (2.1 mM/hr), which are consistent with their proposed role in the transport of nitrogen from peripheral tissues to the liver, where the nitrogen can be converted to urea (Marliss et al., 1971; and Polelsky et al., 1969). In addition the amino acids removed by the liver play an important role in the synthesis of glucose, as will be discussed later. However one of the most significant contributions made by Wolff et al., (1972) is the confirmation that few conclusions on the amino acid nutrition of the ruminant can be obtained from measurements of amino acid concentrations in peripheral plasma (Hogan et al., 1968; Leibholtz, 1969; and Reis and Tunks, 1970).

The second major approach to the study of the glucogenicity of amino acids in ruminants has utilized radioactively labelled ^{14}C -amino acids. This method is expected to yield a minimal estimate for gluconeogenesis in the whole body (liver plus kidneys), whereas the previous technique is a measure of maximal gluconeogenesis from amino acids in the liver.

Subsequently Wolff and Bergman (1972) employed a continuous infusion of each of five glucogenic ^{14}C -labelled amino acids into the vena cava to label the plasma pool and to determine the transfer of carbon from each amino acid to glucose. The ^{14}C data obtained were compared with the maximal glucose synthesis possible from the net hepatic uptake of all plasma amino acids.

Blood was sampled from the aorta, the portal vein and hepatic vein for measurement of blood flow values as well as the plasma amino acid specific activities. From the plateau specific activity levels for both the amino acid and glucose, a percentage conversion was assessed. The overall results of this study show that when sheep are fed a near maintenance ration, they derive at least 11 per cent of their blood glucose from five plasma amino acids (glycine, alanine, serine, asparatate, and glutamate) while net hepatic uptake from the plasma was sufficient to produce a maximum of approximately 30 per cent of the glucose. The former figure is a minimal value for the role of plasma amino acids in whole-body glucose production whereas the latter is the maximal contribution that plasma amino acids could have made to hepatic glucose production. A true value would lie somewhere between these two figures. Of the five amino acids studied, alanine was the most glucogenic, with a conversion rate of 2.45 ± 0.82 mM/hr, and glutamine was the second with a corresponding rate of 1.48 ± 0.40 mM/hr. Together they account for at least 40 per cent of the glucogenicity of all the amino acids.

The finding that alanine and glutamine are the principal amino acids removed by the liver for gluconeogenesis has been confirmed in recent investigations on humans (Felig, 1970; Felig *et al.*, 1970 and Ruderman and Lund, 1972). In addition these two were shown to be the main amino acids released from skeletal muscle. Following these studies, both alanine and glutamine cycles (Figure 16, Appendix) were proposed as an important means of linking amino acid metabolism with the control of

gluconeogenesis. In a recent review article, Felig (1973) examined in detail the metabolism of alanine, particularly as it relates to glucose homeostasis in man. Considering the intimate relationship between the metabolism of alanine and glucose as both precursor and product, the cycle is now described as the "glucose-alanine cycle". Although alanine is the primary amino acid released from peripheral protein stores, a number of points must be considered in order to account for the pattern of amino acid output from muscle in a steady state condition: first, alanine comprises no more than 7-10 per cent of the amino acid residues in skeletal (Kominz et al., 1954) and cardiac muscle proteins (Katz and Carsten, 1963); second, a specific polyalanyl protein has not been identified in muscle; and third, were such a protein in fact present in man, under steady state condition, in small and undetectable amounts, it would not account for the constant alanine output after 5 to 6 weeks of starvation (Felig et al., 1970). Therefore it is apparent that the release of preformed alanine from muscle protein or from the intracellular amino acid pool cannot explain the predominant contribution of this amino acid to the total nitrogen release from muscle. Consequently Felig et al., (1970) suggested a *de novo* synthesis of alanine from the transamination of pyruvate. In support of this conclusion is the finding that there exists a direct linear correlation between circulating concentrations of alanine and pyruvate in man under basal conditions (Felig and Wahren, 1971). In summary, therefore, these studies indicate the existence of a glucose-alanine cycle in which alanine is formed peripherally by transamination of glucose derived pyruvate and is transported to

the liver where its carbon skeleton is reconverted to glucose.

Although attention has been focused on the relation of alanine to glucose homeostasis, the glucose-alanine cycle is of importance in nitrogen metabolism as well. In addition to providing carbon skeletons for gluconeogenesis, the net effect of peripheral synthesis of alanine and its subsequent uptake by the liver is the transfer of amino groups from muscle to hepatic tissues, where they may be disposed of as urea. Carlsten et al., (1967) suggested that alanine provides a nontoxic alternative to ammonia in the transport of nitrogen from peripheral tissues to liver.

The importance of alanine as a glucogenic precursor has been well established in monogastrics as well as ruminants. It is of special interest in the latter case due to their dependence on endogenous glucose synthesis to supply their energy demands.

Techniques Used for Measurement of Alanine and Glucose Metabolism

Throughout the previous discussion the experimental techniques employed in the studies of glucose metabolism and gluconeogenesis have been mentioned briefly. The present section comprises a summary of these various methods with emphasis placed on alanine and its contribution to glucose synthesis. The majority of the research involving metabolic parameters has

centered around glucose. The objective of the present study was to utilize a number of these techniques to measure the metabolic parameters of alanine, as well as assess its contribution to glucose synthesis.

A.) Early Dietary Studies

The first problem in the area of glucose metabolism in ruminants is the absorption of glucose from the gastrointestinal tract. It is well established that negligible amounts of glucose are absorbed under dietary regimes of roughages. However the quantity of glucose absorbed in ruminants fed concentrate or high grain diets is still under investigation. Topps et al., (1968a and b) measured the flow of digesta to the abomasum in sheep and young steers on concentrate diets. To accomplish this the animals were fitted with rumen and abomasal cannulas. Paper impregnated with chromium sesquioxide was administered to the rumen and samples were collected from the abomasum as well as from the feces. By measuring the amounts of dry matter, starch, cellulose, total nitrogen and energy passing through the duodenum and the amounts excreted in the feces, an estimate of the amount of starch escaping fermentation and flow into the small intestines was made. The results demonstrated that under dietary regimes of concentrates very little starch escapes rumen fermentation. By taking jugular vein blood samples they showed that diets had little or no effect on the concentrations of glucose. The major objection to this type of

study is the use of jugular vein blood as an indicator of any glucose fluctuation under the different dietary regimes. As pointed out previously, peripheral blood cannot be used as the sole estimate of fluctuations in blood metabolites (Wolff et al., 1972).

A number of investigators, realizing the inaccuracy of conclusions based on jugular vein blood, conducted similar studies using glucose concentration differences between portal and arterial blood (Schambye, 1951, Roe et al., 1966). The results still showed that no glucose was absorbed into the blood of animals on a diet of hay or one supplemented with 50% grain.

The gluconeogenic effects of amino acids have been estimated using techniques which assess the quantity of total amino acids passing through the abomasum of sheep per day (Hogan and Weston, 1967). These studies are indirect and tend to overestimate the true glucogenic contribution of amino acids in the ruminant. Measurement of urinary nitrogen excretion has been used as an index of protein deamination which, in turn, is utilized as an estimate of the amino acid contribution to glucose synthesis in sheep (Bergman, Roe and Kon, 1966). The results can be misleading because, under some circumstances, urea synthesized as a result of deamination of amino acids may not be excreted in the urine. In sheep that are starved, urea storage in the body pool of urea increases (Packett and Groves, 1965; Cocimano and Leng, 1967) and on low protein-diets some of the urea synthesized may be recycled to the digestive tract and utilized for microbial growth (Schmidt - Nelson et al., 1957).

Nolan and Leng (1970) utilized the rate of entry of urea into the body pool of urea to indicate the upper limit of net amino acid deamination, and from this derived the potential rate of glucose synthesis from deaminated carbon residues. The method consisted of a single injection of radioactively labelled ^{14}C -urea into the jugular vein and the urea entry rate, pool size and urea space were calculated from the decline of specific radioactivity of urea in the blood. The estimates based on the rate of entry of urea are of limited value, and would tend to overestimate due to the considerable amounts of ammonia absorbed from rumen which would be converted to urea in the liver. (Wolff, Bergman and Williams, 1972).

B.) Indicator Dilution Techniques

Indicator dilution is a general term which originally referred to a method of measuring the absorptive ability of a tissue or an organ for a specific nutrient. The indicators presently in use range from simple dyes (Evan's blue) to radioactive isotopes and measure a large number of metabolic parameters such as pool size, pool space, turnover rate, irreversible loss, conversion rate, as well as the standard absorption and production rates.

Meier and Zieler (1954) postulated a working model for indicator dilution techniques which is based on a closed flow system with a single input and a single output. The system

contains a specific volume of fluid which enters and exits at a constant rate of flow. Due to the many branchings and interlacings of blood vessels within the system particles entering at the same instant will require varying amounts of time to reach the single output. The time required is dependent upon the path taken and the velocity with which they travel. Thus there is no single traversal time, but rather a distribution of traversal times. Such a system depends on a number of assumptions which include; (a) the particles entering the system at any time are dispersed when they exit in exactly the same manner as particles entering at any other time; this property is referred to as stationarity of flow; (b) the flow of indicator particles is representative of the flow of total fluid; and (c) recirculation of indicator is not present.

In developing this model, Meier and Zierler, (1954) employed both a single injection as well as a continuous infusion of an indicator. The indicator was administered at the point of entry and its concentration was measured at the output as a function of time. The instantaneous injection technique is characterized by a concentration curve which reaches a maximum quickly and then declines (Figure 17, Appendix). Curve A is the result of a single injection of a dye into the systemic venous circulation. The indicator passes through the pulmonary circulatory tree and the heart and is sampled from a systemic artery. The small rise in the curve at 18 seconds is interpreted to represent the appearance of detectable recirculating dye. In the continuous infusion method, the indicator is injected at a constant rate and its concentration will rise to a plateau level as

illustrated in Figure 17, (Appendix) Curve B. This model proposed by Meier and Zierler is valid on theoretical grounds. One potential weakness lies in the condition that it be closed with a single input and a single output. Such a situation is very difficult to isolate in an intact animal. Therefore for this model to hold further assumptions are necessary, one of which is that the concentration vs time curve is essentially the same in all output branches, and thus the situation reduces to that of many inputs, single output. A second is that the flow from the site of injection becomes mixed in the sense that the fraction of it leaving through any input channel is proportional to the flow in that channel.

The above discussion presents the theoretical considerations behind indicator dilution techniques.

1. Blood Flow Measurements

The net metabolite contribution of an organ or organ system can be estimated by measurements of the venoarterial (V-A) concentration differences, in coordination with the rate of blood with reference to the system of interest. A number of researchers have used this approach to assess the contribution of amino acids to glucose synthesis in ruminants (Wolff and Bergman, 1972 a,b,c; Bergman et al., 1974; and Kaufman and Bergman, 1974).

Quantitative estimation of the absorption of particular end

products of digestion must entail measurement of the portal vein blood flow (Fries and Conner, 1961). Of the indicators used for portal vein blood flow, the method involving para-aminhippuric acid (PAH) as developed by Roe, Bergman and Kon (1966) has a number of advantages. The primary consideration for the use of PAH is that samples can be taken once the plasma PAH has attained a constant concentration after a period of continuous infusion in which the PAH is allowed to equilibrate with the extra cellular fluid. This plateau level is attained since the PAH is rapidly and totally excreted by the kidneys provided that the infusion rate is less than the maximal ability of the kidney to excrete PAH. The two major advantages of this method include; 1) the timing of sample collection is no longer as critical and, 2) a greater sensitivity is achieved due to a greater venoarterial concentration difference. Katz and Bergman (1969) developed a method to measure both portal and hepatic venous blood flows simultaneously by utilizing PAH as the indicator. The calculations for these blood flows shall be dealt with extensively in the experimental section following. The assumptions made are that the PAH should not be excreted or chemically altered in its passage through the portal bed or liver ; and that the infused PAH should be well mixed in the portal vein. Both of these criteria were tested and the results indicated that the assumptions were valid (Katz and Bergman 1969).

In an accompanying paper, Katz and Bergman (1969b) employed this technique to measure the effects of feeding, fasting and pregnancy on the hepatic and portal metabolism of glucose, free

fatty and ketone bodies in sheep. The results indicate the average net hepatic glucose production by fasted nonpregnant and pregnant sheep is 0.13 and 0.19 g/hr kg respectively. Three hours after feeding these values were 0.28 and 0.43 g/hr kg in nonpregnant and pregnant sheep respectively. Thus the mean hepatic glucose production increased nearly 50 per cent 3 hours after feeding which proves that, unlike monogastrics, gluconeogenesis increases after feeding in ruminants.

The figures previously mentioned on the extent of renal gluconeogenesis were arrived at through blood perfusion studies on the kidney using para-aminohippuric acid as the indicator (Kaufman and Bergman, 1971, 1974). The PAH was constantly infused into the posterior vena cava and simultaneous blood samples were taken from the aorta and renal vein to determine renal venoarterial differences for glucose, free fatty acids (FFA), ketone bodies, and PAH. In addition a catheter was inserted into the bladder for continuous urine collection during the period of blood collection. Renal blood flow per minute equals the urinary excretion rate of PAH in milligrams per minute, divided by the difference in blood PAH concentrations between the aorta and the renal vein.

Wolff et al., (1972) investigated the quantity of amino acids added to the plasma by the portal drained viscera and that metabolized by the liver of sheep fed a near-maintenance diet by employing the blood flow technique as described above with PAH as the indicator. The catheters for infusion and blood sampling were surgically implanted into the aorta, caudal vena cava, left

hepatic vein, portal vein and mesenteric vein. The results and conclusions of this study have been discussed in detail previously (page 25).

C) Isotope Dilution Methods

In the field of ruminant metabolism, specifically involving the study of the body pool kinetics of various metabolites, two groups of investigators can be distinguished; those using the single injection method and those employing a continuous infusion of radiocative tracer. Both techniques have been employed to a great extent in measuring the kinetic parameters surrounding glucose metabolism. The application these two techniques have in relation to the assesement of the contribution amino acids to gluconeogenesis, is emphasized in the disscusion section. A list of terms and their definitions is presented in a glossary at the end of this thesis.

1.) Single Injection Technique

The single injection technique involves the rapid intravenous injection of a tracer dose (negligible weight, high specific activity) of a ^{14}C -labelled substrate and the subsequent measurement of the dilution rate of the radioactive material (Cook, 1966). Early work with this method involved measurements of the size and turnover rate of the body glucose pool in the rat (Feller *et al.*, 1950; Baker and Incefy, 1955), the dog (Searle *et al.*, 1954) and man (Baker *et al.*, 1954) indicated that a single injection of ^{14}C glucose gave values for body glucose pool size which seemed to be too high. Steele *et al.*, (1956) discussed the limitations of single injection procedures in studies on glucose pool size and utilization rates in dogs. They concluded that straightforward mathematical treatment of the curve showing the decline in specific activity of plasma glucose after the injection of labelled glucose seriously overestimated glucose utilization rates. In agreement with this conclusion is work by Annison and White (1961) who found large differences in glucose entry rate measured by a single injection, using a single exponential function, and by constant infusion. These large differences are due, in part, to the fact that the analysis of the data from a single injection was oversimplified and lacked a multicompartmental approach (Baker, 1969). Kronfeld and Simesen (1961) investigated glucose kinetics in sheep further by the use of a single injection technique. Considering the problems associated with this technique when applying a single exponential function to the entire decay curve, these workers estimated the glucose entry

rate and pool size by assuming the portion of the curve from 40 to 160 minutes to be linear on a semilogarithmic plot, and obey first order kinetics. Thus they derived a definition of the glucose pool to imply the group of carbon atoms in equilibrium with plasma glucose carbon from 40 to 160 minutes after a single intravenous injection of a tracer of ^{14}C -glucose. The model which follows is that the pool comprised glucose molecules which are situated in the extracellular fluid which are in dynamic equilibrium with glucose molecules entering and leaving this space, to and from the cells. This model proposed by Kronfeld and Simesen (1961) assumes that thorough mixing of the tracer mechanically and by diffusion throughout the space is achieved rapidly, certainly in less than 40 minutes after the injection. In addition re-entry of ^{14}C -glucose, which has undergone metabolic mixing with intermediates, is assumed not to be appreciable before 160 minutes. The turnover rate represents the flow of glucose into and out of this extracellular pool, which Kronfeld and Simesen (1961) regard as an index of the overall rate of glucose metabolism that is, an estimate of the glucose taken up by cells and utilized for both energy and synthesis. An interesting point brought out by these workers is that recycling of ^{14}C from glucose through intermediaries and back into plasma as glucose- ^{14}C would become prominent sooner in rats and dogs than in cows and sheep. This is due to the lower level of gluconkinase and glucose - 6 - phosphatase activities of various tissues of ruminants than in nonruminants. An example is that the major recycling of ^{14}C between plasma glucose and liver glycogen, which markedly affects the plasma glucose level of

nonruminants, is insignificant in sheep which lack hepatic glucokinase activity (Gallagher and Butterg, 1959). Therefore recycling in ruminants would be expected to take longer, and the glucose specific activity re-entering the plasma would be comparatively less than in nonruminants. Wrenshall et al., (1961), have also presented evidence substantiating the use of the single-injection method with extrapolation of a properly chosen time interval to estimate the entry rate of glucose in dogs.

A further advance in the single injection technique was the use of multicompartmental analyses of the decay curve by White et al., (1969). This approach was initiated upon the realization that recycling of ^{14}C is a major process in ruminants. The evidence supporting this includes the catabolism of glucose to lactate and its incorporation within a large pool of potential glucose precursors or entry into the tricarboxylic acid cycle, as well as the fixation of ^{14}CO which is returned to the glucose pool. Therefore the most likely explanation for more than one exponential term in the equation describing the disappearance of radioactivity in plasma glucose after an injection of ^{14}C -glucose is that carbon originally derived from glucose is returned to the sampled pool at two different rates. The first order kinetic approach by Kronfeld and Simesen (1961) was described as an oversimplification and that the whole of the isotope dilution curve should be used in calculating such parameters of glucose metabolism (Rescigno and Segre, 1966). When the method employed by Kronfeld and Simesen (1961) was applied to the single injection results obtained in the study of

White et al., (1969), the estimate of turnover rate was 3 per cent higher and the glucose pool size was 14% higher than that calculated by a multicompartmental approach. Another drawback to this simplified analysis is that this approach would not detect the changes in the isotope dilution curve which occur under varying physiological states. The multicompartmental method, however is designed to interpret the entire curve and thus will detect any of these changes.

The mathematical treatment of the isotope dilution curve will be described in detail in the experimental section following. Briefly, therefore, the curve is described as a sum of exponential terms as follows (White et al., 1969):

$$SR_t = \sum_{i=1}^n A_i e^{-m_i t}$$

where SR_t equals the specific radioactivity of plasma glucose at time (muc/mg.C). A_i equals the zero-time intercept of each component (muc/mg.C), $-m_i$ equals the rate constant of each component (min⁻¹), n equals the number of exponential components, i equals the exponential component number and t equals time (min.). The number of exponential terms is determined by the shape of the observed specific radioactivity time curve and on the basis of a postulated model which is depicted in Figure 18 (Appendix) (Leng 1970). The parameters of the model are arbitrary constants of the functions or equations and can be estimated with the use of computer programs (Baker, 1969).

Most of the work in body pool kinetics has been with infusions either single or continuous of ^{14}C -labelled glucose to measure the parameters of glucose metabolism. The glucogenicity of amino acids has been studied using ^{14}C -labelled amino acids as described previously (Reilly and Ford, 1970 and 1971). However, Black *et al.*, (1968), investigated the role of individual amino acids in glucose synthesis in lactating dairy cows using a single injection technique. A particular advantage in the use of a single injection method for this type of study is that one can assess time relationships among the amino acids as glucose precursors which would not be apparent during a constant infusion. In addition Black *et al.*, (1968) stated that a constant infusion of amino acids is limited due to the much greater inhomogeneity of the amino acid pools. The turnover of amino acids in plasma has been studied previously using this technique in dogs (Elwyn *et al.*, 1968) and in rabbits (Henriques *et al.*, 1955). The latter attempted a multicompartamental analysis of the data and found that three exponential terms were required to fit the specific activity time curve.

Therefore it is with this in mind that a single injection technique combined with a multicompartamental approach was employed to assess, quantitatively, the metabolic parameters of alanine as well as its contribution to glucose synthesis in ruminants under steady state or maintenance conditions.

3.) Continuous Infusion

Under the heading of continuous infusion isotope dilution methods there are two distinct approaches which differ slightly. The first procedure to be developed is the priming dose-constant infusion technique, and the second involves a continuous infusion of the tracer without a priming dose.

The priming dose-constant infusion technique, is based on the assumption that the body pool of a substrate is instantaneously mixed and that a steady state of substrate replacement exists (Cook, 1966). A single injection of the tracer is immediately followed by a constant infusion of the tracer. The ratio of priming dose to infusion rate must be determined such that a relatively constant specific activity of the test substrate is maintained after the initial mixing of the priming dose is complete. With glucose as an example, the rate of change of total ^{14}C in blood glucose with time is the difference between the rate at which ^{14}C enters and leaves the glucose pool the rate at which ^{14}C leaves the pool is dependent on the turnover rate and size of the pool. The specific radioactivity of glucose carbon in plasma at any time (SR) is then predicted by the expression:

$$\text{SR}_t = X - Y e^{-bt} \quad \text{White et al., (1969)}$$

where X equals the asymptotic value for the specific radioactivity of plasma glucose ($\mu\text{C}/\text{mg}$ of C), $X-Y$ equals the intercept specific radioactivity ($\mu\text{C}/\text{mg}$ of C) at $t=0$ and b

equals the rate constant (min^{-1}). The specific activity values between 60 and 180 minutes were used to estimate the values of X , $X-Y$ and b , due to the initial mixing process and due to recycling of ^{14}C after a long time period (Steele et al., 1956). White et al., (1969) determined these values by using a computer program which performed an iterative procedure from the original parameter estimates. Irreversible loss was calculated by dividing the rate of infusion of radioisotope by X . Pool size was obtained by dividing the priming injection (μmC) by the intercept at zero time ($X-Y$) obtained by extrapolation.

This technique has been employed to study the kinetics of glucose metabolism in monogastric animals (Searle et al., 1956; Steele et al., 1956) and in ruminants (Annison and White, 1961; Bergman, 1963) due to the significantly higher glucose entry rates obtained by the single injection technique. However, the multicompartmental analysis of the single injection technique had yet to be applied at this time, consequently the parameter values were high. Bergman and Hogue (1967) utilized a priming dose constant infusion technique to measure the glucose turnover and oxidation rates in sheep during various stages of lactation.

The primed infusion technique, which was evolved by Steele et al., (1956), is based on evidence that the glucose pool of the dog is distributed in two compartments in the extracellular fluid. No account is taken of recycling between these two compartments and other substrate pools. The single injection results obtained by White et al., (1969) indicate that, in sheep, glucose in both plasma and interstitial fluid constitutes

a single entity and that recycling of glucose carbon from the peripheral pool results in a multtexponential curve. Consequently the use of a primed infusion to calculate the pool size of any metabolite, glucose or alanine appears to be unsound on theoretical grounds. (Leng, 1970)

The continuous infusion technique without a priming dose relies on a simple mathematical treatment (Leng, 1970). In studies conducted to measure parameters of glucose metabolism in sheep (Leng et al., 1967 and White et al., 1969) the specific radioactivity of the infused glucose was found to reach a plateau after 180 - 240 minutes. An estimate of the irreversible loss is determined by simply dividing the infusion rate of radioisotope ($\mu\text{C}/\text{mg}$ of C) by the plateau specific radioactivity ($\mu\text{C}/\text{mg}$ of C).

