

**Studies on protein turnover
in chicken breast muscle**

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This thesis was composed by myself and all the results therein are based entirely on my own work, except for the ^{15}N analysis of samples from birds injected with ^{14}C and ^{15}N labelled leucine. This work was carried out at the Scottish Research and Reactor Centre, East Kilbride by Dr. Tom Preston.

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ABSTRACT

The aim of the project was to investigate the turnover of proteins in growing fowls; to this end, the fate of leucine in muscle was studied. Following the injection of ^{14}C - or ^{15}N -labelled leucine into growing broiler chickens, the specific content of this amino acid was measured in plasma, total muscle protein, sarcoplasmic proteins and myofibrillar proteins, and in the separated enzymes aldolase, creatine phosphokinase, phosphoglycerate mutase and lactate dehydrogenase. The excretion of ^{15}N , derived from leucine, was also measured. For these measurements, the two forms of labelled leucine were injected together into 18 birds, and assays performed on muscle samples taken after killing the birds at six different time intervals, the longest being 72 hours after injection. Separation methods were developed that would yield the above-mentioned enzymes in sufficient quantity for stable isotope analysis, and in a high degree of purity. A median purity ranging from 90% (for creatine phosphokinase) to 99% (for aldolase) was achieved. The specific activity of ^{14}C , or atom percent excess of ^{15}N , were calculated for each fraction, over the experimental period. Plots of labelling versus time were of an individual shape for each protein or protein fraction. The results obtained indicate that established techniques of pulse-labelling may seriously underestimate the rate of protein synthesis. The physiological significance of these results is discussed.

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List of abbreviations

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
CM-cellulose	Carboxymethyl-cellulose
CPK	Creatine phosphokinase (EC 2.7.3.2)
EDTA	Ethylene diamine tetra acetic acid
FDP	Fructose 1,6-bisphosphate
FPLC	Fast Protein Liquid Chromatography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (EC1.2.1.12)
LDH	Lactate dehydrogenase (EC1.1.1.27)
MES	N-morpholino ethane sulphonic acid
NAD ⁺	Nicotinamide-adenine dinucleotide (oxidised)
NADH	Nicotinamide-adenine dinucleotide (reduced)
NADP	Nicotinamide-adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced)
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
PEP	Phosphoenolpyruvate
PGM	Phosphoglycerate mutase (EC2.7.5.3)
PK	Pyruvate kinase (EC 2.7.1.40)
PMSF	Phenylmethyl sulphonyl fluoride
tris	Tris(hydroxymethyl) amino methane

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Chapter 1

Introduction

1.1 Early theories on protein metabolism

1.1.1 The theory of Voit

Between 1881 and 1905 a number of theories were proposed on how protein is metabolised by animals. In 1881 Carl von Voit suggested that protein from the diet was absorbed into the blood, becoming "circulating protein". This could be catabolised under the influence of "living protoplasm" without actually becoming an integral part of the protoplasm. The theory envisaged "living protoplasm" to be in a state of suspension, while circulating protein was in solution. Voit thought that a small amount of living protoplasm dies each day and goes into solution, joining the circulating protein derived directly from the diet. An equal amount of circulating protein is taken up by the protoplasm each day to replace that loss. Voit proposed that the loss was due to damage caused by "wear and tear".

Unlike many earlier theories (see Cathcart, 1912) Voit's theory was based on experimental work. Many experiments were carried out on the blood of adult dogs. Arterial/venous (A.V.) differences were carefully studied. It was found that during starvation about one per cent of tissue protein was broken down per day. When protein was eaten in an amount equal to twelve per cent of the body protein, the rate of breakdown was fifteen times greater than during starvation. From this he calculated that the breakdown in tissues was insignificant compared to that of circulating protein introduced as food.