Previous work dealing with amino acid turnover in sheep has involved a constant infusion of a mixture of ^{14}C - labelled amino acids (Reilly and Ford, 1971). The major difficulty with this approach is that the radioactivity of individual amino acids will vary between themselves and different blood vessels of the body. Thus data obtained from the infusion of a mixture of ^{14}C -labelled amino acids must be interpreted with extreme caution. The use of a continuous infusion of individual amino acids was employed by Wolff and Bergman (1972) to evaluate the interconversions and metabolism of plasma amino acids by the portal-drained viscera, liver, and peripheral tissues of fed sheep. Each experiment involved the continuous intravenous infusion of a ^{14}C -labelled amino acid, and sampling of blood

from the aorta, the portal vein and hepatic vein. Simultaneous equations were described to assess the rates of interconversions in the separate groups of tissues. Subsequently these authors (Wolff and Bergman, 1972b) described the use of this technique to assess the contributions of various plasma amino acids to glucose. As described previously, the results from this study indicated that of the five amino acids examined, alanine was quantitatively the most important for blood glucose synthesis.

Recycling of label has been suggested to be the major problem in the interpretation of isotope experiments, whether these be single injection or continuous infusion (Bergman, Katz and Kaufman, 1970, Bergman *et al.*, 1965, Gurdide *et al.*, 1963, and Lindsay, 1970). During a six hour continuous infusion, as was used in the study by Wolff and Bergman, (1972) the label could have recycled through 1) the intracellular pools of the amino acid, 2) synthesis and catabolism of proteins, and 3) metabolically related compounds such as other amino acids, organic acids and glucose. Wolff and Bergman (1972) ignore recycling through intracellular pools since the utilization rates (entry of turnover rate) assess the metabolic processes which led to an irreversible loss of label from the plasma pool, while production rates determine the rate at which the pool was replenished from unlabelled sources. Recycling of label through proteins is probably small since most enzymes (Schmike and Doyle, 1970), all plasma proteins, (McFarlane, 1964) and most structural proteins have half-lives greater than six hours. Amino acid interconversions are expected to be important as an avenue for recycling. However labelled alanine was found to have

little activity in any other amino acid (Wolff and Bergman, 1972a). Thus it would appear that experiments involving the use of ^{14}C -labelled alanine are not as affected by recycling as other amino acids. However, the recent discovery of alanine and glutamine 'cycles', which are important for the transport of nitrogen from peripheral tissue to the liver, (Felig et al., 1970, Marlins et al., 1971) may prove to be definite contributors to the recycling of these labels, although quantitative measurements have not yet been determined.

EXPERIMENTAL

I NET METABOLISM OF GLUCOSE AND ALANINE BY THE PORTAL-DRAINED VISCERA

Introduction

The measurement of portal vein blood flow and arterio-venous concentration differences of specific metabolites has gained importance in the quantitative estimation of the utilization of end products of digestion by animals (Fries and Conner, 1960). By an analysis of the portal blood the amount of material absorbed into the veins draining the gastrointestinal tract as well as the degree of metabolism occurring in the liver may be determined. In order to accomplish both these estimates, Katz and Bergman (1969a) proposed a method by which the hepatic and the portal venous blood flows are measured simultaneously. Consequently, such a technique enabled researchers to distinguish between the production (absorption) and utilization of individual components by the liver from that by the portal bed under varying physiologic conditions. Utilizing this approach, Katz and Bergman (1969b) studied the hepatic and portal metabolism of glucose in both fed and fasted, pregnant and nonpregnant sheep. Work by Bergman et al., (1970) using this method demonstrated that the portal bed of sheep utilizes significant amounts of glucose, regardless of whether they are fed, starved or hypoglycemic. Thus the portal-drained viscera

(gastrointestinal tract, pancreas, and spleen) are more important in terms of glucose utilization than was previously recognized. However this discovery was only possible once distinction could be made between portal utilization and hepatic production, because in terms of total splanchnic metabolism (hepatic plus portal) values show a net production of glucose.

Alanine was investigated in a similar manner by Wolff et al., (1972) to determine the quantity added to the plasma by the portal-drained viscera and that metabolized by the liver of sheep fed a near-maintenance ration. As a measurement of the net gut output, alanine was absorbed into the portal blood to a greater degree than the other amino acids tested. The liver removed large quantities of alanine. In fact the hepatic uptake exceeded the gut output, which was interpreted to indicate a net movement of alanine from peripheral tissues to the splanchnic viscera (liver plus portal bed).

Considering these findings, the objective of the present experiment was to evaluate the use of this technique and study its relative merit in the determination of various parameters pertaining to glucose and alanine in ruminants.

Experimental A.1.

Materials and Methods

1.) Surgical Procedures

A 2 year old wether weighing 42 kg was employed in this experiment. Feed was withheld from the animal for 72 hours prior to surgery. Thirty minutes before the anesthetic was administered, the sheep was given a subcutaneous injection of atropine sulfate (5 ml-dose of 0.6 mg/ml) to decrease the salivary secretions. The ventral abdominal wall was closely shaved and washed with soap and water. The sheep was anesthetized with a 1% pentathol sodium injection into the jugular vein. During the slow administration of the drug the eye reflex as well as the tension of the jaw muscles were frequently tested. Following infusion of 15-18 ml of the pentathol sodium the animal was transferred to a prone position on the operating table. An endotracheal catheter was introduced which was connected to a closed circuit gas anaesthetic machine. The apparatus delivered a controllable amount of the gas halothane mixed with oxygen. Therefore the respiration rate could be monitored and the state of anesthesia controlled carefully.

For portal and mesenteric vein as well as carotid artery cannulation, the sheep was placed in left lateral recumbency. The surgical area was further clipped, washed, and disinfected with weak tincture of iodine. An incision 25 to 30 cm long was

made approximately 3 to 5 cm behind and parallel to the last rib on the right side of the sheep, which penetrated through skin, fascia, and rectus abdominis muscle and into the peritoneal cavity. The peritoneum was secured firmly with hemostats.

The left lobe of the liver was located and moved cephalad (towards head) exposing the portal vein. The duodenum and abomasum were identified by following these structures in an anterior direction, the omasum was found and brought toward the incision site. The omasal vein was readily accessible for cannulation. Silastic tubing (.30 in I.D. x .065 in O.D.) inserted with fine piano wire, was administered into the vein through a 13 gauge needle. While palpating the portal vein, the tubing was carefully manipulated along the omasal vein until it was felt in the portal trunk. The wire was slowly removed and the catheter was secured by silk sutures at the point of entry. The cannula was further anchored by additional sutures to the neighbouring fascia and exteriorized at the dorsal extremity of the incision. A 4-way plastic stopcock (Travenol Laboratory Deerfield, Ill. 60015) was fixed to the end of the catheter and sutured onto the back of the animal.

A second cannulation was performed on a reticular vein using the same material and techniques as described above. It was exteriorized in the same position and secured to the back of the animal in a similar manner. This catheter was intended for infusion purposes only.

In order to implant the carotid artery cannula a 5-6 cm incision was made along the mid-line of the trachea. The artery

on the side of the neck was located and isolated from the surrounding fascia. The polyethylene catheter (P.E.90) was introduced via a 13 gauge needle and secured in place by means of a purse string suture through the tunica adventitia. The cannula was exteriorized and fixed with a 4-way stopcock which was sutured to the back of the sheep's neck.

The abdominal incision was closed in three parts; the peritoneum, the muscle layer and the skin. The latter employed a heavy grade silk suture, whereas the former two used cat gut.

By the time the incisions were being sutured the animal was breathing straight oxygen from the halothane machine. To aid the recovery process and prevent infection, an intravenous infusion of 20 mls of 20% sterile glucose preparation was administered followed by an intramuscular injection of 5 ml of Derafort, ¹ a penicillin - streptomycin preparation. The Derafort injections continued for 3 days following the operation. The Endotracheal tube was removed once the chewing reflex of the sheep became strong. This procedure reduces the chance of the animal aspirating rumen contents. When the sheep began showing signs of voluntary motion, it was carefully transferred to a padded pen and kept under close observation. The animal was able to regain equilibrium within 4 hours and returned to normal within 24 hours of the operation.

During the five day recovery period between the operation

1. Derafort, Ayerst, Montreal.

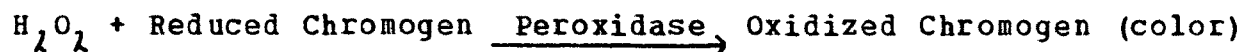
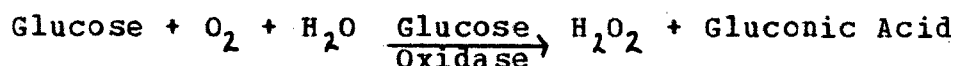
and the blood flow experiment, sterile heparinized saline (100 units/ml) was flushed through each cannula twice daily to ensure their patency.

2.) Nutritional Regime

The animal was placed on a maintenance diet of 1 kg dehydrated grass pellets per day. The pellets were fed to the animal in 200 gm portions every two hours starting at 0900 hours and finishing at 1700 hours. This regime was maintained for four days prior to the experiment day. The infusion began at 1000 hours. The animal was not fed, thus the results are based upon a 17 hour fast (overnight).

3.) Glucose Determination

The glucose concentration was determined by the standard colorimetric glucose reagent, glucostat (Worthington Biochemicals). This mechanism (Washko and Rice, 1961) makes use of the coupled enzyme reactions:



For this, as well as the other concentration assays,

deproteinized plasma was employed. The whole blood plasma was first centrifuged and the resulting supernatant was deproteinized by the use of 10% ZnSO_4 and .5N NaOH in the following proportions:

1 ml plasma or serum + 8 ml distilled H_2O
+ 0.5 ml 10% ZnSO_4
+ 0.5 ml 0.5N NaOH

The ZnSO_4 and NaOH were titrated together in order to ensure neutrality. After thoroughly mixing the above preparation and waiting 10 minutes, each sample was centrifuged again and the clear supernatant was employed for the subsequent assays.

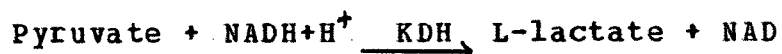
One ml of the deproteinized plasma was added to 4 ml of glucostat preparation and the reaction proceeded for exactly 20 minutes at which time it was stopped by adding 2 drops of 4N HCl. The optical density of each sample reaction was read on a Spectronic 20 spectrophotometer at a wavelength of 420 nm. These readings were converted to mg/100 ml glucose by comparison with standard curve (Appendix, Figure 1) prepared from known glucose concentrations.

4.) Alanine Determination

The concentration of alanine was determined by an enzymatic technique described by Bergmeyer (1965) in which alanine is converted to pyruvate by glutamate pyruvate transaminase (GPT) and oxoglutarate:



This reaction is coupled to a second in which lactic dehydrogenase (LDH) reduces pyruvate in the presence of NADH to lactic acid:



The disappearance of NADH was followed on a UNICAM SP 800 spectrophotometer at a wavelength of 340 nm. Since there is an excess of both enzymes, oxoglutarate and NADH, the rate of the coupled reaction with limited alanine concentrations is strictly proportional to the amount of alanine present. The measurement of the reaction rate permits the determination of alanine within each sample by use of a standard curve prepared with known alanine concentration (Appendix, Figure 2).

5.) Blood Flow Determination

The portal vein blood flow was measured with the use of the indicator para- aminohippuric acid (PAH). A priming dose (15 ml) of a 1.5% PAH solution was administered into the cannulated reticular vein at the beginning of the experiment. This was immediately followed by a continuous infusion of the same PAH solution (1.5%) into the reticular vein by means of a polystatic infusion pump (Buchler Instruments) at a rate of 0.79 ml/min.

After a one hour equilibration period, heparinized blood samples were withdrawn from the portal vein, and carotid artery at 15 minute intervals for one hour to determine the alanine, glucose and PAH concentrations. The plasma levels of PAH were assessed by the method of Smith et al., (1945). The optical densities (O. D.) of the samples were measured on a Spec 20 spectrophotometer at a wavelength of 540 nm. The corresponding PAH concentrations were determined by comparison with a standard curve of known PAH concentrations (Appendix, Figure 3).

Calculations

The portal vein plasma flow (PVPF) and the portal vein blood flow (PVBF) were calculated according to the following equations; (Katz and Bergman, 1969a)

$$PVPF = \frac{CI \times IR}{C_{PV} - C_A}$$

$$PVBF = \frac{PVPF}{(1-PCV)}$$

Where:

CI= concentration of PAH in the infusion solution (mg/100 ml)

IR= infusion rate (mls/min)

PCV= the blood packed cell volume (hematocrit)

C_{PV} and C_A = Plasma PAH concentration in portal vein and arterial blood respectively.

The net portal production rates of both glucose and alanine were determined as follows: (Katz and Bergman 1969b)

$$P = F_{PV} (C_{PV} - C_A)$$

where P represents the portal net production rates of the metabolite; F_{PV} is the whole blood flow (ml/min) in the portal vein; and C_{PV} and C_A are the concentrations of the metabolite in the portal vein and arterial vessels respectively. A negative value for the production rates of either alanine or glucose indicates utilization.

The results were statistically analyzed using the student T test to denote significant differences for both alanine and glucose concentrations between the carotid artery and portal vein plasma. Values preceded by a sign indicate the standard error of the mean.

Results and Discussion

A summary of the mean blood concentrations of glucose, alanine and PAH that were obtained by sampling from the portal vein and carotid artery, as well as the corresponding packed cell volume figures are presented in Table 1(a) (Appendix).

1.) Portal Blood Flow

To calculate the portal vein blood flow (PVBf) and the portal vein plasma flow (PVPF), the PAH was allowed to equilibrate with extracellular fluids and thus the concentration figures employed are those at the plateau level (Appendix, Table 1(a)). In this experiment the plateau or constant PAH concentrations for both the carotid artery and portal vein were attained between the 1 hour 25 minute and 1 hour 45 minute blood collections. Thus the average of these two concentration values were used for the determination of the blood flow figures. A summary of the calculations and results is presented in Appendix, Table 1(b).

A portal vein blood flow value of 2010 mls/min (47.85 ml/min/kg/B.W.^{3/4}) was determined in the present experiment. The result agrees with previously published results which estimate the mean portal vein blood flow in a range from 1800 ml/min (Bergman and Wolff 1971) to 2493 ml/min (Katz and Bergman, 1969a) for fed, nonpregnant sheep. The latter study employed a technique which enabled a measurement of both the

hepatic and portal blood flows. Such a result was not possible in the present experiment since catheters were implanted into the portal vein and carotid artery only, and not into the hepatic vein. The value for portal vein blood flow in the present study appears to fall well within range of established results. Additional support is provided by more recent work by Wolff, Bergman and Williams (1972) who quote portal blood flow values from 1440 to 2240 ml/min in sheep fed a near-maintenance diet. These results are more relevant to the present experiment since the dietary regime as well as the physiological status of the sheep (nonpregnant and nonlactating) are similar.

2.) Net Glucose Metabolism

The negative production value ($-.142$ g/hr/kg B.W) indicates a net utilization of glucose by the portal drained viscera. This finding is in agreement with published work which showed that a net utilization of glucose in the portal bed was almost always obtained regardless of the diet or whether the sheep were hypoglycemic or fasted (Katz and Bergman, 1969b; Bergman *et al.*, 1971). The figure obtained in the present study is slightly larger than the mean portal bed utilization rate of fed sheep and smaller than that in sheep fasted for three days. This appears appropriate since the value of $.142$ g/hr/kg B.W.^{3/4} was determined with a sheep fasted for 17 hours. The net utilization of glucose, as shown here as well as in previous works, is slight. The actual portal-arterial glucose

concentration differences calculated in this experiment were not statistically significant ($P < 0.05$ level) which supports others who have observed the same (Roe et al ., 1966; Schambye, 1951).

3.) Net Alanine Metabolism

The figure for alanine metabolism by the portal bed demonstrated a net production (absorption by the portal blood) of 1.49 mM/hr. The published values indicate a mean portal bed production rate of $2.29 \pm .29$ mM/hr (Wolff Bergman and Williams, 1972), which was determined on ten separate experiments whereas the figure obtained in the present study was based upon a single experiment. Considering this fact, the single value of 1.49 mM/hr arrived at here cannot be regarded as an absolute estimate but undoubtedly is within the range reported by Wolff et al., (1972). It is important to note, however, that the alanine concentrations between the carotid artery and the portal vein proved to be significantly different in the present experiment.

Conclusions

The results from the present determinations of portal vein blood flow, as well as the net metabolism of alanine and glucose by the portal drained viscera aid more in evaluation of the

effectiveness of this method rather than as absolute estimates which would be representative of all sheep. The purpose of this experiment was to assess the value and application of the blood flow technique using para-aminohippuric acid as the indicator. Therefore the results for portal vein blood flow (2010 ml/min), net glucose utilization by the portal bed (.142 g/hr/kg B.W.), and net alanine production by the portal bed (1.49 mM/hr) are within the range of previously published figures and consequently contribute additional support for the use of this approach in metabolic studies.

The sophisticated surgical procedures required by this method are the limiting factor upon which the success of the study is based. Thus in order to state accurately that the arterio-venous concentration differences represent a specific organ or tissue system, the catheters must be implanted precisely. This process requires a great deal of skill. Often the catheters become dislodged or blocked and consequently the entire experiment is destroyed. The chemical analyses as well as the calculations are straight forward and require a minimum of interpretation.

The technique of blood flow measurement along with arterio-venous concentration differences proved to be an effective approach in determining, quantitatively, the net metabolism of specific compounds by individual organs or tissues. This is emphasized through the demonstration by Katz and Bergman (1969b) of a method whereby the net metabolism of the total splanchnic region is divided into that of the liver and portal-drained

viscera. Although highly demanding, their technique was instrumental in recognizing that there is a net utilization of glucose by the portal bed. The results of the present study confirmed their finding that no net glucose absorption occurs in sheep fed a maintenance diet, but a portal glucose utilization appears to be present at all times.

Although the alanine data for net portal output did not agree as closely to published results as that for glucose, the value of 1.49 mM/hr/kg B.W. is still of the magnitude which substantiates its established role as the primary amino acid absorbed by the portal circulation and delivered to the liver.

From the data obtained in this experiment it was not possible to determine the liver's role in the metabolism of these two metabolites due to the difficulty in cannulating the hepatic vein. This was never accomplished successfully, thus the results are based on the portal bed and its relation to alanine and glucose.

In summary therefore, the results obtained from this study indicated that the technique employed is effective and accurate to be applied "in vivo" metabolic studies. The major advantage to this method is that once the catheters have been properly implanted, an organ or tissue system can be completely isolated and consequently the results obtained are directly attributable to the functioning of that system. The primary limitation to this technique is the complex surgical skills required around which the entire study revolves.

II Single Injection of Labelled ^{14}C -Alanine

Introduction

The single injection technique of a labelled tracer is regaining acceptance as an effective and accurate procedure in measuring body pool kinetics for various metabolites. The majority of the work done has involved labelled ^{14}C - glucose to study glucose metabolism. Numerous models have been developed to describe the movement of glucose carbon in the body, with a 2 or 3 compartmental model as the most widely established to fit ^{14}C -glucose isotope dilution data in rats (Baker et al., 1959; Steele, 1964) and in sheep (White et al., 1969; Skinner et al., 1959; Rescigne and Segre, 1966). The fitting of data to such models has involved a number of difficulties which, in the early stages, had persuaded many workers to doubt the validity of this approach. Annison and White (1961) stated that the parameters governing the entry and outflow of glucose from the proposed multi-compartment system cannot be adequately defined for a mathematical treatment of single-injection data. The difficulties involved with defining components suggest the use of curve-fitting computer techniques in which the individual component slopes within a complex curve need not be clearly defined (Berman, 1963). This approach has been utilized by a number of investigators in defining parameters of glucose metabolism in monogastrics (Baker et al., 1959; Segal et al., 1961). Work with ruminants using a single injection technique combined with a multicompartmental analysis has been sparse and the results variable (White et al., 1969; Leng, 1970). The

latter author attributes the variability to the fact that insufficient blood samples were taken over too short a time period in many experiments for accurate analysis of the data.

The multicompartamental approach to the analysis of single injection data has been used to a limited degree in ruminants with the major emphasis upon glucose metabolism studies. Thus the field of amino acid research, specifically their metabolic parameters and contribution to glucose synthesis, has been sparsely touched by the use of this technique (Black, 1968; Egan and Black, 1968). It is only recently that work on the determination of such parameters as turnover rates of individual amino acids as well as their relation to gluconeogenesis has been conducted (Wolff, Bergman and Williams, 1972, Wolff and Bergman, 1972a and b). The methods employed by these researchers involve blood flow studies, as described in Experiment A, and continuous infusion of labelled amino acids, which will be discussed in the following section. In one study reported, a compartmental analysis was attempted on the data of a single injection of glycine and the existence of at least three pools for the metabolism of glycine revealed (Henriques et al., 1955).

The objective of the following experiments was to assess the value of the single injection of ^{14}C -labelled alanine along with a multicompartamental analysis of the data as a means of quantitatively estimating the metabolic parameters of this amino acid as well as its contribution to glucose. Incorporated within this assessment are the relative advantages and disadvantages of this technique. Such factors as physiological assumptions, ease

of application and calculation of results as well as time and expense are included in evaluating the effectiveness of this method.

The study consists of three separate experiments, each comprising of a single injection of ^{14}C -alanine into the jugular vein. Blood samples for analysis were collected from the jugular vein, except in the third experiment where carotid arterial blood was also withdrawn. The analysis for all three comprised inspection of the fall in the specific activity of plasma alanine with time.

The parameters for alanine metabolism were determined from a multiexponential function applied to the data to generate a line of best fit for the decay curve. This procedure involved the use of a computer. The details surrounding the analysis shall be discussed in the following materials and methods section.

Experiment B.1.

Materials and Methods

A three year old wether weighing 35 kg was used for this experiment. The surgical preparation consisted of inserting a polyvinyl catheter (P.E. 90) into the right jugular vein 24 hours prior to the start of the experiment. The catheter was maintained operative by frequent flushings with a solution of heparinized saline (100 units/ml).

The nutritional regime consisted of a roughage diet of 1 kg

alfalfa hay fed twice daily at 0800 hours and 1600 hours. On the day of the experiment the animal was given its morning feed and the injection began 4 hours later at 1200 hours. Thus the sheep was in a fed (4 hr fast) condition. The experiment was performed with the animal in a raised metabolism cage which enabled the collection of urine and feces for the proper waste disposal. The building was well ventilated thus adding a further control measure to the use of isotopes in living animals

1.) Isotope and Blood Collection

L-Alanine U-L- ^{14}C was obtained from ICN Chemical and Radioisotope division with the following specifications; specific activity 100-120 mC/mm, packaged in 0.01N HCl at a concentration of 0.1 mC/ml.

A single injection of .05 ml (50 uCi) of the isotope was administered into the jugular vein and 10 ml of blood plasma was collected from the same jugular vein at the following time intervals:

Sample No.	Collection Time
1	Control (before infusion)
2	5 min
3	10 min
4	20 min
5	30 min
6	52 min
7	60 min
8	120 min
9	180 min
10	1110 min (0830 hours following day)

The time required for collection of plasma was held constant at between 80-100 seconds for each sample.

2.) Chemical Methods

a) Separation of Plasma Components

The separation of the plasma constituents was accomplished by ion exchange chromatography. The adsorption and retention of the charged molecules by the resin, either positive (cation exchange column) or negative (anion exchange column), permit the separation of the plasma constituents into a basic fraction (positively charged amino acids), an acidic fraction (negatively charged organic acids) and a neutral fraction (sugars).

i) Cation Exchange Resin

The cation resin (DOWEX 50W-X8) employed was obtained from the Bio-Rad Laboratories. It was in the hydrogen form (200-400 mesh) with a total capacity of 5.1 meq/dry gram or 1.7 meq/ml wet resin. Approximately 2 dry grams of resin were used for separation. To ensure the resin was in the hydrogen form 40 ml 2N HCl was passed through the column followed by a wash with deionized water until the eluent is neutral. Three ml of the deproteinized plasma sample were applied to the top of the column and allowed to stand for 15 minutes before being passed through. The sample was run slowly through the column and all non-adhering compounds (sugars and acids) were washed through with 50-60 ml water. The basic substances (amino acids) which are adsorbed to the resin were eluted by passing 60-70 ml 2N HCl through the column. The amino acids

retain their charge and were eluted by the greater concentration of hydrogen. The resin was discarded and fresh resin was regenerated for the next sample.

ii) Anion Exchange Resin

The anion resin was obtained from J. T. Baker Chemical Company and was in the chloride form (200-400 mesh). It is a strong basic resin with a total exchange capacity of 4.4 meg per dry gram. Approximately 2.5 dry grams of the resin was utilized for each plasma sample. The resin was converted to the formate form by passing through 60 ml of 1M sodium formate. At this stage the chloride test was negative when tested with AgNO_3 . Following the sodium formate, 20-30 ml of 0.1N formic acid was added and the column was washed with distilled water until neutral. The sample from the cation column was administered and all non-adhering substances (sugars) were washed through the column with water. The anions (acids) were eluted by passing 40 ml of 4N formic acid followed by 30 ml of 8N formic acid through the column. Once again the resin was discarded with a fresh quantity regenerated for each subsequent sample.

Once separated, each fraction was evaporated to dryness with the use of a Buchi Rotovapor. These samples as well as the original undiluted plasma samples were counted using an ISOCAP/300 liquid scintillation counter (Nuclear Chicago). The counting efficiency was detected by using the external standards ratio (ESR). Figure 4 (Appendix) is the quench

correction curve using the ESR method in conjunction with the quenched ^{14}C standards provided by Nuclear Chicago. It is from this Figure that the counting efficiency for each sample was detected. In all of the counting throughout this project, Phase Combining System (PCS) solubilizer was used as the fluor solution. The standard mixture was 200 μl of sample in combination with 10 ml PCS.

In order to evaluate the efficiency of the ion exchange chromatography in separating plasma constituents, a preliminary run was undertaken using a prepared mixture of the following:

0.01 ml L-Alanine- ^{14}C

1 ml plasma

0.1 mg lactate

0.1 mg lactate

This solution was passed successively through the cation and anion exchange column as described above and the fractions counted yielding the figures listed in Table 4 (Appendix). These recovery figures indicate that ion exchange chromatography can be used effectively for the separation of plasma into acidic, basic and neutral fractions.

To confirm further that the radioactivity corresponded to the metabolites under investigation, the basic and neutral fractions were spotted (50 μl) on a paper chromatogram. A two directional paper chromatography was used for the separation

of alanine in which the solvent system for the first direction consisted of n-butanol, glacial acetic acid and water in a ratio of 80:20:20 (v/v) respectively. For the second direction liquified phenol:H₂O was used in the proportions 94:28 (v/v) respectively. Single directional paper chromatography was employed to separate the sugar fraction. The solvent system consisted of iso-propanol:n-butanol:water in a 7:1:2 (v/v) ratio. The amino acid and sugar chromatograms were compared to standards consisting of L-Alanine UL-¹⁴C and glucose-UL-¹⁴C respectively. The standards and the sample chromatograms were cut into identical strips 3 cm wide. The strips were put through a radio-chromatography scanner (Neuclear Chicago, Actigraph II). By comparing the peaks of radioactivity of the sample to that of the standard, an accurate evaluation can be made regarding the location of activity. Consequently this procedure provides the information as to whether the radioactivity detected in the basic and neutral fractions is due to alanine and glucose respectively. The results of this technique are illustrated in Figure 5 (Appendix) for alanine. The activity of glucose was not strong enough for the use of the Actigraph scanner, which has an efficiency maximum of approximately 20 per cent. Therefore a slight variation of the above technique was developed utilizing the liquid scintillation counter (ISOCAP 300). This method consists of cutting the above strips into smaller sections and placing them into vials immersed in the liquid scintillation fluid (PCS). The individual vials were counted and by comparison to

the standard U- 14 C-Glucose chromatogram strip, the peaks of radioactivity were located. Table 3 (Appendix) illustrates the results for a neutral fraction sample and a standard.

3.) Blood Analysis

Whole blood samples were collected in heparinized test tubes and centrifuged on a Sorvall RC 2-B automatic refrigerated centrifuge. The resulting supernatant was deproteinized with 10 per cent ZnSO₄ and 0.5N NaOH in a similar manner described previously. Glucose and alanine assays were performed on the deproteinized supernatant by methods outlined in the preceding experiment.

Calculations

Specific activity decay curves were fitted with a function which was the sum of exponential terms having the form:

$$SA = \sum_{i=1}^n A_i e^{-m_i t} \quad (\text{Leng, 1970})$$

where:

SA = specific activity of plasma glucose at time t (nC/mg C)

A_i = zero-time intercept of each component (nC/mg C)

$-m_i$ = rate constant of each component (min⁻¹)

n= number of exponential components

i= exponential-component number

t= time (min)

Fitting was accomplished by iterative minimization of the error sum of squares for the fitted function. In practice, a Fortran IV subprogram written by R. Fletcher (1972) was used. The number of exponential terms is determined by the shape of the observed specific radioactivity decay curve. On the present experiment a two term function was fitted to the curve.

Pool size and metabolic turnover estimates were calculated from the parameters of the least squares fitted functions using the formulae of Leng (1970):

$$\text{Pool size, } Q \text{ (mg C)} = \frac{P}{\sum_{i=1}^n (A_i)}$$

where P is the injected dose of radioactivity.

$$\text{Irreversible Loss (mg C/min)} = \frac{Q}{\sum_{i=1}^n \frac{A'_i}{m_i}}$$

$$\text{Total entry rate (mg of C/min)} = Q \sum A'_i m_i$$

where A'_i are fractional zero-time intercepts; therefore

space is defined as;

$$\text{Pool Size, } Q \text{ (mg)} \times \frac{100}{\text{plasma Alanine concentration (mg/l)}} \times \text{Body Weight (kg)}$$

Recycling rate is defined as total entry rate minus irreversible loss. The percent conversion of ^{14}C -Alanine to glucose was estimated by the method of Kreisburg *et al.*, (1972) by using the equation;

$$\% \text{glucose from Alanine} = \frac{G \times SA \times G^S}{I} \times 100 \quad \text{where}$$

G = glucose concentration (umoles/ml)

SA = specific radioactivity of glucose (dpm/umole)

G^S = the glucose space (ml)

I = injected dosage of $\text{U-}^{14}\text{C}$ -alanine (dpm)

The value of alanine recovered in glucose is determined from the maximum glucose specific activity achieved during the 3 hour time interval following the infusion of ^{14}C -alanine. For the purpose of this calculation, the glucose space was assumed to be equivalent to $0.3 \times \text{body weight in kg}$ (Monugian *et al.*, 1964).

Results and Discussion

Before proceeding into the specific values for the parameter estimates, it was confirmed by paper chromatography that the radioactivity in the basic and neutral fractions was

due to alanine and glucose respectively. From the close proximity of the peak of radioactivities for the alanine and glucose samples as compared to the standards of each, (Figure 5 Table 3, Appendix) it is obvious that the radioactivity in the basic and neutral fractions is a result of labelled ^{14}C -alanine and ^{14}C -glucose respectively.

In addition to the raw data, Table 4 (Appendix) shows individual values for pool size, space, total entry rate, irreversible loss and recycling of alanine in sheep estimated from the change of specific radioactivity of plasma alanine after an injection of 50 μCi of $\text{U-}^{14}\text{C}$ -alanine. Figure 6 (Appendix) illustrates the curve for ^{14}C -glucose activity. The line of best fit for the ^{14}C -alanine data is pictured in Figure 7 (Appendix). It is from the latter that the calculations for the metabolic parameters are made. The per cent of injected ^{14}C -alanine appearing in glucose is also included in Table 6 (Appendix).

The metabolic parameters of alanine (Table 6, Appendix) show a total entry rate of 8.61 mg C/min (5.80 mM/hr), an irreversible loss of 4.96 mg C/min (3.34 mM/hr), a recycling rate of 3.65 mg C/min (2.46 mM/hr) and a conversion percentage to glucose of 3.57%. Wolff and Bergman (1972a) quote a total plasma alanine turnover rate of between 8.3 and 11.3 mM/hour. The technique used to measure these latter figures was a continuous infusion of $\text{L-U-}^{14}\text{C}$ alanine without a priming injection. The total entry rate and the irreversible loss are lower than the values reported by Wolff and Bergman (1972a).

It should be emphasized that the term total turnover rate, as used by Wolff and Bergman, has also been given different titles by various workers, such as, transfer rate (Kronfeld and Simesen, 1961), utilization rate (Annison and White, 1961) and entry rate (Leng et al., 1967). All of these terms imply the same concept, namely the rate of entry of all alanine carbon into the sampled compartment. As pointed out by White et al., (1969), this individual parameter, when measured by a primed or continuous infusion represents the irreversible loss concept as defined by the single injection technique. Irreversible loss does not include alanine carbon which has recycled between the sampled compartment and any peripheral compartment. The difference between total entry rate and irreversible loss is regarded as the rate of recycling of alanine between the sampled and peripheral compartments. Based on this concept the difference between the irreversible loss determined by the present experiment and published values of entry or turnover rate for alanine would appear to be even greater.

The per cent conversion of alanine to glucose of 3.57% is, again, lower than the relatively established values of between 6 and 8% (Black et al., 1968). Wolff and Bergman (1972b) have estimated that of the existing glucose 5.4% comes from alanine by the use of the continuous infusion approach, which has been said to account for the assumptions and inaccuracies of the single injection method. Kreisburg et al., (1972) estimated a per cent transfer of alanine to glucose of 12.5%. These workers along with others (Annison and White, 1961) suggest that the

problems of rapid metabolism of the labelled metabolite as well as recycling yield overestimates of metabolism values arrived at by the single injection technique. Thus they recommend the use of a continuous infusion which eliminates these sources of error and provides an accurate assessment of metabolic parameters. The present results illustrate a conclusive underestimation of these figures when compared to the values of continuous infusion experiments. However a partial answer to the discrepancies is provided by Leng (1970) who stated that for accurate analysis of single injection data, an adequate number of samples have to be taken in the initial 5 to 10 minutes since the decline in activity of amino acids, and especially alanine, is most dramatic within this time interval. Clearly, by inspection of Table 6 and Figure 7 and 8 (Appendix) this was not adhered to in this experiment. There are not enough points within the first 5-10 minutes of the decay curve to permit accurate interpretation by the computer for the calculation of the parameters.

Experiment B.2.

Materials and Methods

The experimental conditions as well as the sheep employed were indential to that described in Experiment B.1.

The surgical preparation consisted of implanting polyvinyl catheters (P.E.90) into both of the jugular veins. One was employed for infusion and the other for blood collection.

The nutritional regime was also the same as outlined

previously. The sheep was fed 1 kg alfalfa hay twice daily at 0800 hours and 1600 hours. The infusion began at 1130 hours therefore similar to Experiment b.1 the results are based on fed conditions (3.5 hr fast).

1.) Isotope and Blood Collection

The isotope used, L-Alanine U-L- ^{14}C was obtained from ICN and adheres to the specifications stated previously. The major source of variation between the present experiment and the first one is in the amount of isotope administered as well as the time periods for blood collection.

0.085 mCi (85 uCi) of l-alanine-U- ^{14}C was administered as a single injection into the left jugular vein and 10 ml of blood plasma was collected from the right jugular vein at the following time intervals:

Sample no.	Collection Time (min.)
1	CONTROL (Before Infusion)
2	3.0
3	3.5
4	4.0
5	4.5
6	5.0
7	10.0
8	15.0
9	20.0
10	30.0
11	45.0
12	60.0
13	75.0
14	90.0
15	105.0
16	120.0
17	135.0

In order to accomplish the collection of blood for the time period of 5 minutes following infusion, a continuous sampling technique was devised. This method utilized the two jugular veins, such that at the exact time when the labelled alanine was injected into the left jugular, blood was drawn continuously from the right jugular by means of heparinized syringes.

As soon as 10 ml of blood was withdrawn into the first syringe, a second was immediately affixed to the catheter and the next sample was collected. This procedure continued for 5 minutes following the infusion of the isotope and represents a continuous collection of blood over this time interval. The remainder of the blood samples were withdrawn at the specified intervals described above. This approach to blood sampling was developed in an attempt to assess the initial decline of alanine specific radioactivity more accurately since the majority of the decay curve is completed within the first 10 minutes following injection.

2.) Chemical Methods

The procedure for fractionation of plasma components, glucose and alanine analysis have all been described in detail previously.

Another chromatography experiment was performed to test that the activity of the neutral and basic fractions was due to glucose and alanine respectively.

The solvent system for the two-directional separation of alanine and the for the single-directional separation of glucose were the same as stated in the preceding experiment. The results from this study are also included in Figure 5 and Table 3 (Appendix).

Calculations

A two term exponential function was employed to produce the line of best fit for the decay curve of alanine specific activity. A detailed description of the calculations used in determining the metabolic parameters is provided in the calculations section of the previous experiment.

Results and Discussion

In Table 5 (Appendix) the specific activity values for both alanine and glucose at each time interval, as well as the individual values for pool size, space, total entry rate, irreversible loss and recycling are presented. The percent transfer of alanine carbon to glucose is also defined. The results of the present experiment are based on the change of specific radioactivity of plasma alanine with time after an injection of 85 μ Ci of U- 14 C-alanine.

Figure 8 (Appendix) outlines the linear curve for glucose specific activity. The line of best fit estimated by the two

term exponential function is illustrated in Figure 9 (Appendix).

The parameters of alanine metabolism as illustrated in Table 5 (Appendix) demonstrated a total entry rate of 14.52 mg C/min (9.77 mM/hr), an irreversible loss of 7.60 mg C/min (5.12 mM/hr), a recycling rate of 6.91 mg C/min (4.65 mM/hr) and a percent conversion to glucose of 5.00%. When compared to the results of the previous experiment, the above values correspond much closer to the published estimates of total alanine turnover reported by Wolff and Bergman (1972a). As stated in the Discussion of Experiment B.1, total plasma turnover as measured by a continuous infusion technique is the same as irreversible loss determined by the single injection method. Therefore the value for irreversible loss 7.60 mg C/min (5.12 mM/hr) is comparatively close to the figures of 8.3 and 11.3 mM/hr for total plasma turnover as stated by Wolff and Bergman, (1972a).

It is significant that the results of this experiment are much closer to reported values than the figures of the preceding experiment. This fact indicates that the changes in techniques made, specifically the continuous blood collection over the first 5 minutes, improved the efficiency and accuracy of this approach. In other words the computer analysis proved more reliable since there were more points, especially during the initial rapid decline of alanine specific activity, to compute the line of best fit. Thus the degree of interpolation of the points in Figure 9 (Appendix) is reduced considerably in this experiment as compared to the previous one.

The glucogenicity of alanine was estimated to be 5.00%, which is in close agreement with the values of 5.4% as determined by Wolff and Bergman (1972b) using a continuous infusion approach on sheep. The 5.00% figure is also less than previous estimates of between 6 and 8% in lactating cows (Black et al., 1968) and 12.5% in man (Kreisberg et al., 1972).

As additional support to the validity of this multicompartmental approach for the analysis of single injection data, a three term exponential function was also employed to determine the line of best fit for the decay curve of alanine specific radioactivity in the present experiment. Since the results outlined in Table 5 (Appendix) are based on a two term fit, an approach of this nature would indicate whether in fact, the number of exponential terms is determined by the shape of the observed specific radioactivity decay curve. The results of applying a three term function to the decay curve of alanine which has already been fitted to a two term analysis are presented in Table 5 (Appendix). The values for the metabolic parameters are identical to those of a two term exponential function (Table 5), which demonstrates that the data, or the shape of the decay curve is what determines the number of exponentials that can be used.

The results of the paper chromatography separation of alanine and glucose are presented in Figure 5 and Table 3 (Appendix) respectively. Similar to Experiment B.1, the peaks of radioactivity of the plasma fractions corresponded to those of the standards for alanine and glucose.

The overall significance of this experiment is that the improvements made in the present techniques over those of the previous experiment have yielded results which correspond well to the estimates determined by a continuous infusion approach. Thus the method of a single injection of an isotope combined with a multicompartmental analysis has improved its status as a means to study the kinetics of alanine metabolism in ruminants.

Experiment B.3.

Materials and Methods

A 1.5 year old wether weighing 37 kg was used for this final single injection experiment.

The surgery performed on the animal varied slightly from the previous two experiments in that polyvinyl catheters (P.E.90) were inserted into both right and left jugular, but in addition one was also implanted into the right carotid artery. The left jugular cannula was used for infusion and blood was collected from both right jugular and carotid. The catheters were maintained patent by flushings with a solution of heparinized saline (100 units/ml) twice daily.

The nutritional regime was similar that of the first two experiments in that 2 kg alfalfa hay was fed to the sheep twice daily; once at 0800 hours and once at 1300 hours and again the animal is under fed conditions (5 hour fast) during the experiment.

1.) Isotope and Blood Collection

L-Alanine U-L- ^{14}C obtained from ICN was infused into the left jugular vein as a single injection of 0.095 mCi (95 uCi). 10 ml of blood plasma samples were withdrawn from the right jugular vein and the right carotid artery at the following time intervals:

CAROTID ARTERY

Sample no.	Collection Time (min)
1	CONTROL
2	CONTROL
3	.67
4	1.33
5	1.66
6	2.25
7	2.83
8	3.83
9	4.83
10	5.00
11	10
12	15
13	25
14	35
15	45
16	60
17	75
18	90
19	120

JUGULAR VEIN

Sample No.	Collection Time (min)
1	CONTROL
2	CONTROL
3	.33
4	.58
5	.83
6	1.95
7	2.17
8	2.45
9	2.75
10	10
11	15
12	25
13	35
14	45
15	60
16	75
17	90
18	120

The continuous blood sampling technique developed in Experiment B.2 was employed in the present experiment.

2.) Chemical Methods

The procedures for fractionation of blood plasma, and the determination of plasma glucose and alanine have been outlined in detail previously. Due to the positive results obtained for the paper chromatographic separation of ^{14}C -labelled alanine and glucose in Experiment B.1 and B.2, no such analysis was conducted in this the final single injection experiment.

Calculations

The calculations for the present experiment duplicates those used by the first two. However instead of a two term exponential function, the decay curve of ^{14}C -alanine required a three term function to produce a line of best fit which incorporates all of the points.

Results and Discussions

Table 6 (Appendix) outlines the data for glucose and alanine specific activities in both the jugular vein and carotid artery. In addition the values for the parameters of alanine metabolism which include pool size, space, total entry rate, irreversible loss, recycling and the conversion percentage of alanine to glucose are also found in this table. Therefore these results are based on the change of alanine specific activity with time after a single intravenous injection of 95 μCi of $\text{U-}^{14}\text{C}$ -alanine.

The specific activity curves of glucose in the jugular vein and carotid artery respectively, are diagramed in Figure 10a and b (Appendix). Figure 11a and b (Appendix) illustrate the fitted

line to the logarithmic decay data of alanine by the three term exponential function in the jugular vein and carotid artery respectively.

The results of the glucose and alanine concentration for the carotid artery and jugular vein were compared statistically by using the students' t test for significance at a level $P > 0.05$.

The values for the parameters as described in Table 6 (Appendix) show total entry rates of 49.78 mg C/min (33.52 mM/hr) and 61.37 mg/min (41.32 mM/hr) for the jugular vein and carotid artery respectively. The rate of irreversible loss for jugular vein and carotid artery are 10.01 mg C/min (6.75 mM/hr) and 9.33 mg C/min (6.28 mM/hr) respectively, and recycling was determined from the difference which amounts to 39.75 mg C/min (26.77 mM/hr) for the jugular vein and 52.04 mg C/min (35.04) mM/hr) for the carotid artery. The estimates of irreversible loss for the carotid artery and jugular vein (6.28 mM/hr and 6.75 mM/hr) are extremely close and both correspond relatively well to the estimates of total turnover rates of between 8 and 11 mM/hr as reported by Wolff and Bergman (1972a). The recycling values of the present experiment indicate that this process is occurring to a high degree and obviously yields correspondingly high values for total entry rates. This factor plays the major role in determining the number of exponential terms required to assign a line of best fit to the decay curve. By inspecting Figure 11 a and b three distinct slopes may be noted. An initial rapid

decline, followed by a second more linear phase and ending with a elongated curvilinear section. Thus by visual inspection of the logarithmic decay curve it is obvious that a two term exponential function, which was used in the two previous experiments, is not adequate to account for the third component. Therefore a three term fit is necessary in order to account for the large amount of recycling which occurred. An interesting point to note is that the values for irreversible loss are not influenced by the high degree of recycling. Although the calculations are designed to separate these two parameters, it is encouraging to note that the system is functioning according to the pattern set.

The glucose concentration in the carotid artery is significantly higher than that in the jugular vein ($P < 0.05$). The corresponding values for alanine concentration in these blood vessels proved to be not significantly different. Thus from this statistical analysis one can state that glucose is being utilized by the brain tissue. No estimate of quantity is valid since the present experiment was performed on one sheep only and no estimate of blood flow was made.

The per cent conversion of alanine carbon to glucose for the jugular vein (6.78%) and carotid artery (6.74%) agree well with each other as well as with the published values of 5.4% for sheep (Wolff and Bergman 1972a), and 6 to 8% for lactating cows (Black et al., 1968).

The differences between the carotid artery and jugular vein sampling in terms of irreversible loss and the glucogenic role

of alanine are insignificant. Therefore the place of blood collection appears to be of minimal importance for the determination of total body or total plasma metabolic parameters. However the variation between arterial and venous plasma becomes more significant when considering such values as the total entry rate and the rate of recycling which deviate depending upon the vessel from which blood is collected. Such a result has been demonstrated before and (Wolff and Bergman 1972a&b) and is also evident in the present experiment with the estimates of total entry rate (61.3 mg C/hr) and recycling rate (52.04 mg C/min) of the carotid artery being considerably larger than the total entry rate (49.78 mg C/min) and recycling rate (39.74 mg C/min) determined in the jugular vein. This is reasonable by examination of the alanine decay curves for each of these blood vessels (Figure 11 a and b, Appendix). By visual inspection the third component or section of the curve for the decline in specific radioactivity of ^{14}C -alanine in the carotid arterial plasma levels off and does not fall to the abscissa as it rapidly as does in the jugular vein. By the shape of the decay curves it appears that recycling is more pronounced in the carotid artery plasma than the jugular vein plasma. This postulation is given support from the fact that the linear parameter (A), which represents the zero-time component, for the third term is larger in the carotid artery than in the jugular vein. So the level of specific radioactivity remaining in the blood of the carotid artery is more extensive than that in the jugular vein which implies a higher degree of recycling. Although it is difficult to speculate upon the exact reason why

recycling is more prominent in the carotid artery, a possible explanation arises from consideration of the anatomy of the area in question.