1.1.2 The theory of Pflüger

In 1893 Pflüger put forward an opposing theory, which was a modification of a much older one advanced by Liebig (1842). Pflüger believed tissue proteins were readily catabolised, while food proteins were not. That is, circulating protein was fairly stable towards oxidising agents whereas living protoplasm was unstable and susceptible to oxidation. The greater lability of tissue proteins, according to Pflüger, was the result of the presence of cyanogen radicals in their molecules. He concluded that all protein broken down must first become an integral part of the bioplasm or living tissue.

Most of Pflüger's theory was based on work carried out in his laboratory by Schöndorf. Blood was taken from a starving dog and circulated through the liver and hind limbs of a well-fed dog. The concentration of urea in the blood was increased. If blood from a well-fed dog or starved dog was circulated through the liver and hind legs of a starving dog no increase in urea was observed. From this it was suggested that it is the state of nutrition of the muscle cell, and not the circulating protein, which is the determining factor in protein breakdown. However present views would suggest that the enzymic state of the liver, and particularly its content of the enzyme arginase, would be the determining factor in producing these results. (For review see Cohen and Brown, 1961).

1.1.3 The theory of Folin

At the time that Voit and Pflüger were formulating their theories, it was the accepted view that quantitative changes in total protein metabolism had no appreciable effect on the percentage

composition of urine. It was not until 1905 that Otto Folin seriously challenged this idea. Folin carried out a large number of experiments on urine from human subjects on standard diets either rich in nitrogen, or poor in nitrogen, but all relatively free from purine, creatine and creatinine. He found that whatever the amount of protein in the diet the amount of creatinine and neutral sulphur remained constant in the urine. However when the amount of dietary protein was altered changes in the excretion of urea and inorganic sulphates were observed and Folin proposed that there might be two types of protein metabolism, one that he called endogenous protein metabolism and the other exogenous protein metabolism. Endogenous protein metabolism was thought by Folin to set the limit for the lowest level of nitrogen equilibrium attainable and was related to the creatinine and free sulphur concentrations in urine. This type of metabolism was thought by Folin to be indispensable for the continuation of life. The protein involved in exogenous metabolism was related to the urea and inorganic sulphate concentrations in urine and was not essential to the organism or to be more specific its nitrogen not needed. The organism excretes this excess nitrogen as urea leaving the carbon chains to be used as fuel.

Folin compared his two types of protein metabolism with those of Voit and Pflüger - did Pflüger's views cover Folin's endogenous metabolism and did Voit's views represent the variable exogenous metabolism? Folin thought the endogenous metabolism was, more or less, identical to Pflüger's idea of protein metabolism, since it represents the breakdown of living protoplasm. It differs, however, because it yields much less end product, i.e. Folin's endogenous

metabolism accounts for only a very small part of total protein metabolism, whereas Pflüger thought all protein was metabolised in this way.

In the case of exogenous metabolism, Folin saw nothing in common between his and Voit's ideas on circulating protein. However, taken as a whole, both theories are not so different. Both use the idea of a small but constant breakdown and replacement of tissue protein due to "wear and tear". Both thought that the majority of dietary protein was not incorporated into the bioplasm but was broken down elsewhere (in the blood according to Voit or in the intestine and liver according to Folin).

Folin had shown that changes in protein metabolism could be reflected in the composition of urine. He went on to say "this fact constitutes, it seems to me, definite proof that neither the theory of Voit nor that of Pflüger can be correct". However both Voit and Pflüger could have interpreted Folin's data according to their own theories. Folin did not disprove either theory by his findings, although he presented his paper as though he had. In fact, Folin had himself interpreted the data wrongly; it is now known that creatinine is a metabolite of creatine and not protein, and that in man uric acid (which Folin associated with protein) is a product of nucleic acid degradation. However Folin's theory was generally accepted for many years.