Blood samples are withdrawn from the carotid artery and jugular vein simultaneously and by the time recycling takes place the tracer dose of ^{14}C -alanine has virtually mixed and traversed through the entire circulatory system. Therefore the labelled alanine has been taken up by the tissues which metabolize this amino acid. Two potential sources of alanine utilization are the liver and the kidneys for the purpose of gluconeogenesis. Thus the radioactive tracer has left the amino acid pool and has been incorporated into the glucose pool. However re-entry of the labelled carbon can occur through the established "glucose-alanine cycle" (Felig *et al.*, 1970) where alanine is synthesized *de novo* in the muscle tissues by transamination of glucose derived pyruvate. Blood carrying the labelled glucose from the liver is transported to the muscle tissues where the conversion to ^{14}C -labelled alanine occurs. By the time this process becomes pronounced blood entering the carotid artery after mixing in the heart is the best estimate of the recycling effect since it has yet to perfuse any additional tissues. However the jugular vein blood collected in the present experiment is influenced by the degree of metabolism occurring in the brain tissue. Although the statistical analysis of the arterio-venous alanine concentration differences have shown no significant difference a dilution effect of the tracer may be at work. That is the brain tissue is in a dynamic state where the input equalizes the output in terms of alanine. Thus the tracer

may be taken up or diluted by addition of nonlabel or a combination of both. Consequently the dilution effect of the brain tissue may be the influencing factor which causes the recycling value to be larger in the carotid artery than in the jugular vein.

This final single injection experiment contributes further evidence to the use of this approach in evaluating metabolic parameters of alanine. The slight modifications introduced in this experiment such as using a three term exponential function, did not affect the values of irreversible loss and per cent conversion to glucose observed previously. Therefore the technique of employing a multicompartamental analysis can be adjusted to any changes occurring within the animal which might influence the results. Consequently the technique employed here and in the previous two experiments is flexible.

Conclusions

The metabolic parameters of alanine as determined in the above three experiments are summarized in Table 7 (Appendix). The results for the rate of irreversible loss and percent transfer (glucogenicity) of alanine conform reasonably well with the published values as mentioned previously. The overall trend for the three studies is a slight underestimation of these values as compared to the results from continuous infusion experiments (Wolff and Bergman, 1972a). This finding is interesting since the major complaint of the single injection

technique is its proposed overestimation of turnover rates of the metabolite under investigation (White et al., 1969).

The multi-exponential approach in determining a line of best fit to the decay curve of alanine also calculated the parameters of alanine pool size and space. The former represents the quantity of body alanine with which the injected ^{14}C -alanine mixes and the latter implies the volume of fluid through which the alanine pool is distributed. Of the two measurements, the alanine space in terms of per cent body weight is the most informative and comparable for it incorporates the sheep's individual variation in body weight. Leng (1970) in a review article presents a summary of the glucose metabolism values of nonpregnant and nonlactating sheep. The glucose space (as per cent of body weight) ranged from 18 to 35 per cent depending upon the technique used as well as the food intake, diet and feeding regime of the sheep. On the average the higher estimated values of glucose space were determined through the use of a single injection technique. However none of the workers employed a multicompartmental approach to analyze the decay curve of ^{14}C -glucose. Although these results do not bear direct relevance to the present work which investigates these parameters of alanine, they give some insight into the overall pool size and space of glucose within sheep. This knowledge helps in assessing the significance of the values determined in the present series of experiments since the reported work on alanine is extremely sparse.

The figures for alanine space as a per cent of the body

weight range from 13.27% for the first experiment down to 3.71% and 4.44% for the second and third experiments respectively. All of these values are determined on jugular or peripheral venous blood which is the only true estimate of the total body pool size or space. Thus the alanine space of 10.88% as determined from the carotid artery plasma in the third experiment is not a valid estimate of this parameter in terms of the entire body. The value for the first experiment is high relative to those for the second and third experiment may be explained adequately because of the lack of blood samples in the first 5 to 10 minutes post injection. As mentioned previously this approach depends on frequent sampling of blood following the single infusion. Therefore the values of 3.71 and 4.44% reflect closely the actual alanine pool space, in sheep under these dietary conditions. The justification of this statement stems from the fact that the alanine pool is regarded as one of the precursor compartments which is connected to the large glucose pool. From the values of the per cent contribution of alanine to glucose synthesis (6-8%) in nonpregnant, nonlactating, steady state sheep it appears that the relationship of alanine pool space (mean of 4.07%) to glucose pool space (range of 18-35%) is close to what would be expected.

The multicompartmental approach to the analysis of decay curves of isotopes was first used in the study of glucose metabolism. Besides the numerous parameters isolated, this method ascertained the existence of three compartments; a large body pool of glucose which is interconnected with two precursor pools (Leng 1970). The proposed model (Figure 18, Appendix)

depicts the relationships of the glucose pools. The results from the preceding series of experiments indicate a two compartment and possibly a three compartment model for alanine metabolism. It is not the purpose of the present study to speculate on the exact number of compartments with reference to alanine, nor to predict what these consist of. Answers to these questions can be formulated through more extensive work with this technique in the future.

The primary objective of the present series of experiments was to study the effectiveness of the single injection technique coupled with a multicompartmental analysis of the resulting decay curve, as a means of quantitatively assessing the parameters of alanine metabolism in nonpregnant, nonlactating sheep. The results presented in Table 7 (Appendix) along with the above discussions indicate that this method has the potential to be used extensively for in_vivo metabolic studies.

III CONTINUOUS INFUSION OF LABELLED ^{14}C -ALANINE: WITHOUT A PRIMING DOSE

Introduction

The continuous infusion of a radioactively labelled tracer has been widely used for metabolism studies in animals. There appears to be two schools of thought governing the use of this technique, the first involves a continuous infusion preceded by a priming dose of the tracer (Steele, et al., 1956 and Steele 1964) and the second comprises the use of a continuous infusion of label without a priming injection (Leng et al., 1967 and White et al., 1969). The latter technique has been employed intensively in the study of glucose metabolism in sheep (Bergman, 1973).

Leng (1970) has indicated that due to the extensive recycling occurring between the glucose pool and peripheral substrate pools in sheep, the use of a primed infusion technique for the determination of glucose pool size is unreliable on theoretical grounds. The large amount of recycling of alanine as determined by the single injection experiment previously, indicate that a primed-infusion approach would not be an efficient method to measure these parameters of alanine.

The continuous infusion technique used by Leng et al., (1967 and White et al., (1969) has a distinct advantage in that the calculation of irreversible loss (total entry rate) relies on a simple mathematical treatment. The infusion rate of

radioactivity divided by the mean plateau level of specific radioactivity of the metabolite, which is normally attained between 3 and 4 hours for glucose, gives an estimate of this parameter. This is indeed a simplification of the actual isotope dilution curve which is described by the following equation (Steele et al., 1956; Steele, 1964):

$$SR = \frac{R}{Q} \sum_{i=1}^n \frac{A'_i t}{m_i} (1 - e^{-m_i t})$$

Where R is the infusion rate (nCi/min), Q is the pool size, A'_i is the fractional zero-time intercept, $-m_i$ is the rate constant and SR is the plasma specific radioactivity at time t (nC/mg C). As time approaches infinity during a continuous infusion of isotopically labelled materials a plateau specific radioactivity is obtained which is described as;

$$SR = \frac{R}{Q} \sum_{i=1}^n \frac{A'_i}{m_i}$$

Therefore if the specific radioactivity of the metabolite at the plateau level (as described above) is used to calculate irreversible loss then

$$\text{irreversible loss} = \frac{\text{infusion rate (nCi/min)}}{\text{SR (nCi/mg C)}}$$

Though the continuous infusion of labelled tracer has been used extensively in the measurement of the parameters surrounding glucose metabolism, it is relatively recently that this approach has been used to measure amino acid metabolism in sheep fed alfalfa hay (Wolff et al., 1972a,b,c). These workers investigated the turnover of plasma amino acids and their contribution to gluconeogenesis. One of the objectives of the above studies was to assess the metabolism of plasma amino acids by portal-drained viscera, liver and peripheral tissues of sheep. In order to accomplish this each experiment consisted of a continuous intravenous infusion of a ^{14}C -labelled amino acid and blood was sampled from the aorta, the portal vein, and a hepatic vein. Blood flow in both the portal and hepatic veins was determined simultaneously. The calculations devised by these researchers for determining the rates of plasma amino acid turnover by each tissue shall be outlined and discussed in a later section.

This work is based upon the assumption that when a continuous infusion of labelled amino acid is given, while the animal is in a steady state, the plasma and tissue pools attain plateau specific activities. The value of the specific activity therefore depends upon the rate of renewal or turnover from unlabelled precursors (Gan and Jeffay, 1967). These precursors can be amino acids absorbed from the gastrointestinal tract, compounds of intermediary metabolism, or the proteins of body tissues. With this in mind Wolff and Bergman (1972a) estimated

the turnover rates for alanine, aspartate, glutamate, glycine and serine in the portal-drained viscera, liver, peripheral tissues as well as the total plasma turnover for the body pool of each amino acid.

The present sequence of experiments utilizes the continuous infusion technique as described above to evaluate its effectiveness for the study of alanine metabolism and to compare it with the two previously described methods. The parameters of alanine center around its rate of irreversible loss and its contribution to glucose synthesis in sheep under steady state conditions. By utilizing the blood flow data from the first experiment an estimation of the portal metabolism of both alanine and glucose has been made.

Experiment C.1.

Materials and Methods

1.) Surgical Procedures

The experiment was conducted on a 3.5 year old wether weighing 45 kg. Surgery was performed on the animal one week prior to the commencement of the experiment. The preparation of the sheep for surgery, the method of implantation of catheters as well as the post-operative care are described in detail under the surgical procedures section of the first experiment involving blood flow studies. Catheters were placed into the

portal vein, mesenteric vein and carotid artery, and were maintained open by infusing heparinized saline (100 units/ml).

Unfortunately the day before the experiment the portal vein catheter was caught on an unknown object and had to be removed. Thus only the mesenteric vein and carotid artery cannula remained patent. The experiment was carried out as planned with the continuous infusion of labelled ^{14}C -alanine into the mesenteric vein and blood collection from the carotid artery.

The nutritional regime consisted of a maintenance diet of 200 gm dehydrated grass pellets fed every two hours beginning at 0900 hours and ending at 1700 hours. The infusion began at 1100 hours and the animal was last fed the previous evening at 1700 hours thus the results are based on an overnight fast (18 hr).

2.) Isotope and Blood Collection

200 μCi of L-U- ^{14}C -alanine obtained from ICN conforming to the specifications described previously was dissolved in 400 ml sterile saline and infused into the mesenteric vein at a rate of 0.475 $\mu\text{Ci}/\text{min}$. Blood (10 ml) was collected from the carotid artery at the following time intervals:

Sample No.	Collection Time (min)
1	CONTROL (Before Infusion)
2	5
3	15
4	30
5	60
6	90
7	120
8	150
9	180

10	210
11	240
12	270
13	300
14	330
15	360

3.) Chemical Methods

The separation of plasma components, as well as the plasma analysis for alanine and glucose concentrations were the same as outlined in the previous experiments.

Calculations

Due to the loss of the portal vein catheter, the production and utilization rates of alanine and glucose by the portal drained viscera could not be determined. Therefore the apparent total plasma turnover of alanine was the only figure calculated. The apparent turnover of this amino acid is defined, in a similar manner to that for glucose (Bergman, 1963), as the rate at which the arterial concentration of ^{14}C -alanine is diluted by unlabelled alanine and thus:

$$\text{apparent turnover, } \mu\text{moles/hr} = \frac{I}{SA}$$

Where I is the infusion rate ($\mu\text{Ci/hr}$) of ^{14}C -alanine and SA is the specific activity ($\mu\text{Ci}/\mu\text{mole}$) of alanine in the arterial plasma. Wolff and Bergman (1972a) have modified this apparent turnover rate to account for the continual absorption of unlabelled amino acids from the gut, some of which the liver immediately removes. Therefore a correction factor is required to find the true turnover of amino acid. The term was obtained

by multiplying the production of the amino acid from the portal-drained viscera by the fractional uptake of ^{14}C -amino acid by the liver. Thus the corrected turnover is calculated as follows:

$$\text{Corrected Turnover} = \text{Apparent Turnover} + \frac{\text{Portal Production}}{\text{Fractional Uptake amino acid by Liver}} \times \text{Fractional Uptake amino acid by Liver}$$

If the arterial concentrations remain constant, then the corrected turnover provides an estimate of plasma amino acid utilization by all tissues of the body and it follows that:

$$\text{Peripheral Utilization} = \text{Corrected Turnover} - (\text{Portal} + \text{Hepatic Utilization})$$

$$\text{Peripheral Production} = \text{Peripheral Utilization} + \text{net Peripheral Production}$$

A value for corrected turnover is not possible in the present experiment and therefore only the apparent turnover of alanine can be assessed. Table 8 (Appendix) presents the data for this continuous infusion experiment and the specific activity versus time curves for both alanine and glucose are pictured in Figure 12 (Appendix).

In addition to the turnover rates a percent glucose production from alanine was determined by dividing the plateau specific activity of glucose in the carotid artery by that of alanine in the same vessel.

The statistical treatment of the data consisted of a

correlation coefficient analysis of both the glucose and alanine plateau levels to detect if the x and y variables are not correlated over this time interval.

Results and Discussion

As illustrated in Figure 12, (Appendix), the glucose and alanine specific activity curves reached plateau levels between 5 and 6 hours after the start of the experiment. The statistical analysis proved that there is no significant slope in this time period for either alanine or glucose. The apparent turnover value for alanine of 7.23 mM/hr (10.75 mg/min), which can be equated to irreversible loss as determined by the single injection experiments (White *et al.*, 1969) agrees well with that reported by Wolff and Bergman (1972b) of between 8.3 and 11.3 mM/hr for two sheep weighing 45 and 59 kg respectively. The former value is a much closer comparison to the present result since the sheep used by both experiments are of equal body weight (45 kg). The per cent of glucose production from alanine in the present experiment was assessed to be 5.07%, which is comparable to the value of 5.4% reported by Wolff and Bergman (1972b).

The major significance of the present experiment is that the continuous infusion of ^{14}C -labelled alanine without a priming dose yielded a statistically significant plateau level of specific activity for both alanine and glucose at

approximately five hours after the start of the infusion.

Additional calculations such as turnover time and pool size were not possible in the present experiment since the infusion was terminated during the plateau interval and thus the half-life of ^{14}C -alanine could not be determined.

Experiment C.2.

Materials and Methods

This final continuous infusion experiment was conducted on a 2 year old wether weighing 42 kg. Surgery was performed in a similar fashion as in the preceding experiment with polyvinyl catheters (P.E.90) being implanted into the portal vein, carotid artery and mesenteric vein. The catheters were maintained in a functional state by frequent flushings with the solution of heparinized saline (100 units/ml). This time all the catheters remained patent during the post-operative recovery phase (5 days) and during the experiment.

The animal was placed on a roughage diet similar to the one employed in the previous experiment. The sheep was not fed in the morning of the experiment, thus the results are also based on an overnight fast (17 hr) with the infusion commencing at 1045 hours.

1.) Isotope and Blood Collection

L-U¹⁴C alanine at a concentration of 0.40 uCi/ml was infused into the mesenteric vein at a rate of 1.1 ml/min (.44 uCi/min). Blood plasma samples were collected simultaneously from the carotid artery and portal vein at the following time intervals:

Sample No.	Collection Time (min)
1 -P.V.	CONTROL-1
2 -C.A.	CONTROL-1
3 -P.V.	CONTROL-2
4 -C.A.	CONTROL-2
5 -P.V.	30
6 -C.A.	30
7 -P.V.	60
8 -C.A.	60
9 -P.V.	90
10 -C.A.	90
11 -P.V.	120
12 -C.A.	120
13 -P.V.	150
14 -C.A.	150
15 -P.V.	180
16 -C.A.	180
17 -P.V.	210
18 -C.A.	210
19 -P.V.	270
20 -C.A.	270
21 -P.V.	300
22 -C.A.	300
23 -P.V.	330
24 -C.A.	330
25 -P.V.	345
26 -C.A.	345
27 -P.V.	360
28 -C.A.	360
29 -P.V.	375
30 -C.A.	375
31 -P.V.	390
32 -C.V.	390

INFUSION STOPPED

2.) Chemical Methods

Plasma specific activities of alanine and glucose were determined from the techniques described earlier which involved separation of plasma constituents as well as assays for alanine and glucose concentrations.

Calculations

The techniques described in the previous experiment were employed to measure apparent turnover rate of alanine. Once again the corrected turnover rate of alanine cannot be assessed since the hepatic vein blood flow as well as the concentration of this amino acid in the hepatic vein were not determined. However a value for the metabolism of both alanine and glucose by the portal-drained viscera was assessed using the equation:

$$P = F_{PV}(C_{PV} - C_A) \quad (\text{Katz and Bergman 1968})$$

where P represents portal net production rates of the metabolite, F_{PV} is the whole blood flow (ml/min) in the portal vein, and C_{PV} and C_A are the concentrations of the metabolite in the portal vein and arterial vessels respectively. The value for portal vein blood flow of 2010 ml/min as estimated in Experiment I on blood flow, was used for the calculations.

The glucogenicity of alanine was determined in a similar manner as in the preceding continuous infusion experiment.

A statistical analysis of the plateau levels (correlation coefficient) and the concentration differences between the carotid artery and portal vein (students' t test) was conducted.

Results and Discussion

The data as well as the results of the calculations are presented in Table 9 (Appendix). Figure 13, (appendix) diagrams the specific activity curves of alanine and glucose in the carotid artery and portal vein respectively.

The plateau levels for both alanine and glucose specific activities (Figure 13, Appendix) were reached at the same interval as in the preceding experiment (5-6 hours post infusion). From the mean value for alanine specific activity at this level, the apparent plasma turnover rate was assessed at 8.5% mM/hr (12.76 mg/min). The figures agree extremely well with that determined by the work of Wolff and Bergman (1972b) which estimated this rate to be in the range of 8.3 and 11.3 mM/hr as reported earlier.

The statistical analysis of the concentration differences of both alanine and glucose between the carotid artery and portal vein proved significant variations in both cases. Therefore it was decided to incorporate another arterio-venous concentration study into the present experiment. Thus the portal vein blood flow value of 2010 ml/min from Experiment A.1 was used to determine the net metabolism of alanine and glucose by the portal drained viscera. Employing the equation described above (Katz and Bergman, 1969) a net utilization of glucose (.190 g/hr/kg) was again calculated. The value derived presently conforms reasonably to the previously estimated figure of 0.142 g/hr/kg and to the range of values determined by Katz and Bergman (1969). The estimate for alanine metabolism by the

portal bed demonstrated a net production rate of 2.29 .29 mM/hr (Wolff, Bergman and Williams, 1972), which is close to that determined in the present experiment (2.02 mM/hr). From the result of the Experiment A.1 dealing with blood flow a net alanine production rate of 1.49 mM/hr was obtained. Both of the estimates derived in the present series of experiments (1.49 and 2.02 mM/hr) concur and are valid assessments since the blood flow figure was determined on the same sheep as was used in this second continuous infusion experiment. The blood flow value from Experiment A.1 can be employed in the present experiment since both studies maintain the sheep on a similar roughage diet and conduct the experiment following an overnight fast.

The percent conversion of alanine carbon to glucose was estimated to be 7.20% by using the technique described previously. This value is again well within the previously stated values of 5.4% (Wolff and Bergman, 1972) and 6-8% (Black et al., 1968)

The preceding figures for the parameters surrounding alanine and glucose metabolism serve as a guide in an attempt to assess the value of the continuous infusion experiment in relation to the two previously mentioned procedures, the blood flow study and the single injection technique.

GENERAL SUMMARY AND CONCLUSIONS

The three experimental techniques employed in this study are designed to measure various parameters pertaining to the metabolism of specific plasma constituents in animals. The primary aim of the present research was to assess the effectiveness of each method in studying the metabolic parameters of alanine and its relationship to gluconeogenesis. Since alanine has been established as the primary amino acid extracted by the liver for endogenous glucose production in sheep (Black et al., 1968; Reilly and Ford, 1971 and Wolff and Bergman 1972b), the results from such a comparison serve a useful purpose in ruminant physiology.

The first method used was the traditional technique of a blood flow study combined with measuring the arterio-venous concentration differences of the metabolites under investigation. As previously reported this approach is an accurate means of estimating the metabolism of a blood component by an organ or tissue system. The results obtained here and their close approximation to reported values attest to this statement.

The major drawback to the use of this technique is the sophisticated surgical procedures which are mandatory. Thus its effectiveness relies upon accurate implantation and maintenance of catheters in specific veins and arteries. An example of this is illustrated in the present study. The metabolism of the portal drained viscera was estimated through catheterization of

the carotid artery and the portal vein. However the activity of the liver in relation to alanine and glucose could not be assessed since no hepatic vein catheter was implanted, which is required as indicated by Katz and Bergman (1968). The reason for this arose from the extreme difficulty of introducing a polyvinyl catheter into the hepatic vein which is not readily accesible in the abdominal cavity. Although attempts were made during the numerous operations performed in this project, successful hepatic vein cannulation remained elusive. The point is that the initial objectives of such blood flow study may become obscured due to the outcome of the surgery. This approach can and does prove effective only when accurate surgical procedures are conducted successfully. Such technical skill takes a great deal of practice and may not always be available under normal laboratory conditions. A distinct advantage to this technique is the simple mathematical analysis which follows. In addition the number of assumptions as well as the total expense involved have limited effects upon the application of blood flow techniques to in_vivo metabolic studies.

The second technique attempted in this thesis project was a single injection of ^{14}C -labelled alanine. In the literature review a history of the various approaches used to analyze the resulting decay curve of specific radioactivity with time was presented and the conclusion was that a multicompartmental analysis is the most efficient and accurate method. The procedure, in brief, consists of utilizing a 2 or 3 term exponential function to determine a line of best fit by iterative minimization of the error sums of squares for the

fitted function. This complicated mathematical treatment was accomplished through the use of a computer program. From the calculations outlined by Leng (1970) the following metabolic parameters of alanine were assessed; pool size, space (% of body weight), total entry rate, irreversible loss and recycling.

The majority of applications using the above multiexponential analysis have been involving glucose metabolism in animals, with sparse work conducted on ruminants. The present study investigated the use of this approach with alanine metabolism in sheep and consequently the results have little in the literature to compare with. However the results of three single injection experiments present evidence suggesting that this technique is valid and accurate for the conditions described here. One of the major advantages of this method is its ability to separate and identify the parameters of total turnover, irreversible loss and recycling. As the third experiment indicated recycling and consequently total turnover rates were high, but the estimate of irreversible loss remained comparable to the previous two experiments as well as to the literature values. In addition to this, but in a more practical sense, the single injection technique requires simple surgical procedures which consist of two jugular vein catheters. Therefore such a study could be conducted in a laboratory or out in the field. The expense involved is certainly less than the continuous infusion of radioactive isotope.

Previous workers have doubted the validity of the single injection approach due to the numerous assumptions which must be

made which include, rapid metabolism of the tracer, incomplete mixing, recycling as well as instantaneous infusion. These points are not overlooked or ignored but become accounted for by the use of a multi-compartmental analysis. In conjunction with the above a possible drawback is the extremely complex mathematical analyses which accompanies this type of study. However once derived the analysis becomes straightforward to apply and can be adjusted to suit the decay curve in question. The third series of experiments reported on previously consist of a continuous infusion of labelled ^{14}C -alanine without an initial priming dose. A priming dose-continuous infusion approach was not considered here due to the theoretical objections to the technique as pointed out previously. A plateau or constant specific activity level for both glucose and alanine was achieved some 5 hours after the start of the infusion. From this level the calculations of total turnover rate of alanine as well as the per cent contribution of this amino acid to glucose synthesis were made with the results being comparable to those cited in the literature. Thus the procedure employed in the present experiments yield results which conform to previously published data and consequently these methods proved accurate in representing continuous infusion techniques in general.

Of the drawbacks affecting this type of an approach, the skilled surgical procedures influence the outcome in a similar fashion as indicated for blood flow studies. This is especially true if following Wolff and Bergmans's (1972a) modification of using the difference in radioactivity of a substance between two vessels to determine an estimate of tissue utilization or

production. An additional negative feature of this technique is the expense required to obtain the necessary radioactively labelled compounds. However the major problem is recycling.