The chief defect of Folin's theory was that it could not be used to predict the minimum protein intake needed to maintain nitrogen equilibrium in an adult species. Experiments over many years have shown conclusively that as the protein intake in (human) diets is

increased, the nitrogen output increases, and equilibrium is only established at a much higher level than would be predicted (Beattie et al 1947; Keys et al, 1955; Munro, 1964; Keis et al, 1965; Elwyn et al, 1979). The current interpretation of this phenomenon would be that (a) food proteins are broken down completely to amino acids before being absorbed; (b) the hepatic enzymes of nitrogen catabolism are partially inducible, and that their concentration responds to some as yet unidentified component of the circulating amino acid pool. Thus as the absorption rate of this signal increases, so (after a delay) does the rate at which all the amino acids are abstracted from the circulating pool and irreversibly converted to urea, or uric acid in fowls. Consequently the amount of amino acids supplied in the diet to maintain long-term equilibrium is greater than would be predicted from the basal rate of protein degradation (the "wear and tear breakdown").

In addition to this, these experiments refer only to adults. Waterlow and Jackson (1981) have shown that an increased rate of growth in infants is accompanied by an increase in the rate of protein synthesis that is greater than that required to account for the net increase in weight, i.e. turnover of protein is increased. The reason for this is unknown. Conversely, in a wasting disease, such as Duchenne's muscular dystrophy, it may be the protein synthesis rate (in muscle) that decreases rather than the degradation rate that increases (Schneible and Young, 1984) although this view does not go unchallenged (Kay, 1984; Millward et al, 1980; Simon et al, 1962; Garber et al, 1980).

1.1.4 The theory of continuing metabolism - The dynamic state

One of the first studies to propose the concept of a "continuing metabolism" was that of Borsook and Keighly (1935). They envisaged a system in which exogenous and endogenous nitrogen contributed equally to the dynamic state of nitrogen metabolism. However, their conclusions were based on indirect evidence from experiments on the rate of metabolism of nitrogen and sulphur. They reasoned that if the total protein metabolised on any day is mainly from the diet, then when nitrogen equilibrium is maintained with a non-sulphur containing nitrogenous substance (e.g. glycine, ammonia, alanine) the sulphur excretion will fall to the endogenous level. Moreover on resumption of a normal diet it will correspond to the sulphur intake. On the other hand, if there is a large and continuing metabolism, the sulphur excretion will be above the endogenous level on a sulphur diet. On the resumption of a normal diet the sulphur excretion on succeeding days will be less than on the days prior to the interruption due to the replenishment of the tissue store of sulphur. This assumption, although tentative, was then used to conclude that nitrogen metabolism in tissues is not due to "wear and tear" as outlined by Folin but is due to the level of nitrogen balance, which is in turn set by the animal. In Borsook and Keighley's experiments the "animal" was adult men.

One unanswered question is, what is the endogenous level of sulphur excretion? This information is essential to be able to say that in continuing metabolism the sulphur excretion should be higher than the "endogenous level". Moreover the workers acknowledged the conclusions of Lee and Lewis (1934) that if a protein diet deficient

in sulphur is fed to an animal, assuming turnover, the composition of tissue protein (i.e. liver) does not change. If this were the case why do they assume that changes in the rate of sulphur excretion are a reflection of protein turnover rates in tissue.

1.1.5 Schoenheimer and ^{15}N studies

It was not until isotopic labelling techniques were used that nitrogen fluxes could be monitored directly. Schoenheimer's group, from 1938 to 1941, carried out a series of experiments using the isotope of nitrogen of mass 15 (^{15}N).

Rittenberg and Schoenheimer (1939) established that in non-growing adult rats dietary glycine labelled with ^{15}N could be involved in hippuric acid synthesis, although the majority of glycine is supplied by the tissues.

The conditions of the experiment were designed to stimulate hippuric acid synthesis and make it possible to trap glycine as hippuric acid. They found that the abundance of ^{15}N in hippuric acid was roughly one third of that administered as ^{15}N glycine. Unfortunately the conditions of the experiment were very artificial. The rats were starved for twelve hours prior to the experiment and then given a large dose of benzoic acid (300 mg) and an excess of isotopic glycine (2 moles per mole of benzoic acid). Under more natural conditions the results might have been very different, which makes it difficult for the authors to prove dietary glycine is normally involved in hippuric acid synthesis. In addition, no attempt was made to discover where the remaining two thirds of labelled glycine had gone.