In theory the continuous infusion of a labelled substrate allows enough time for the tracer to equilibrate with all of the body pools and thus recycling has little significance. This is especially true when the recycling of ^{14}C is due only to the fact that there is a dilution of this carbon in a pool of substrate and the ^{14}C is randomly converted back to the originally labelled tracer. Consequently the value for total entry or turnover rate has been considered synonymous with the irreversible loss as defined by a single injection system. (White et al., 1969). However under certain circumstances this recycling becomes more prominent, specifically in starvation and other physiological stress conditions such as, ketosis in the bovine and pregnancy toxemia in the ovine. In terms of amino acid metabolism and specifically alanine in ruminants, recycling of label through amino acid interconversions was expected to provide negligible error due to the multiplicity of pathways available for the utilization of alanine carbon (Wolff and Bergman 1972a). Such assumptions have come under considerable scrutiny since the establishment of the "glucose-alanine cycle" (Felig, 1973). Under conditions where recycling becomes pronounced the continuous infusion technique is unable to detect this and thus the turnover rate no longer corresponds to the rate of irreversible loss. The result of this will be an overestimation of the actual turnover rate of the metabolite which in the present investigation is alanine.

Of the three techniques investigated during the present project, the single injection with a multi-compartmental analysis appears to have the greatest potential for further research in ruminant physiology and metabolic studies.

GLOSSARY OF TERMS

1) Specific Activity

The specific activity of a substance containing a labelled atom is the amount of radioactivity per unit of substance. This is often expressed as the number of radioactive units (microcuries, counts per minute, disintegrations per minute) per milligram or millimole of the substance.

2) Steady State

In a situation where the rate of entry of a molecule by synthesis or transport equals the rate of exit by breakdown or transport, the concentration of the molecule remains constant and a steady state exists whenever the influx of material does not balance the outflux.

3) Model

A model represents any set of equations or functions that describe the behavior of a tracer in the system. The parameters of a model are the arbitrary constants of the function or equations.

4) Turnover

Turnover refers to the process of renewal of a substance in the

body or in a given tissue.

5) Turnover_Rate

The Turnover Rate is the rate at which a substance is turning over in a given compartment or metabolic pool. The meaning of turnover rate is explicit only when a steady state exists, that is, when the rate of synthesis and transport into a compartment equals the rate of breakdown and exit.

6) Entry_Rate

The rate parameters of turnover estimated from primed or continuous infusions or single infections have been termed turnover rate, transfer rate, utilization rate, entry rate, inflow-outflow rate, flux, renewal rate, or irreversible disposal. These measurements are probably all synonymous with irreversible loss or disposal (White et al., 1969).

7) Total_Entry_Rate

The total entry rate is the rate at which a metabolite appears in the sampled pool.

8) Irreversible_Loss

Irreversible loss is the rate at which a metabolite, which does not return to the sampled pool during the course of the

experiment, leaves this pool.

9) Recycling

Recycling is the rate at which the labelled ^{14}C returns to the plasma metabolite pool, which has previously left the sampled pool.

10) Alanine Pool Size

Alanine pool size is the quantity of body alanine with which injected ^{14}C -labelled alanine mixes.

11) Alanine Space

Alanine space is the volume of fluid through which the alanine pool is distributed.

12) Turnover Time

The turnover time of a substance is the time that is required for the turnover of a quantity of substance equal to that present in the compartment.

13) Precursor

The term precursor refers to a compound which gives rise to another by chemical transformation or by transport from one

organ to another.

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APPENDIX

Figure 1.) Standard Curve for Glucose Determination.

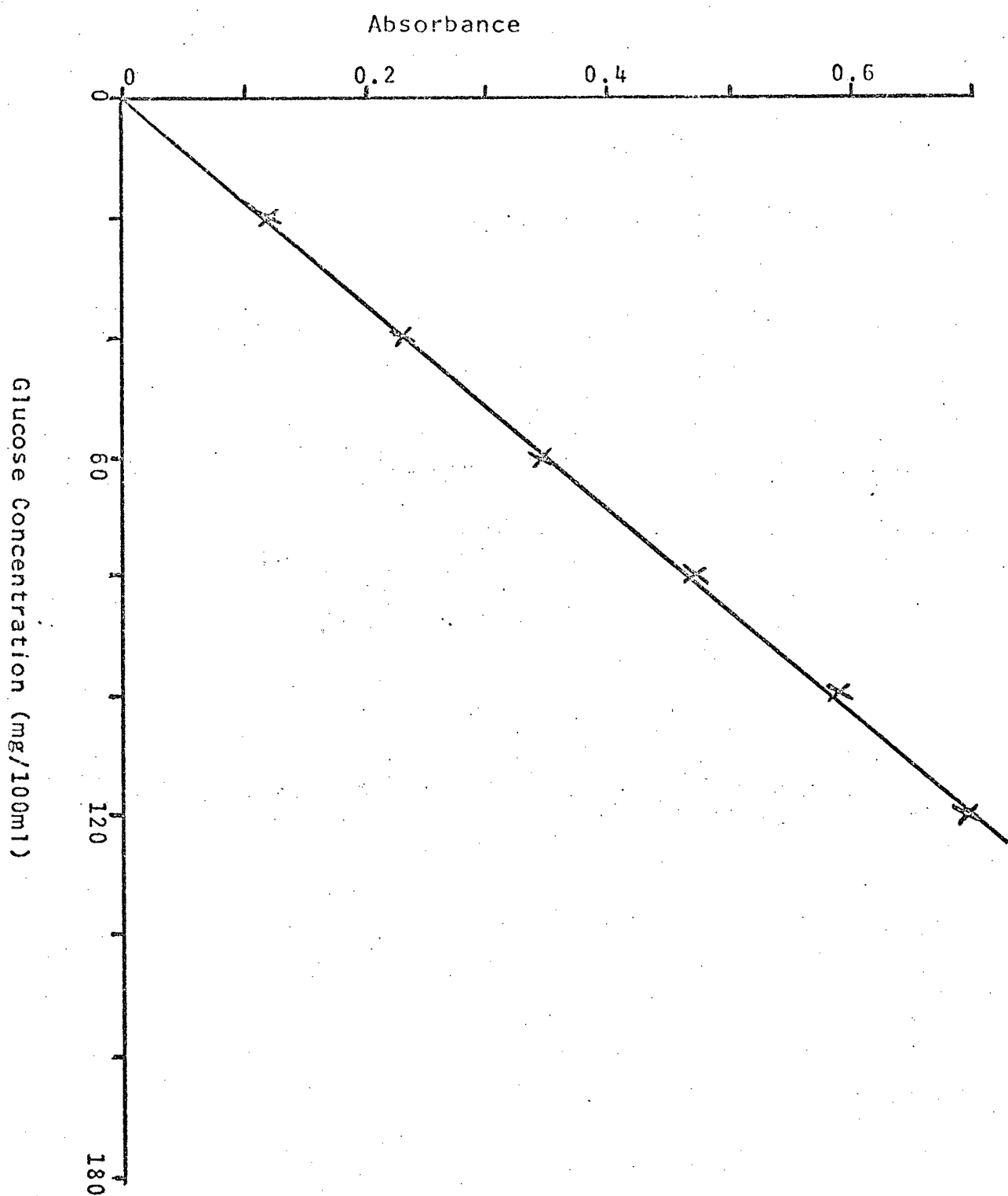


Figure 2.) Standard Curve for Alanine Determination

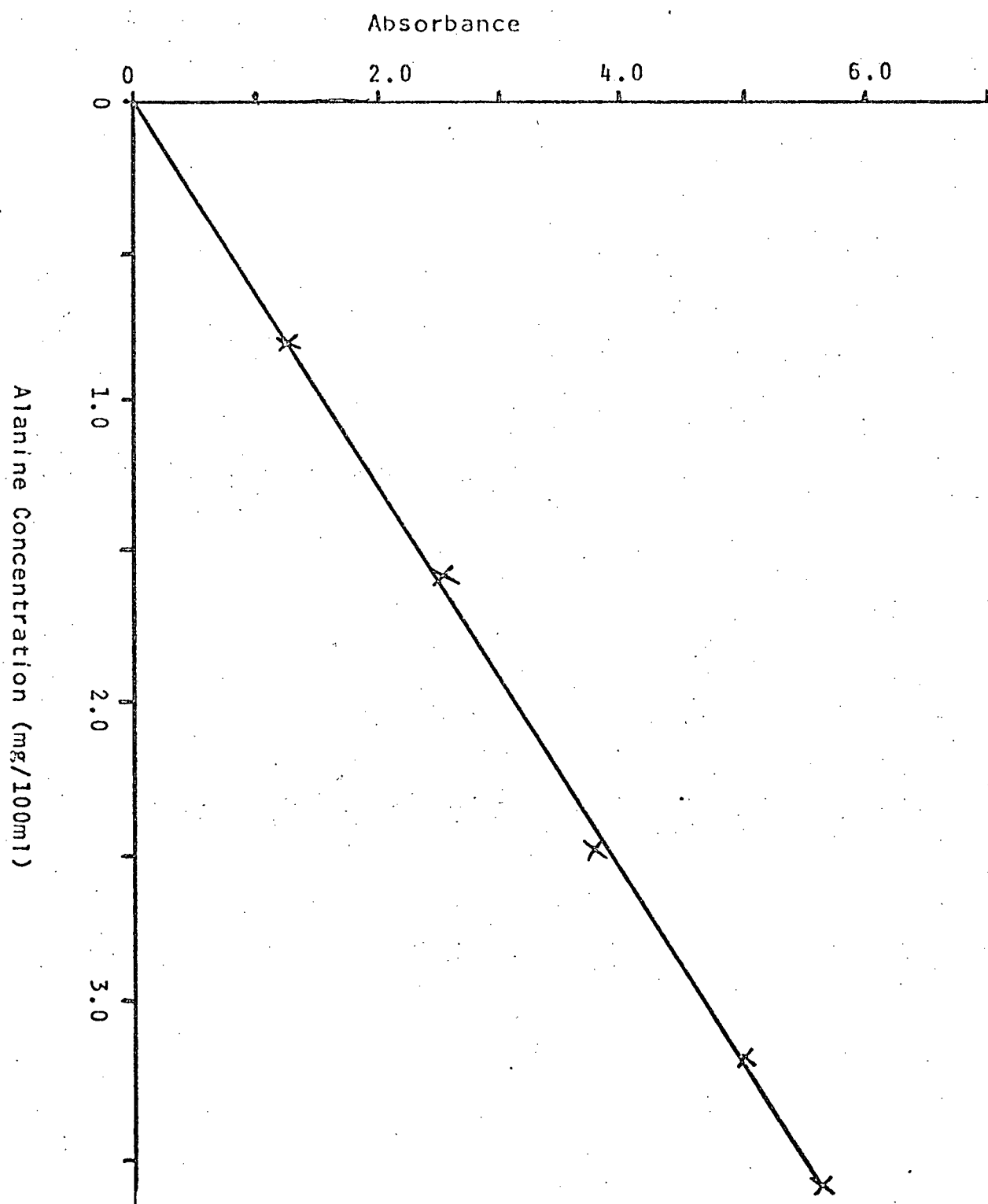


Figure 3.) Standard Curve for Para-aminhippuric Acid (PAH)
Determination

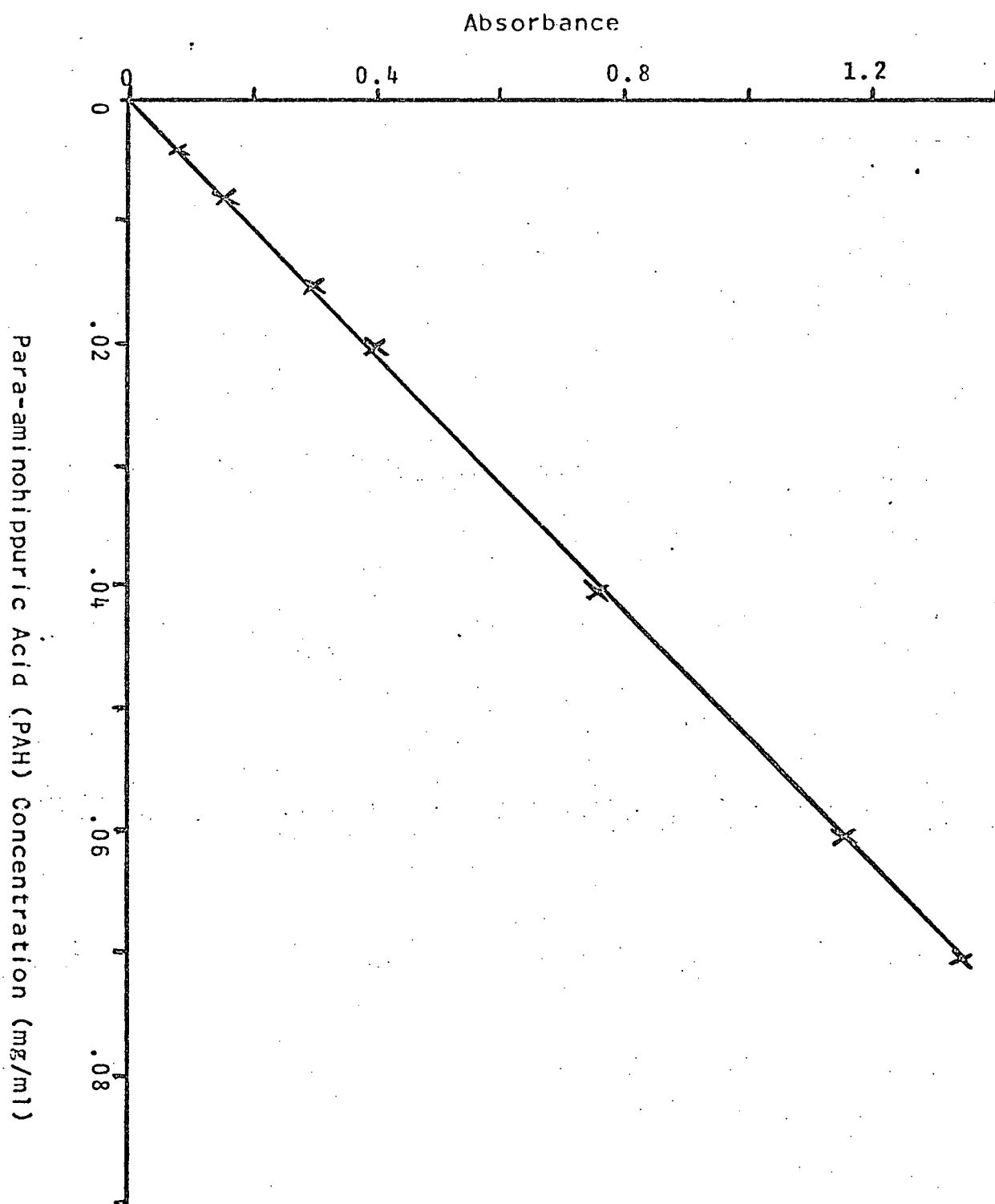


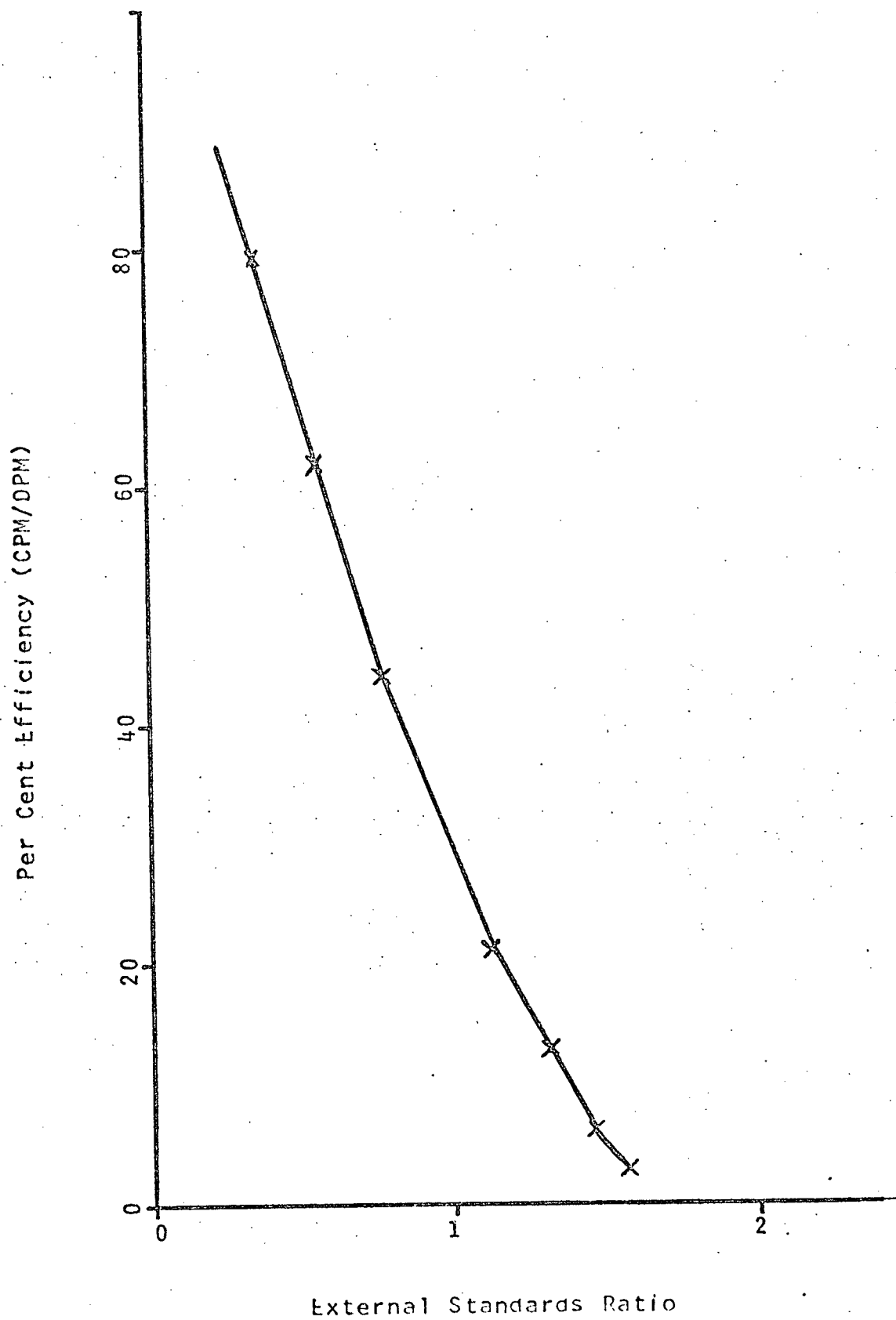
Figure 4.) Quench Correction Curve for ^{14}C .

Figure 5.) Paper Chromatography Separation of ^{14}C -Alanine
with Use of the Actigraph Scanner.

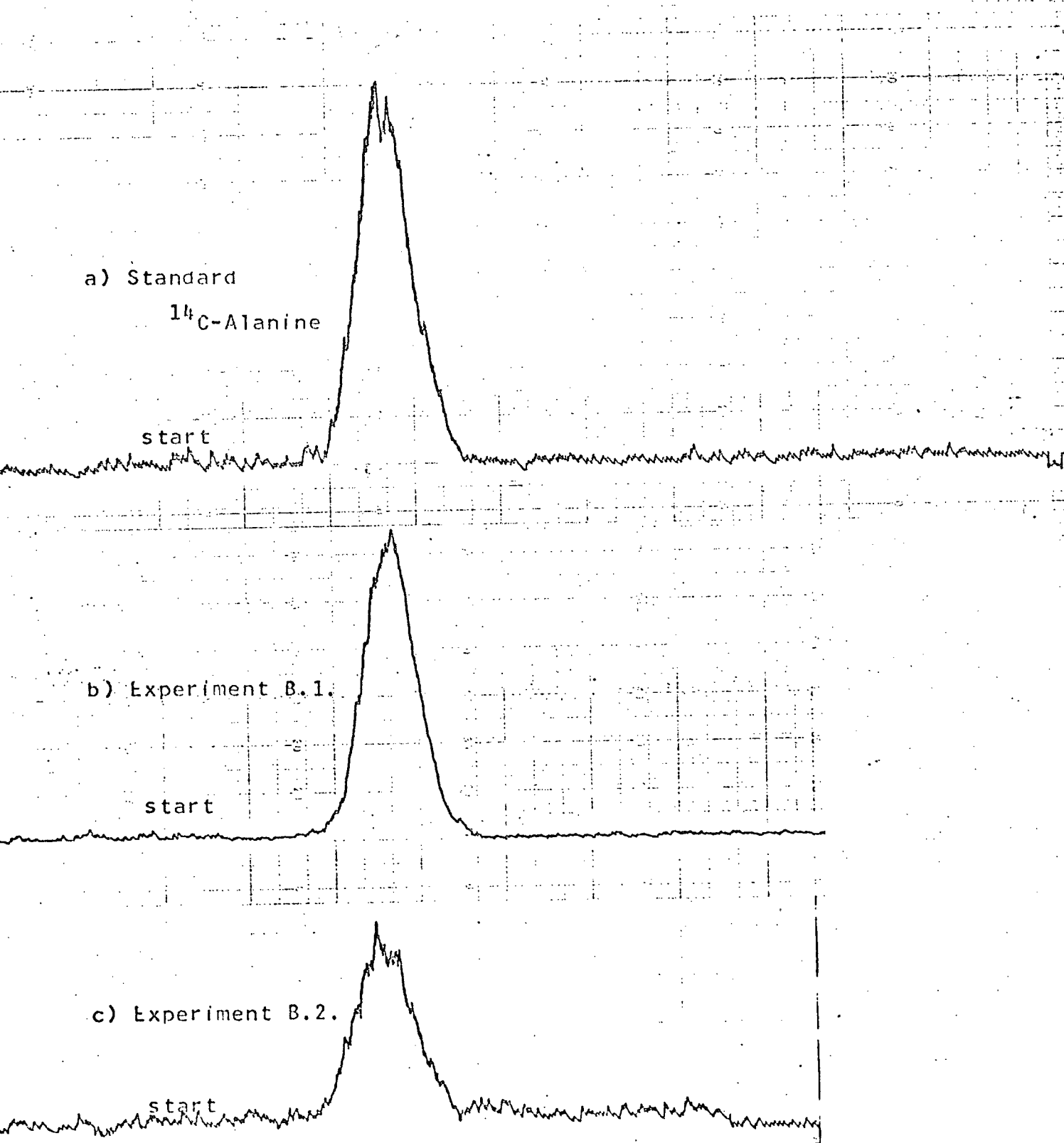


Figure 6.) Activity Curve for ^{14}C -Glucose in Jugular Vein
) Blood (J.V.) Experiment B.1.

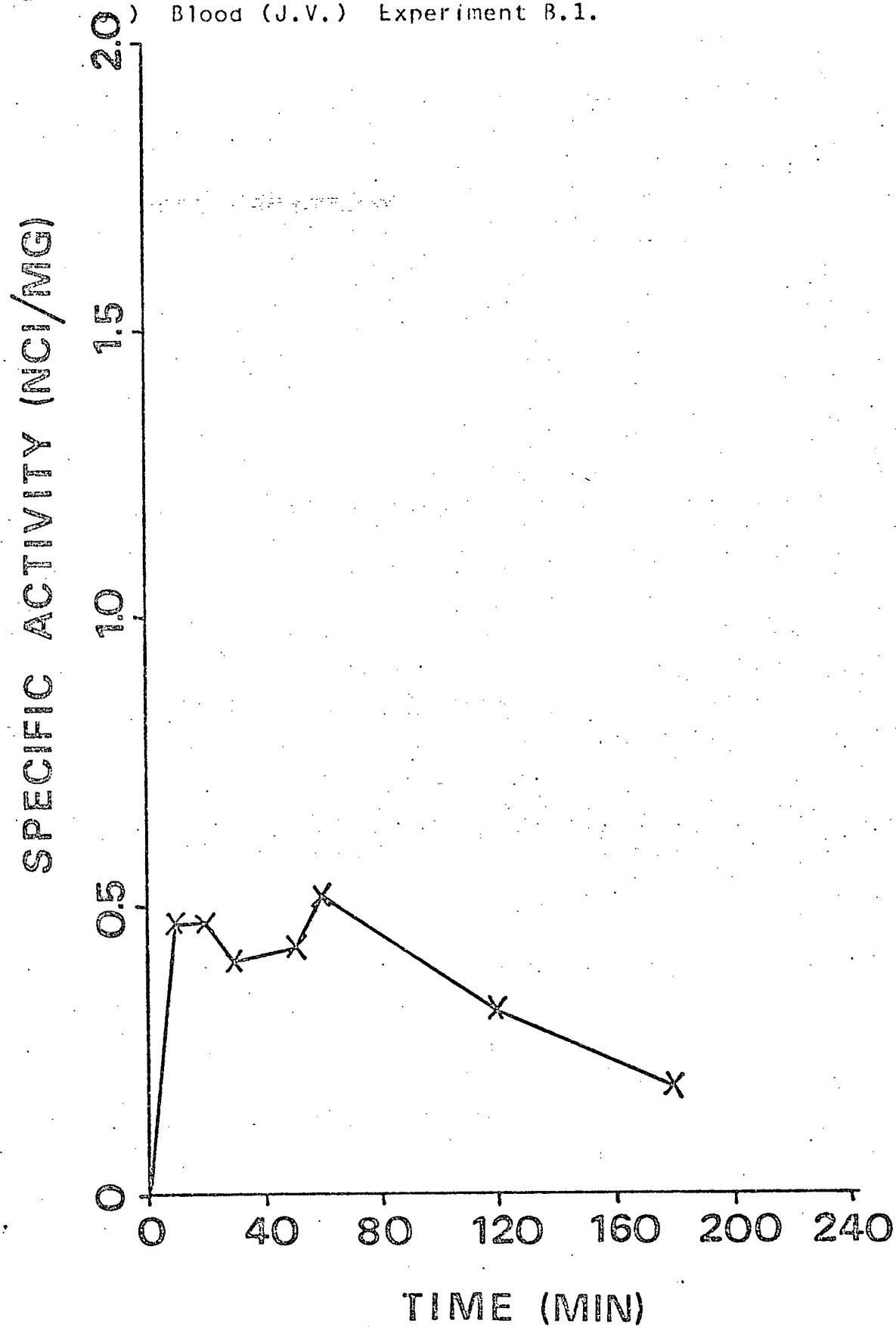


Figure 7.) Activity curve for ^{14}C -Alanine in Jugular Vein (J.V.)
Experiment 8.1.

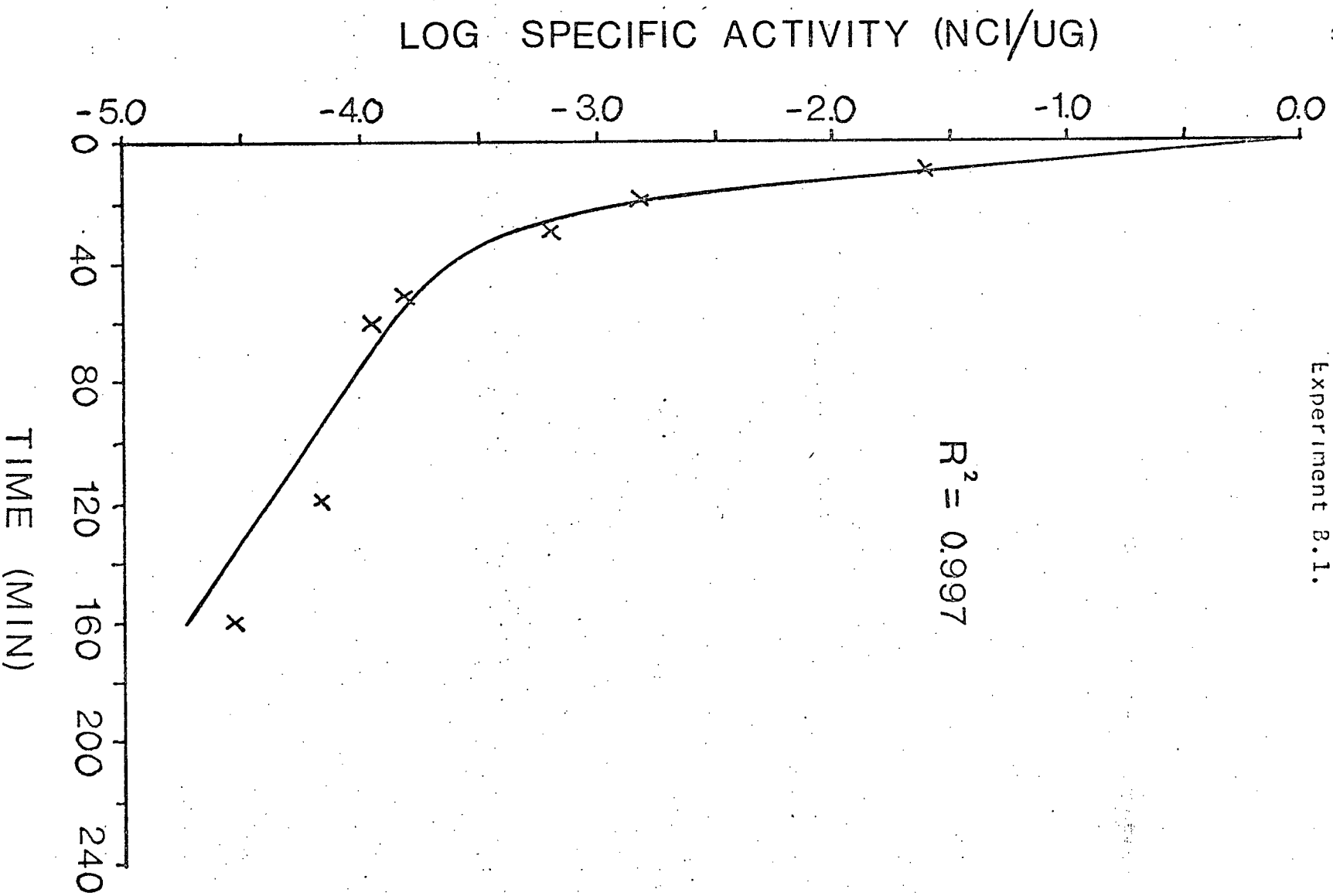


Figure 8.) Activity Curve for ^{14}C -Glucose in Jugular Vein (J.V.)

Experiment B.2.

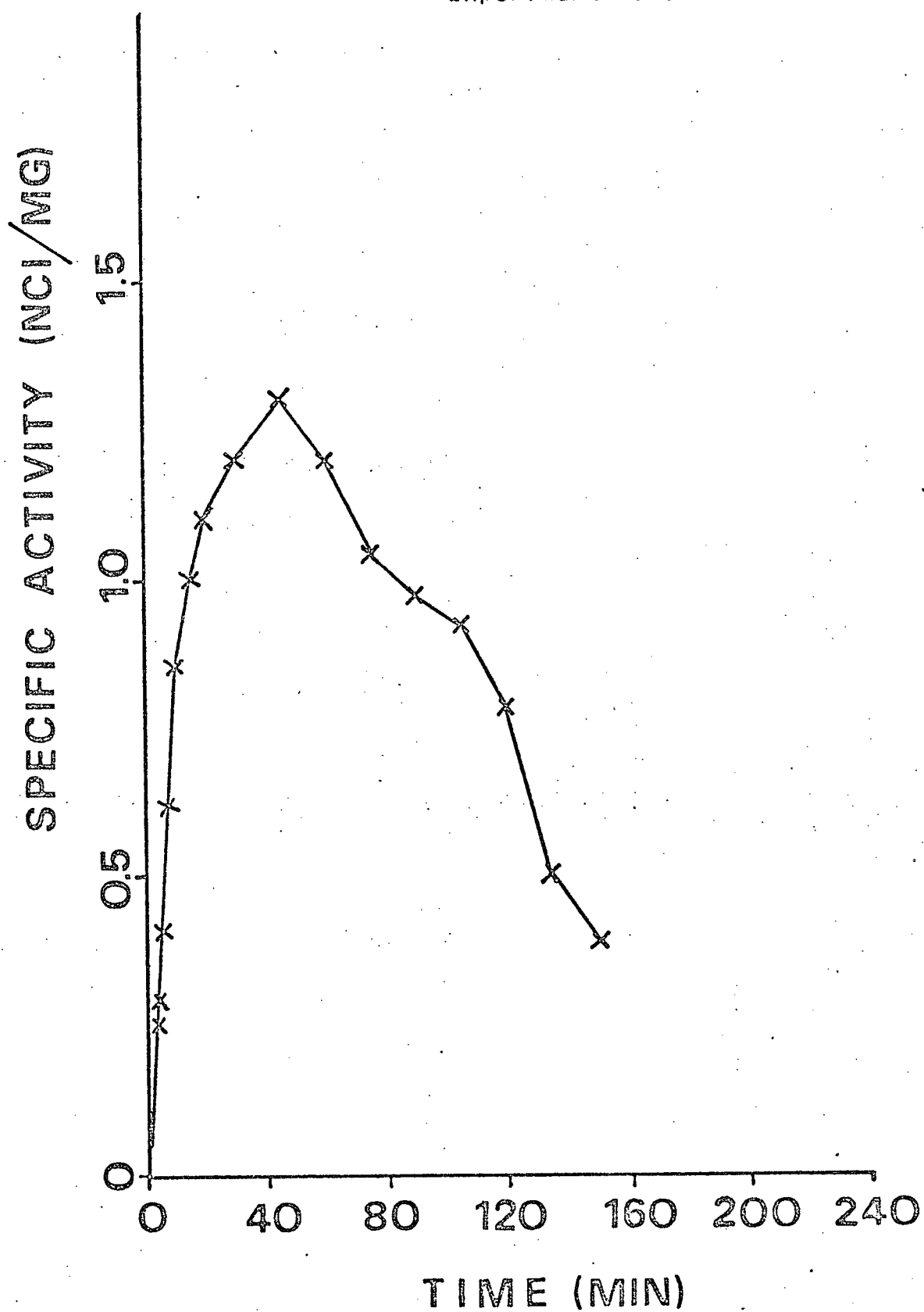


Figure 9.) Activity Curve for ^{14}C -Alanine in Jugular Vein (J.V.)

Experiment B.2.

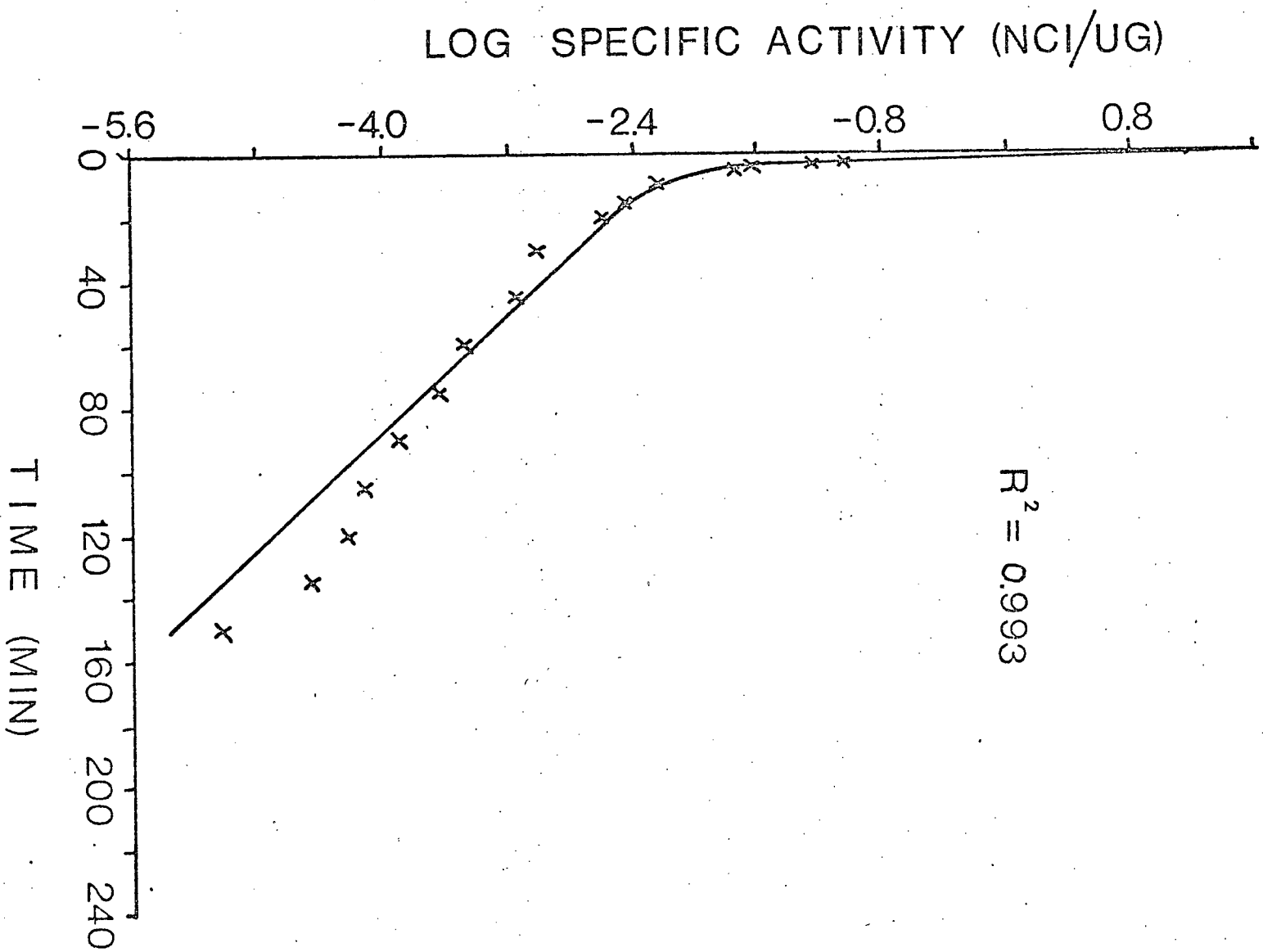


Figure 10a) Activity Curve for 14 -Glucose in Carotid Artery (C.A.)
Experiment P.3.

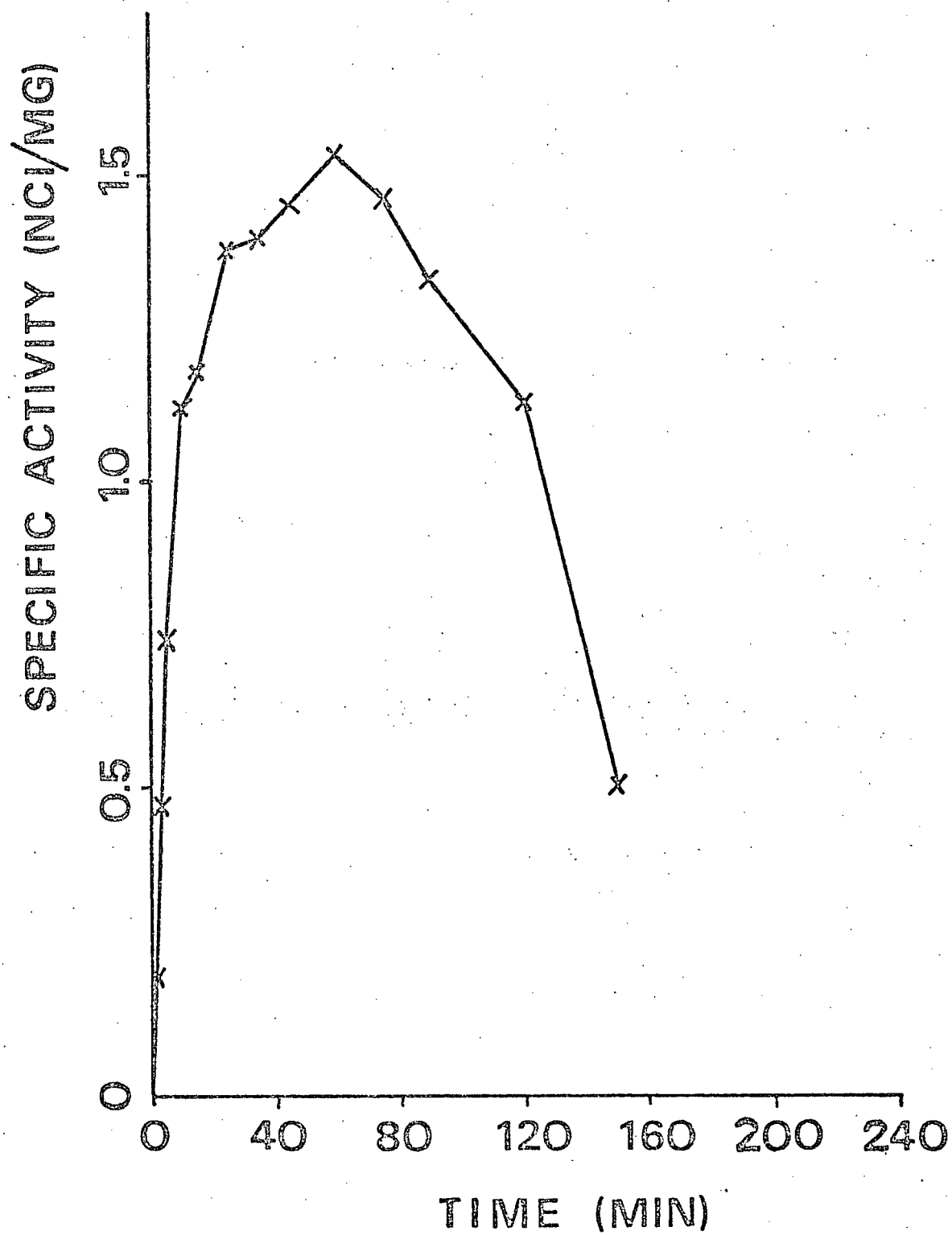


Figure 10b) Activity Curve for ^{14}C -Glucose in Jugular Vein (J.V.)
Experiment B.3.

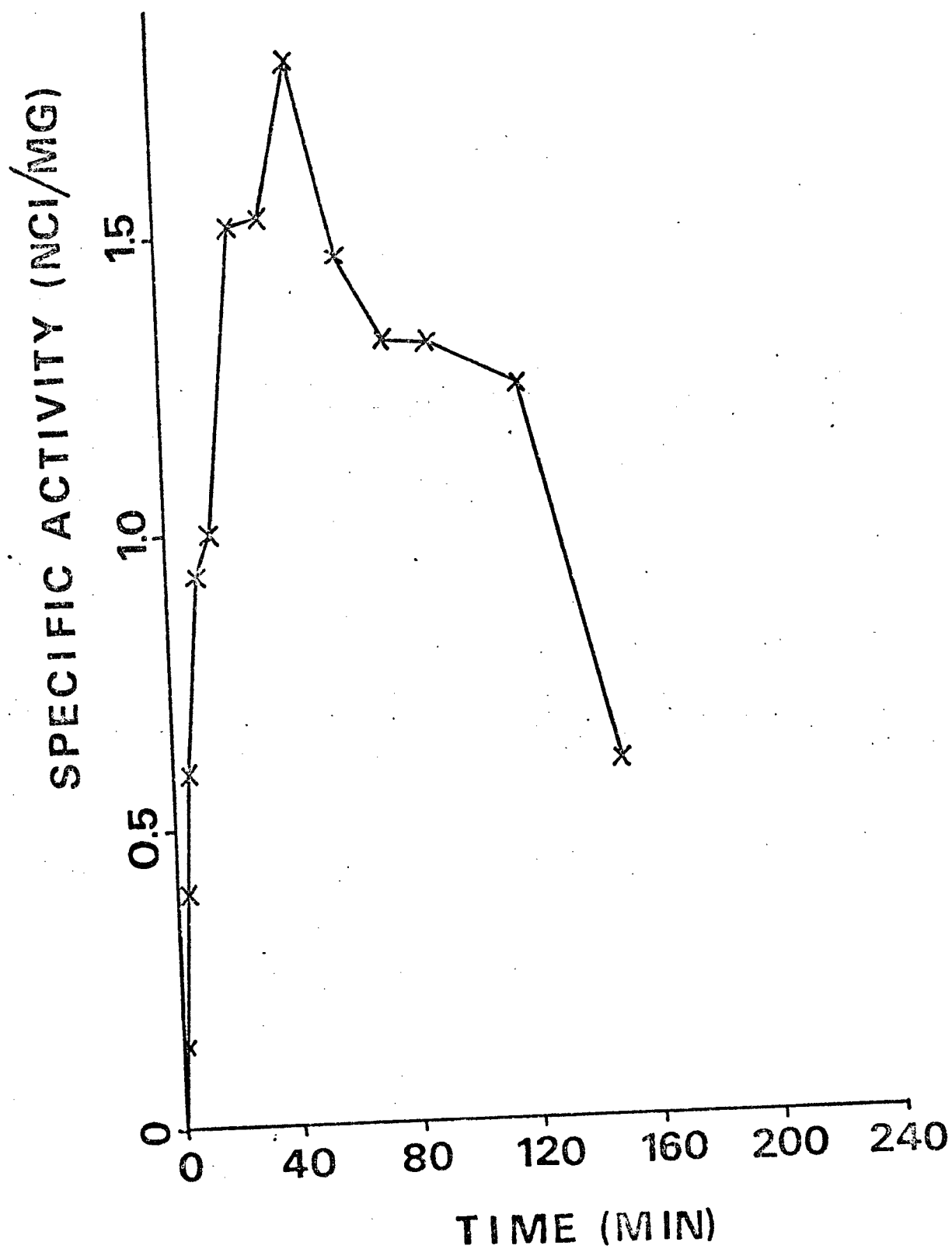


Figure 11 a) Activity Curve for ^{14}C -Alanine in Carotid Artery (C.A.)

Experiment B.3.