In an earlier experiment the group had proved that isotopic

nitrogen - labelled ammonium citrate was used in glycine synthesis (Forster et al, 1939). They also found that glutamate, aspartate, proline, histidine and arginine had increased ^{15}N concentrations. Lysine was the only amino acid isolated which did not contain an increased heavy nitrogen concentration. Glutamate and aspartate had the highest ^{15}N concentrations of all.

These two experiments, taken together, established that the amino group of several amino acids can, at least in the artificial conditions of feeding ammonium salts, arise from the dietary ammonia. One of these amino acids is glycine. They also established that both free exogenous glycine and glycine coming from endogenous sources is lost from the body when a metabolic "trap" (i.e. benzoic acid) is provided.

These data foreshadowed the idea of a "pool" of free glycine (and by implication of other amino acids too), although the authors did not interpret their findings in these terms. Perhaps in retrospect the outstanding contribution of these papers was to focus attention on the amino acid pools rather than concepts, such as "protein", "nitrogen", or "sulphur".

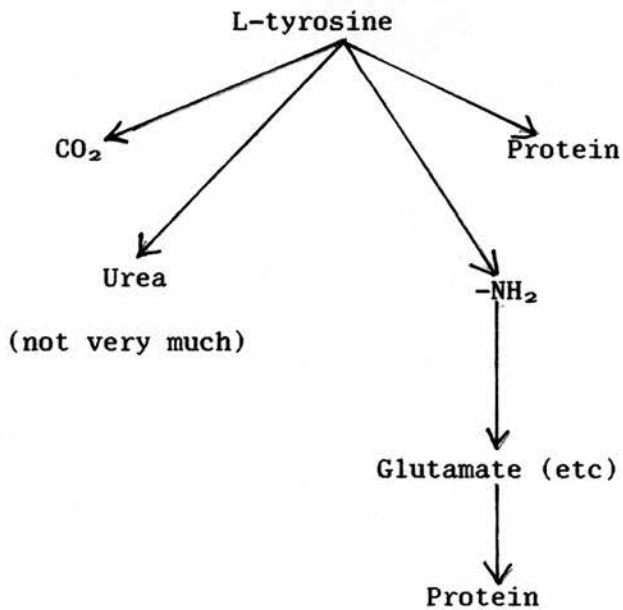
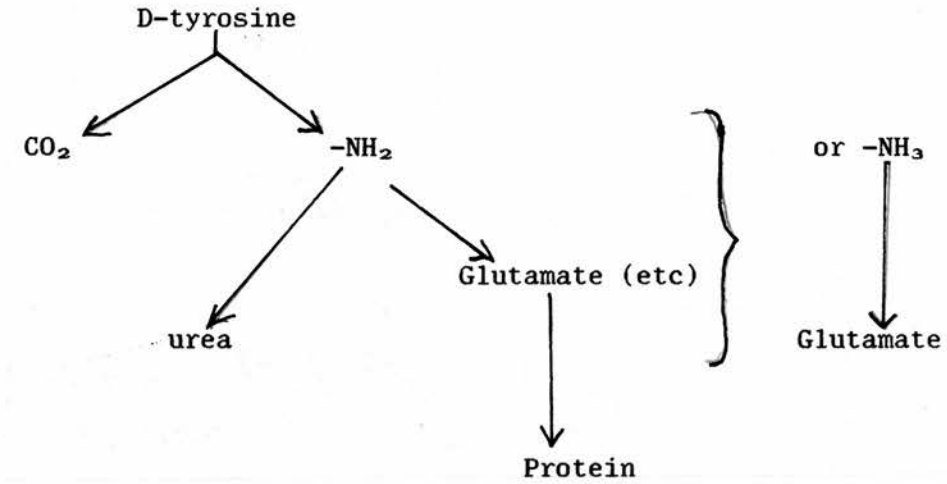
The next question addressed by Schoenheimer was whether dietary amino acids could be used in tissue protein synthesis. That is, in a state of nitrogen equilibrium, is the nitrogen in urine derived entirely from food protein or is dietary nitrogen deposited, with the liberation of tissue nitrogen for excretion?