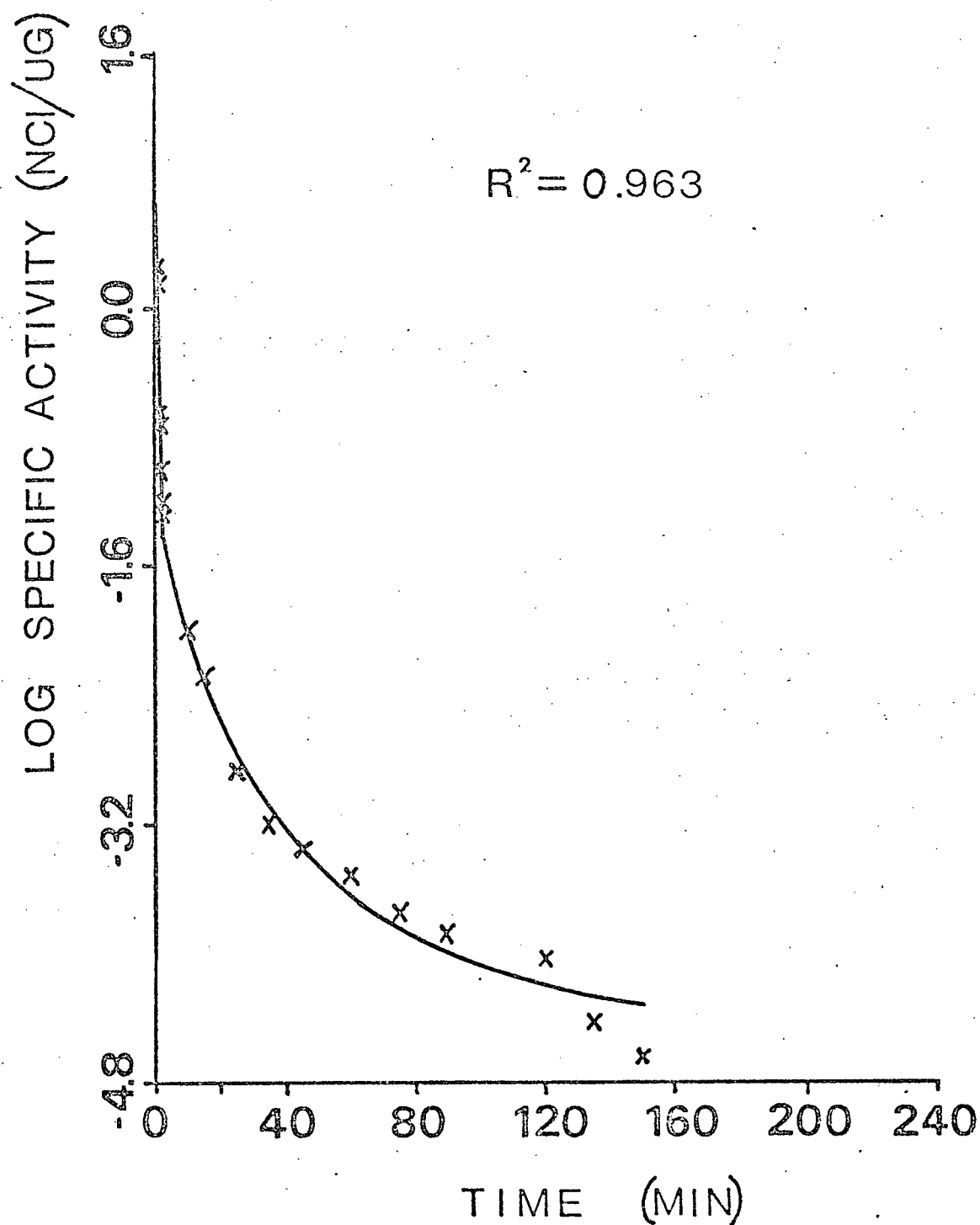


Figure 11b) Activity Curve for ^{14}C -Alanine in Jugular Vein (J.V.)

Experiment B.3.

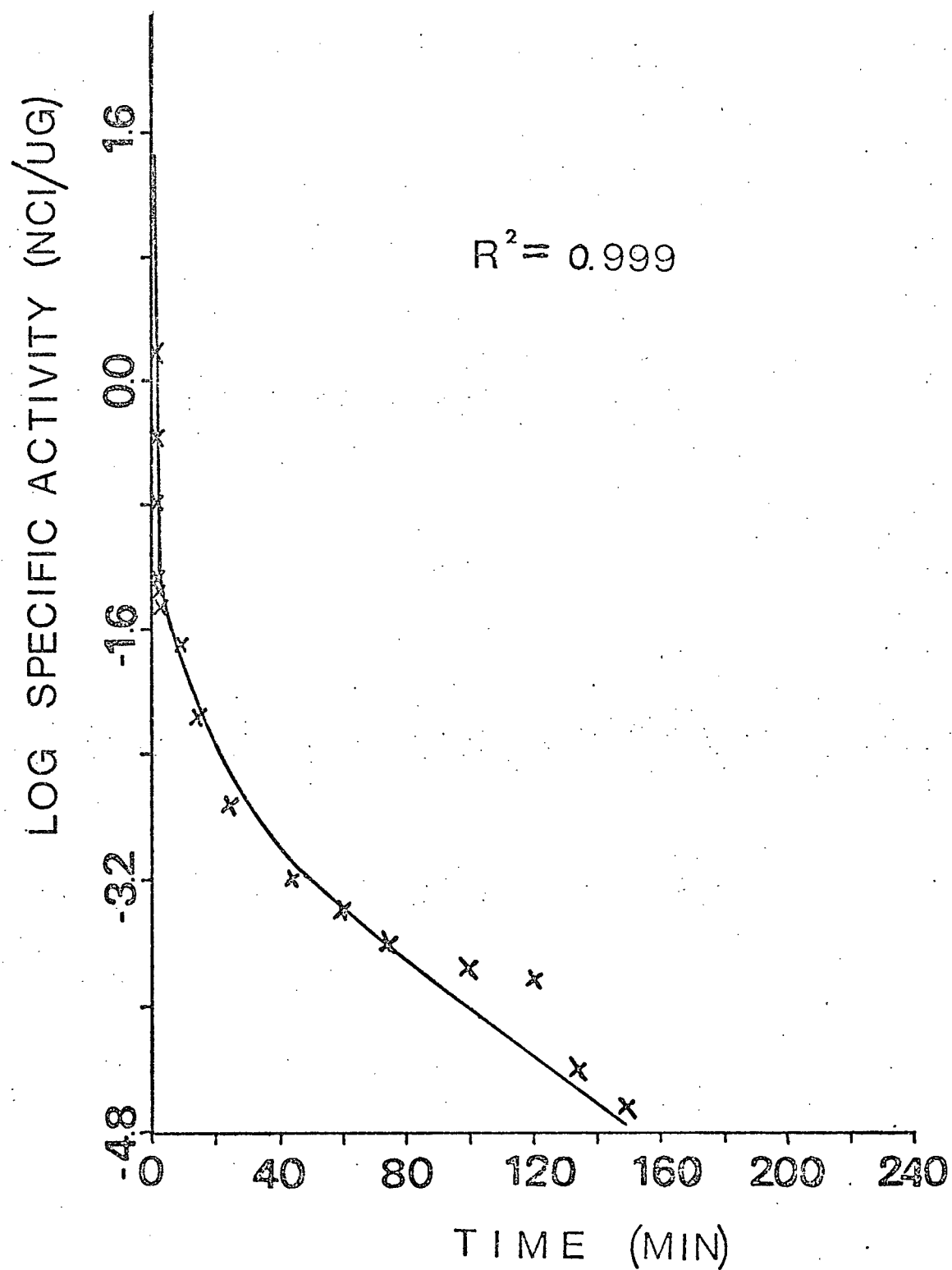


Figure 12.) Activity Curves for ^{14}C -Alanine and ^{14}C -Glucose.

Experiment C.1.

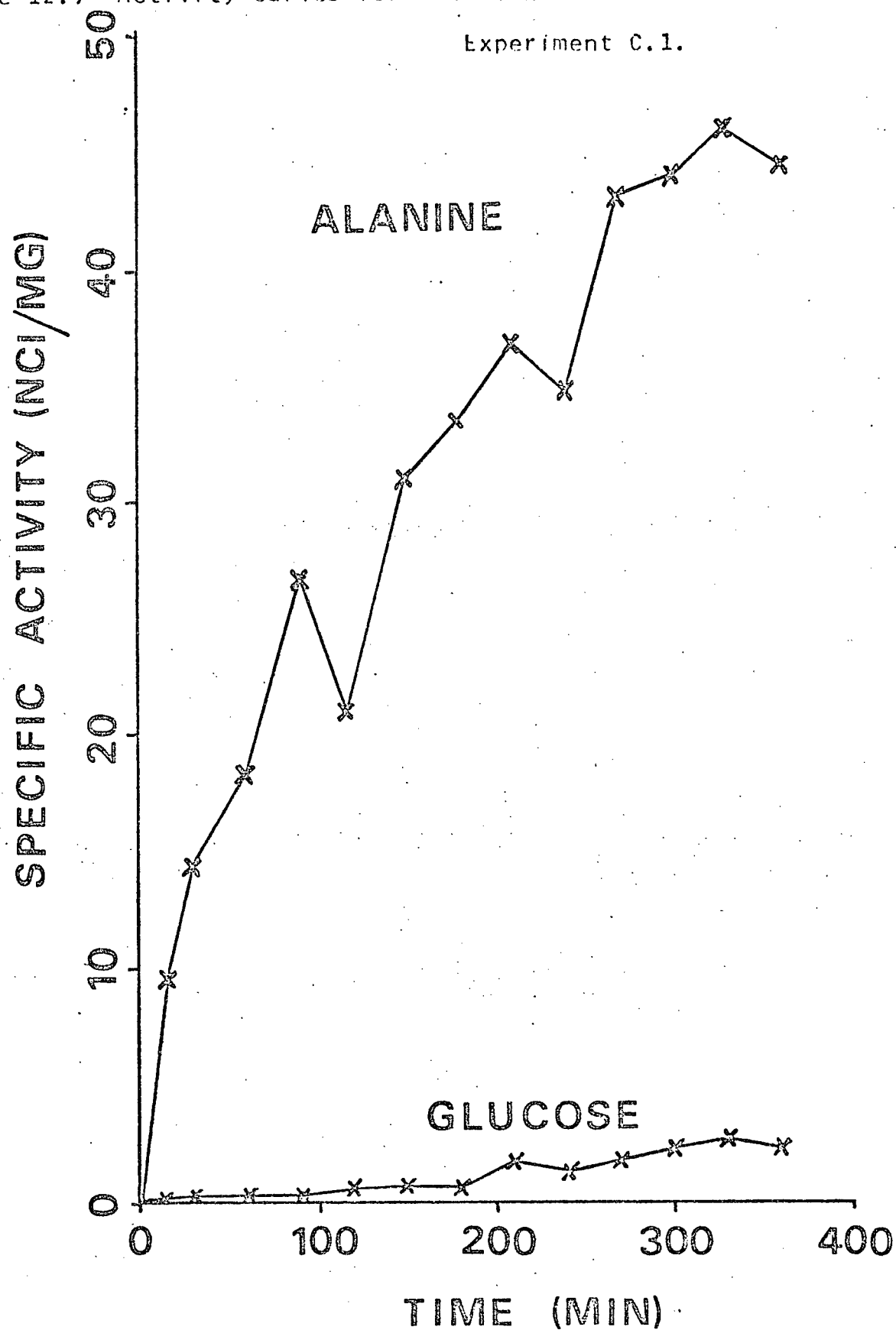


Figure 13.) Activity Curves for ^{14}C -Alanine and ^{14}C -Glucose.

Experiment C.2.

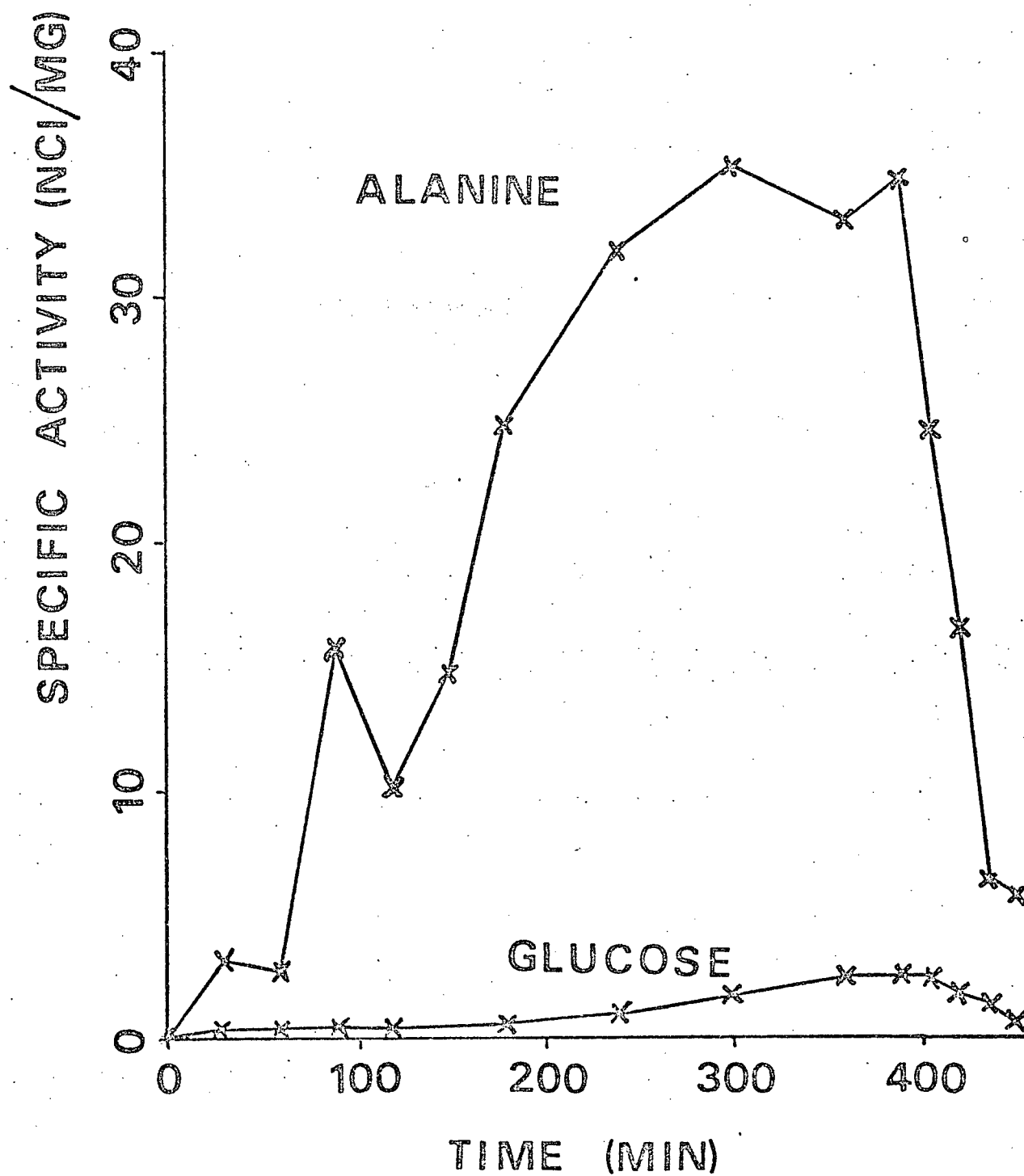


Figure 14. Major Metabolic Pathways In The Liver And Kidneys
Of Ruminants. (Bergman, 1973)

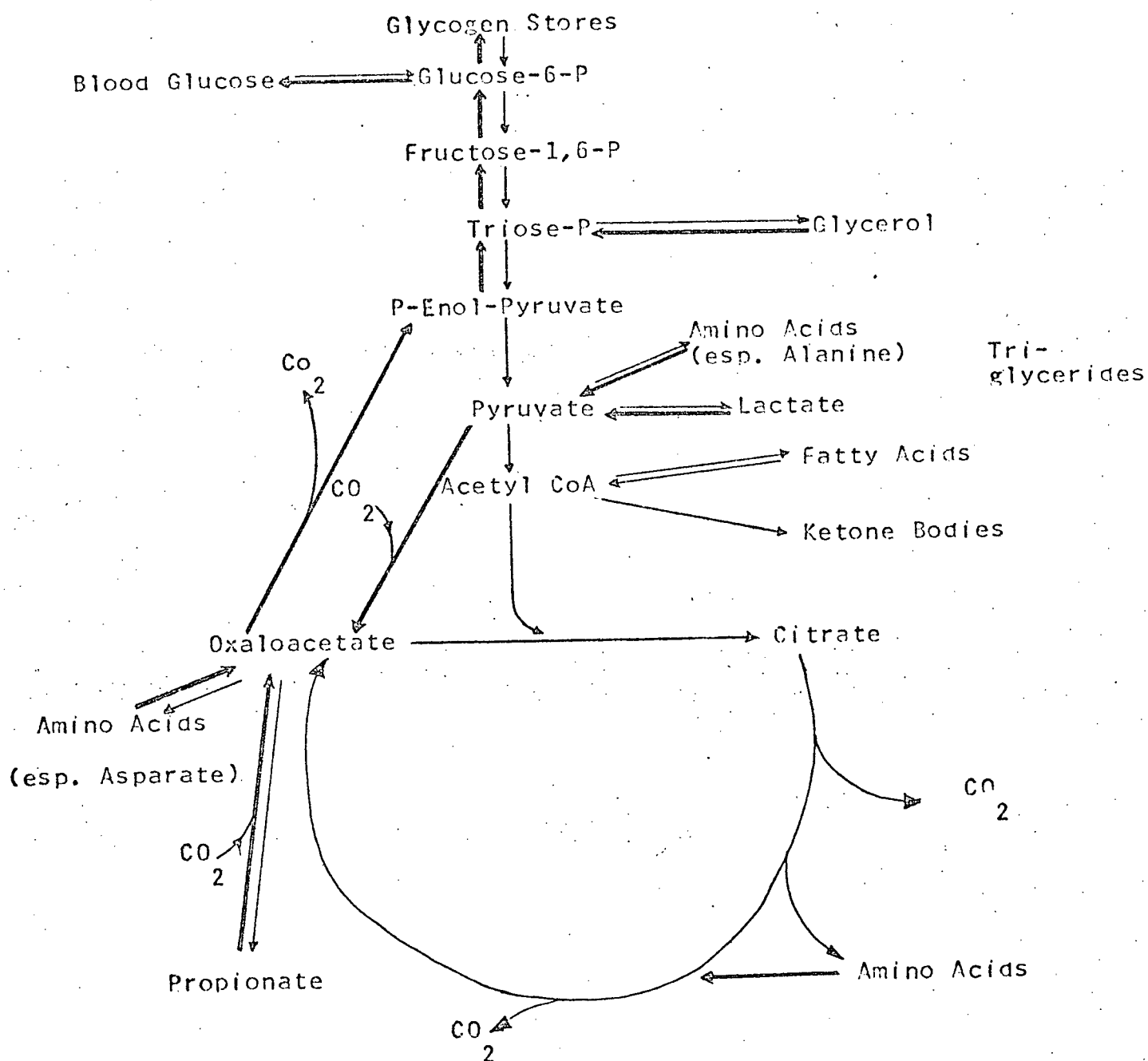


Figure 15.) Schematic Diagram Illustrating the Position of the Sampling and Infusion Catheters. (Katz and Bergman, 1969, pg. 948).

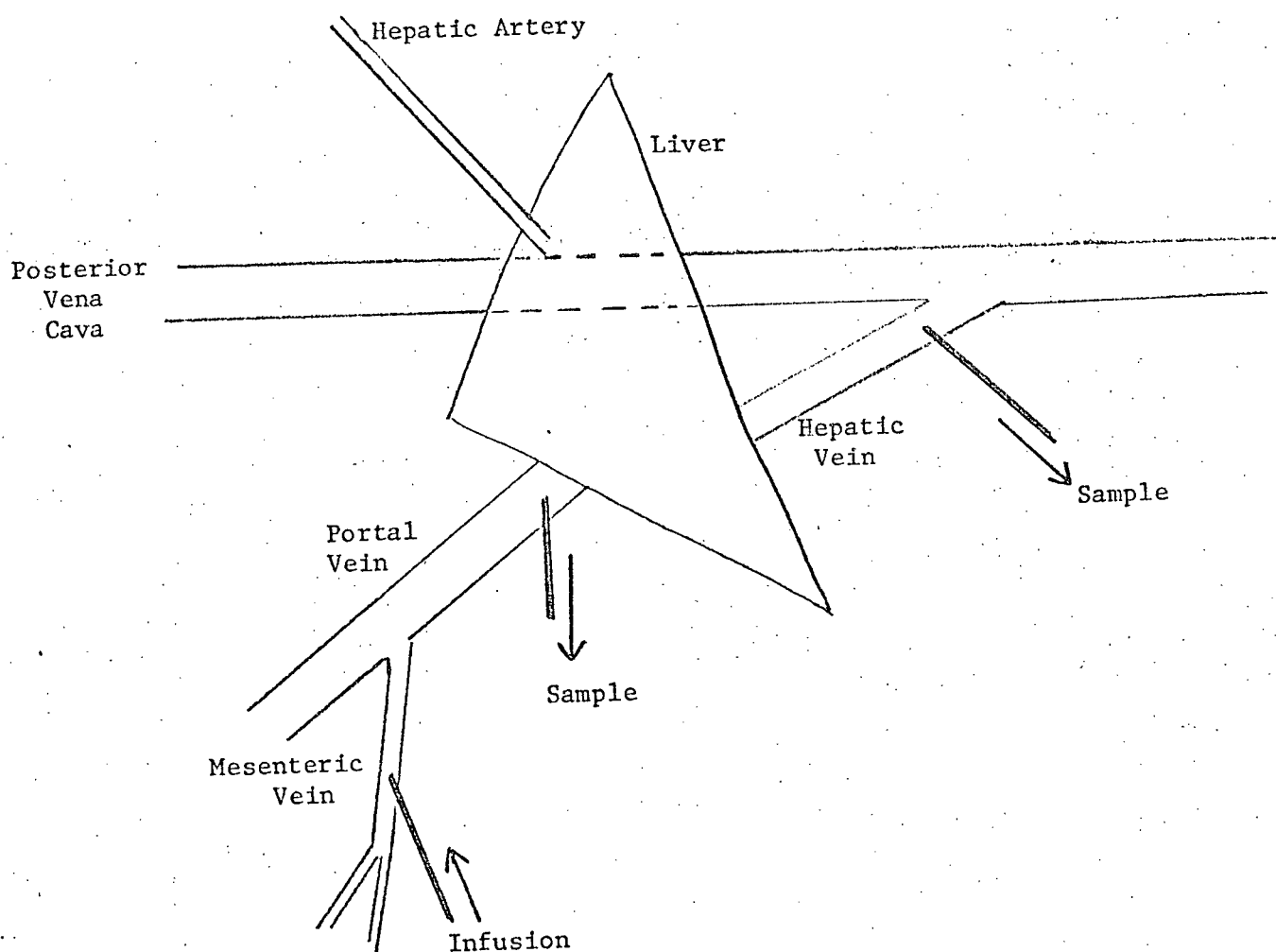


Figure 16. The Glucose Alanine Cycle. (Felig, 1973)

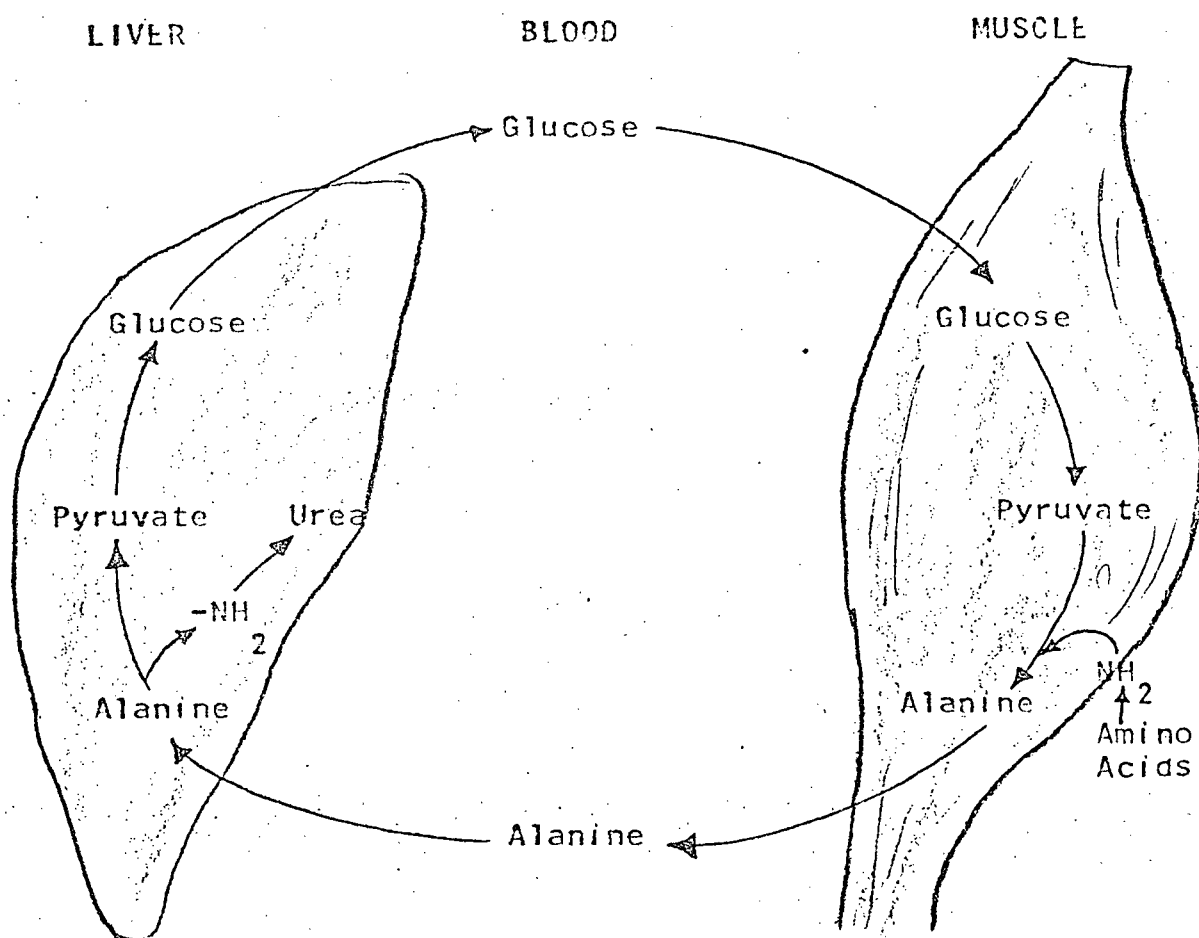


Figure 17. Model for Single Injection (Curve A) and Continuous Infusion (Curve B) of a Dye.
Meier and Zierler, 1954, pg 732.

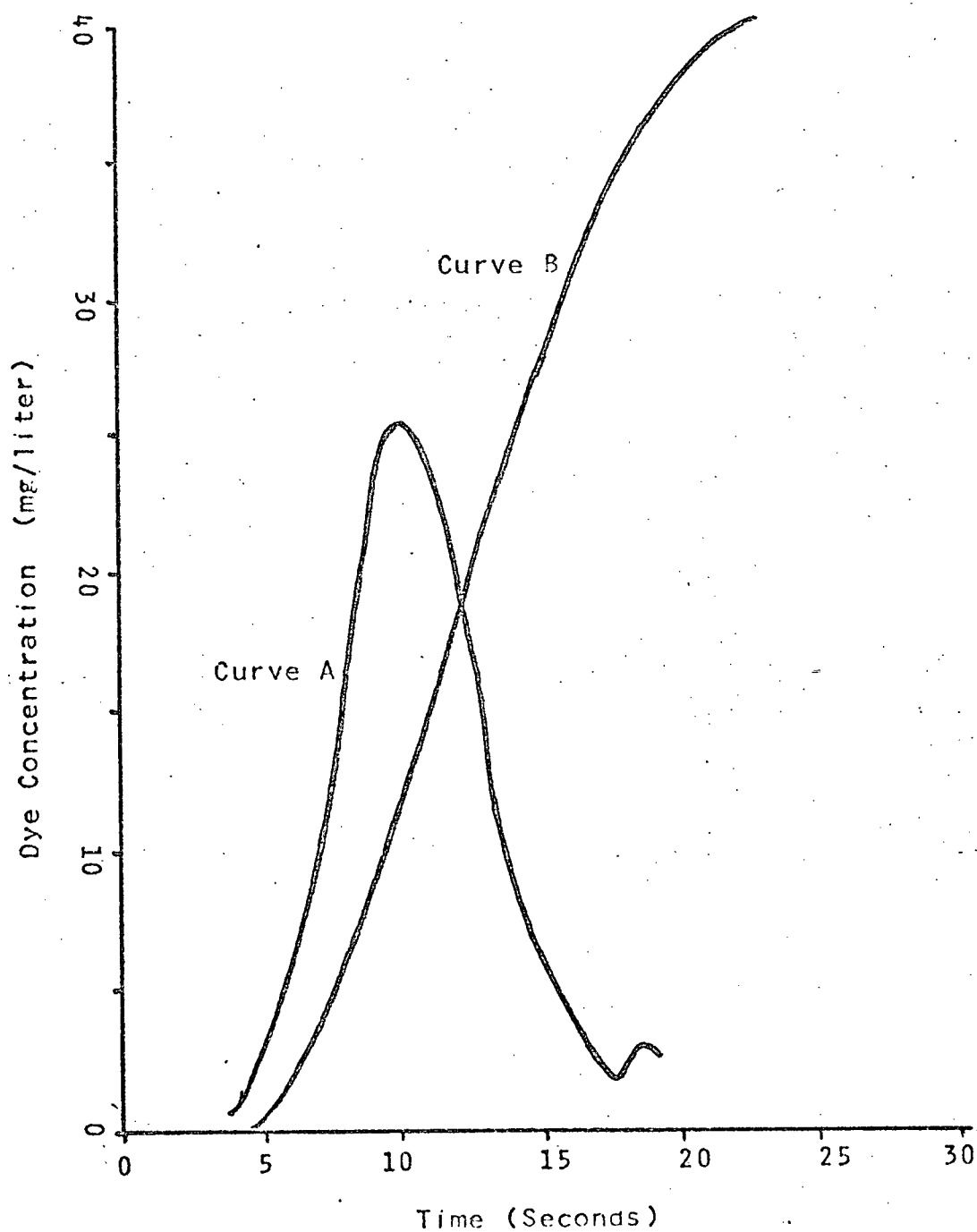
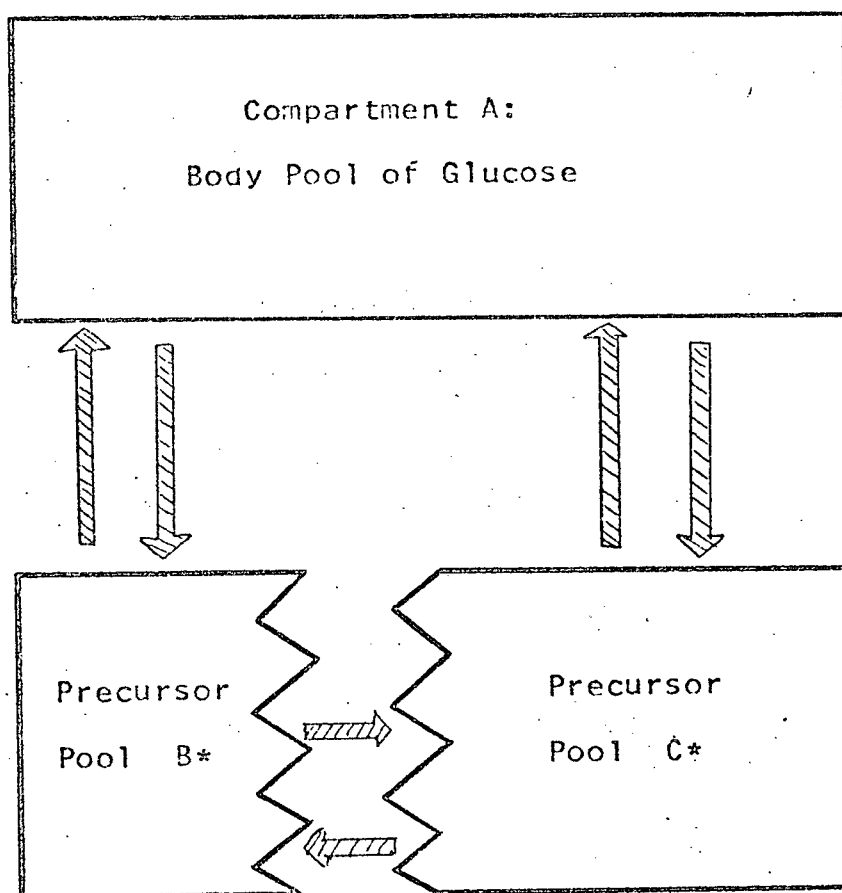


Figure 18. Model for Glucose Metabolism in Sheep.

(Leng, 1970, pg. 216)



*Pools B and C are probably made up of all glucogenic precursors, i.e., glucogenic amino acids, lactate, propionate, and glycerol, and CO_2 and glycogen.

(White et al, 1969)

Table 1(a). Packed Cell Volume Values, Glucose, Alanine and PAH Concentrations in Portal and Carotid Arteries.

Sample Time	Collection Site	Mean PCV (% RBC)	Glucose (mg/100 ml)	Alanine (ug/ml)	PAH (mg/ml)
Control	Carotid Artery	19.25	76.5	10.8	-
1 hr	Carotid Artery	19.75	80.5	10.6	0.006
1 hr 15 min	Carotid Artery	19.8	78.5	10.2	0.0085
1 hr 25 min	Carotid Artery	23.5	77.0	10.4	0.0080
1 hr 45 min	Carotid Artery	29.3	81.0	10.8	0.0080
mean SE		22.32 1.90	78.70 1.28	10.6 0.1	0.0080
Control	Portal Vein	24.0	74.0	11.4	-
1 hr	Portal Vein	23.25	75.0	11.6	0.040
1 hr 15 min	Portal Vein	24.0	79.5	11.5	0.068
1 hr 25 min	Portal Vein	28.0	76.0	12.0	0.0155
1 hr 45 min	Portal Vein	27.6	79.3	11.8	0.0165
mean \pm SE	Portal	25.37 \pm 1.00	76.76 \pm 1.12	11.71 \pm 0.12	0.016

Table 1(b). Calculations for Experiment A.1. (Blood Flow)

Blood Flow : PVPF = $\frac{CI - IR}{C_{PV} - C_A}$ where PCV = 25.37%

$CI = 15.0 \text{ mg/ml}$

$IR = 0.8 \text{ ml/min}$

$C_{PV} = 0.016 \text{ mg/ml}$

$C_A = 0.008 \text{ mg/ml}$

Portal Vein Plasma Flow (PVPF) = $\frac{12 \text{ mg/ml}}{0.0160 - 0.008 \text{ mg/ml}}$

= 1500 ml/min

= 35.01 ml/min/kg BW^{3/4}

Portal Vein Blood Flow (PVBF) = $\frac{1500 \text{ ml/min}}{1 - 0.2537}$

= 2010 ml/min

= 48.08 ml/min/kg BW^{3/4}

Net Portal Metabolism : $P = F_{PV}(C_{PV} - C_A)$

a) Glucose Utilization

$P = 2010 \text{ ml/min}(0.7676 - 0.7870 \text{ mg/ml})$

= -38.99 mg/min = -0.142g/hr/kg BW^{3/4}

b) Alanine Production

$P = 2010 \text{ ml/min}(0.0117 - 0.0106 \text{ mg/ml})$

= +2.21 mg/min = +1.49 mMoles/hr

Table 2. Preliminary Experiment to Test Efficiency of Ion Exchange Chromatography for Separation of Plasma Components.

Sample Fraction	DPM	% Alanine Recovered
U- ¹⁴ C-Alanine (control)	167,971	-
Basic (amino acids)	158,657	94.5
Neutral (sugars)	712	0.4
Acidic (acids)	261	0.2

Table 3. Paper Chromatography Separation of ^{14}C -Glucose

Strip Number	Section Number	DPM		
		^{14}C -Glucose Control	Plasma Sample Exp. B.1	Plasma Sample Exp. B.2
4	1	-	-	-
4	2	-	-	-
4	3	-	-	-
4	4	3.5	-	-
4	5	50.0	4.0	3.0
4	6	1850.0	65.0	58.0
4	7	520.0	2.5	1.0
4	8	25.0	-	-
4	9	2.0	-	4.0
4	10	-	-	-
4	11	-	-	-

Table 4. Data and Metabolic Parameters of Alanine following a Single Injection of U-¹⁴C-Alanine: Experiment B.1.

A) Data

Collection Time (min after injection)	Glucose		Alanine	
	Concentration (mg/100 ml)	Specific Activity (nC/mg)	Concentration (ug/ml)	Specific Activity (nC/mg)
control	54	-	11.2	-
5	38	1.5921	10.5	1.970
10	54	0.4759	12.2	0.410
20	57	0.4702	10.4	0.256
30	58	0.4086	11.4	0.211
52	56	0.4321	11.3	0.142
60	62.5	0.5152	11.7	0.130
120	55	0.3218	11.0	0.081
180	51.5	0.1864	11.7	0.047
mean \pm SE	52.70 \pm 2.40		11.26 \pm 0.17	

B) Metabolic Parameters:

Pool Size (mg of C)	52.88
Space (ml)	4,645.16
Space (% of BW)	13.27
Total Entry Rate (mg C/min.)	8.61
Irreversible Loss (mg C/min)	4.96
Recycling (mg C/min)	3.65
Per cent Glucose from Alanine	3.57
Isotope Dose (uCi)	50.0
Body Weight (kg)	35.0

Table 5. Data and Metabolic Parameters of Alanine Following
A Single Injection of U-¹⁴C-Alanine : Experiment B.2.

A) Data

Collection Time (min after injection)	Glucose		Alanine	
	Concen- tration (mg/100ml)	Specific Activity (nC/mg)	Concen- tration (ug/ml)	Specific Activity (nC/mg)
control	39.8	..*	13.6	..
3.0	26.5	0.39	12.7	1.668
3.5	45.8	0.25	13.6	0.354
4.0	52.0	0.29	13.3	0.289
4.5	48.0	0.41	14.6	0.195
5.0	42.0	0.61	13.0	0.185
10.0	53.0	0.85	14.2	0.106
15.0	53.3	1.00	12.6	0.112
20.0	61.8	1.10	13.8	0.075
30.0	61.0	1.20	14.0	0.050
45.0	60.0	1.30	13.4	0.044
60.0	64.8	1.20	12.9	0.031
75.0	65.0	1.04	13.0	0.027
90.0	62.5	0.97	14.2	0.021
105.0	65.0	0.92	14.6	0.017
120.0	60.0	0.78	13.9	0.014
135.0	66.5	0.50	13.7	0.012
150.0	62.8	0.39	13.5	0.007
mean \pm SE	54.94 \pm 2.52		13.59 \pm 0.95	

Table 5. cont'd.

B) Metabolic Parameters : Two Term Exponential Function

Pool Size (mg of C) = 17.69
 Space (ml) = 1296.85
 Space (% of BW) = 3.71
 Total Entry Rate (mg C/min) = 14.52
 Irreversible Loss (mg C/min) = 7.60
 Recycling (mg C/min) = 6.91
 Per cent Glucose From Alanine = 5.00
 Isotope Dose (μCi) = 50.0
 Body Weight (kg) = 35.0

Three Term Exponential Function

Pool Size (mg of C) = 17.69
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 Recycling (mg C/min) = 6.91
 Per cent Glucose From Alanine = 5.00
 Isotope Dose (μCi) = 50.0
 Body Weight (kg) = 35.0

Table 6. Data and Metabolic Parameters of Alanine Following
A Single Injection of U-¹⁴C-Alanine : Experiment 8.3.

A) Data		Carotid Artery		
Collection Time (min after injection)	Concen- tration (mg/100ml)	Glucose	Alanine	
		Specific Activity (nC/mg)	Concen- tration (ug/ml)	Specific Activity (nC/mg)
control	64.0	-	10.8	-
control	62.5	-	10.5	-
0.33	61.5	0.193	9.5	1.324
0.67	71.0	0.190	10.6	1.177
0.83	66.0	0.197	10.8	0.533
1.13	62.5	0.476	10.6	0.512
1.42	66.0	0.569	12.0	0.361
1.92	64.0	0.565	11.4	0.302
2.17	69.0	0.746	11.6	0.277
5.00	69.0	0.710	9.5	0.259
10.0	59.0	1.124	10.3	0.133
15.0	69.0	1.178	10.4	0.115
25.0	67.0	1.394	10.8	0.065
35.0	71.0	1.382	10.6	0.052
45.0	69.0	1.454	10.6	0.040
60.0	71.0	1.538	10.6	0.033
75.0	71.5	1.467	10.0	0.027
90.0	73.0	1.330	10.3	0.023
120.0	77.0	1.124	10.6	0.020
mean \pm SE	67.52 ± 1.04		10.59 ± 0.14	

Table 6. cont'd

A) Data		Jugular Vein		
Collection Time (min after injection)	Concen- tration (mg/100ml)	Glucose	Alanine	
		Specific Activity (nC/mg)	Concen- tration (ug/ml)	Specific Activity (nC/mg)
control	51.0	-	11.6	-
control	54.0	-	11.3	-
0.33	52.0	0.138	10.8	0.702
0.58	59.0	0.216	10.0	1.184
0.83	62.0	0.233	10.9	0.699
1.95	60.0	0.392	10.3	0.278
2.17	61.0	0.490	10.6	0.264
2.45	59.0	0.532	10.1	0.257
2.75	62.0	0.593	10.3	0.229
10.0	59.0	0.930	9.5	0.181
15.0	54.0	1.018	11.3	0.103
25.0	61.0	1.521	10.3	0.058
35.0	61.0	1.534	10.8	0.042
45.0	61.0	1.800	10.3	0.035
60.0	66.0	1.465	11.3	0.030
75.0	67.0	1.319	11.8	0.023
90.0	66.0	1.316	11.6	0.021
120.0	74.0	1.244	10.3	0.018
mean \pm SE	60.28 ± 1.36		10.73 ± 0.15	

Table 6. cont'd

B) Metabolic Parameters

Carotid Artery

Pool Size (mg of C) =	42.69
Space (ml) =	4026.53
Space (% of BW) =	10.8
Total Entry Rate (mg C/min) =	61.37
Irreversible Loss (mg C/min) =	9.32
Recycling (mg C/min) =	5.20
Per cent Glucose From Alanine =	6.74
Isotope Dose (uCi) =	95.0
Body Weight (kg) =	37.0

Jugular Vein

Pool Size (mg of C) =	17.49
Space (ml) =	1640.91
Space (% Of BW) =	4.44
Total Entry Rate (mg C/min) =	49.78
Irreversible Loss (mg C/min) =	10.03
Recycling (mg C/min) =	39.75
Per cent Glucose From Alanine =	6.78
Isotope Dose (uCi) =	95.0
Body Weight (kg) =	37.0

Table 7. Summary of Metabolic Parameters for the Single Injection Experiments.

Experiment number and type	Blood Source	Sheep Weight (kg)	Plasma Alanine concentration (ug/ml)	Pool Size (mg)	Space (% of BW)	Irreversible Loss (mM/hr)	Total Entry Rate (mM/hr)	Recycling Rate (mM/hr)	% Conversion to Glucose	
S.I.-1	Jugular Vein	35	11.26	.17	52.9	13.27	3.34	5.80	2.46	3.57
S.I.-2	Jugular Vein	35	13.59	.95	17.7	3.71	5.12	9.77	4.65	5.00
S.I.-3	Jugular Vein	37	10.73	.15	17.5	4.44	6.75	33.52	26.77	6.78
	Carotid Artery	37	10.60	.14	42.70	10.88	9.33	61.37	52.04	6.74
mean \pm SE				32.70 ± 17.92	8.07 ± 4.73	6.13 ± 2.54	27.61 ± 25.61	21.48 ± 23.14	5.52 ± 1.54	

Table 8. Data and Metabolic Parameters of Alanine Following a Continuous Infusion of ^{14}C -Alanine: Experiment C.1.

A) Data Carotid Artery

Collection Time (min after start of infusion)	Glucose		Alanine	
	Concentration (mg/100ml)	Specific Activity (nC/mg)	Concentration (ug/ml)	Specific Activity (nC/mg)
control	83.0	-	13.2	-
5	68.0	0.151	12.8	8.4
15	68.0	0.205	14.8	9.6
30	67.0	0.255	14.2	14.4
60	64.0	0.289	15.5	18.3
90	61.0	0.310	14.0	26.7
120	62.0	0.577	16.7	21.0
150	62.0	0.824	13.3	31.1
180	90.0	0.708	14.0	33.7
210	46.0	1.74	14.3	36.8
240	62.0	1.26	14.6	34.6
270	55.0	1.69	15.9	43.1
300	55.0	2.24	15.7	44.0
330	56.0	2.27	15.3	46.2
360	60.0	2.20	16.2	43.4
mean \pm SE	63.96 ± 2.81		14.7 ± 0.29	

B) Metabolic Parameters

Irreversible Loss (mM/hr)	7.23
% Conversion to Glucose	5.07

Table 9. Data and Metabolic Parameters of Alanine Following
A Continuous Infusion of ^{14}C -Alanine. Experiment C.2.

A) Data		Carotid Artery		
Collection Time (min after injection)	Glucose		Alanine	
	Concen- tration (mg/100ml)	Specific Activity (nC/mg)	Concen- tration (ug/ml)	Specific Activity (nC/mg)
control	55.0	-	13.8	-
30	62.0	0.11	13.0	0.615
60	48.0	0.335	12.0	2.50
90	53.5	0.323	15.4	8.57
120	53.5	0.33	14.4	17.92
150	53.5	0.38	14.9	15.37
180	53.5	0.47	14.7	31.97
240	50.0	0.86	13.4	39.70
300	46.5	1.67	14.3	34.75
360	51.0	2.43	15.2	34.56
390	56.0	2.46	14.5	34.21
405	48.0	2.37	14.8	34.41
420	46.4	1.76	14.0	28.70
435	53.0	1.33	14.2	9.36
450	51.0	0.66	14.0	5.57
mean \pm SE	52.06 ± 1.06		14.40 ± 0.26	

Table 9. cont'd

A) Data :

Portal Vein

Collection Time (min after injection)	Glucose		Alanine	
	Concen- tration (mg/100ml)	Specific Activity (nC/mg)	Concen- tration (ug/ml)	Specific Activity (nC/mg)
Control	53.0	-	14.4	-
30	46.5	0.097	14.0	3.07
60	48.0	0.30	13.8	2.61
90	52.0	0.54	15.3	15.7
120	50.0	0.41	15.8	10.0
150	52.0	0.40	14.5	14.6
180	48.0	0.913	15.5	24.7
240	47.0	1.52	15.0	31.8
300	46.5	1.75	14.9	35.2
360	49.0	2.41	16.7	32.9
390	44.0	2.55	20.5	34.9
405	54.0	2.44	17.2	24.4
420	52.0	1.51	14.9	16.6
435	48.0	1.18	21.3	6.2
450	52.0	0.727	14.6	5.7
mean \pm SE	49.46 ± 0.77		15.89 ± 0.53	

B) Metabolic Parameters

Irreversible Loss (mM/hr) = 8.59

% Conversion to Glucose = 5.07

Table 10. Summary of Parameters from Single Injection and Continuous Infusion Experiments.

Parameters of Alanine Metabolism in Sheep

Experiment number and type	Sheep WT (kg)	Plasma Alanine Concen- tration (ug/ml)	Pool Size (mg)	Space (% of BW)	Irre- versible Loss (mM/hr)	Total Entry Rate (mM/hr)	Recycling Rate (mM/hr)	% Conversion to Glucose	
S.I.-1	35	11.26	.17	52.9	13.27	3.34	5.80	2.46	3.57
S.I.-2	35	13.59	.95	17.7	3.71	5.12	9.77	4.65	5.00
S.I.-3	37	10.73	.15	17.49	4.44	6.75	33.52	26.77	6.78
C.I.-4	45	14.70	.29	-	-	7.23	-	-	5.07
C.I.-5	42	14.40	.26	-	-	8.59	-	-	7.20
mean \pm SE			31.69 ± 14.10	7.14 ± 3.07	6.21 ± 0.91	16.36 ± 8.65	11.27 ± 7.74		5.52 ± 0.66