Adult rats were fed a stock diet for ten days, containing an excess of ^{15}N -labelled (dl) tyrosine (Schoenheimer et al, 1939a). Fifty to sixty per cent of the label was recovered in the urine as

urea. Most of the remainder was found in protein in the tissues with a small amount in non-protein nitrogen. The concentration in the liver was found to be high compared to that of the remaining carcass, although the liver was the only organ to be analysed separately. Red blood cells contained almost no label.

In their calculations no account was taken of the fact that the isotopic tyrosine was racemic. In their conclusion they acknowledge this and accept that the unnatural D tyrosine may have been metabolised differently. They suggest that the isomer may be converted to the natural form or that the ^{15}N may have been removed and replaced by normal nitrogen, thus diluting the isotopic concentration of deposited tyrosine. It has subsequently been shown that the rat can utilise both D and L tyrosine although the pathways for each differ (Bubl and Butts, 1948). It would appear that, since the group already knew that D-tyrosine would not be found in protein, they may have been thinking of a pathway similar to that in figure 1.1.1.

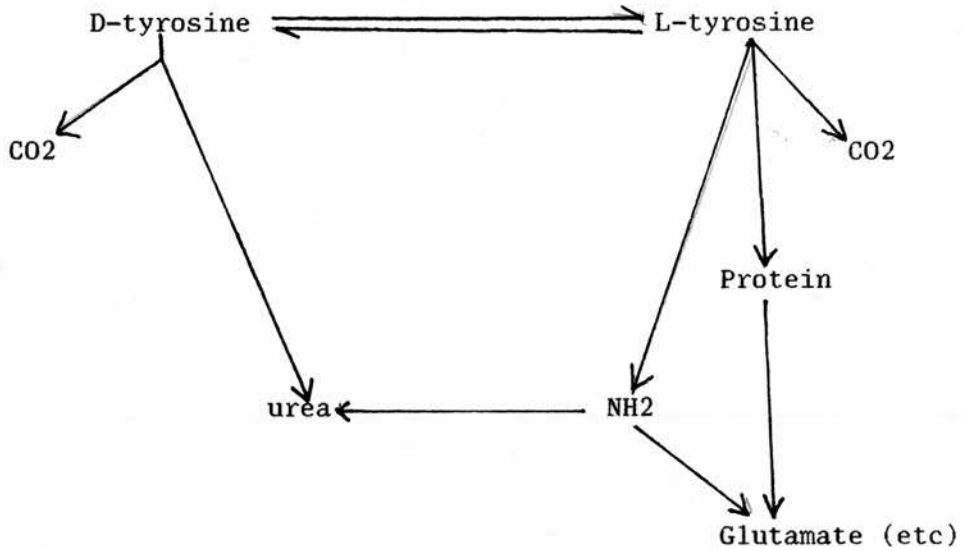
Figure 1.1.1



1.1.1 Pathway of D and L tyrosine metabolism perhaps envisaged by Schoenheimer and co-workers.

However with the knowledge that the rat has an active tyrosine racemase the pathway was probably similar to figure 1.1.2.

Figure 1.1.2



1.1.2 Pathway of D and L tyrosine metabolism incorporating a D,L racemase.

Nevertheless this experiment reached important conclusions. Firstly, that dietary tyrosine was not entirely responsible for urinary nitrogen and that some dietary tyrosine is used in normal protein synthesis. Secondly, only 25% of the total recovered label found in tissue protein was in the form of tyrosine. The remainder was found in glutamate, aspartate, histidine and arginine. The only amino acid analysed which was found not to contain ¹⁵N was lysine. It is now known that lysine is "metabolically inert" in animals, that is, it does not participate in reversible transamination (Weissman and Schoenheimer 1941).

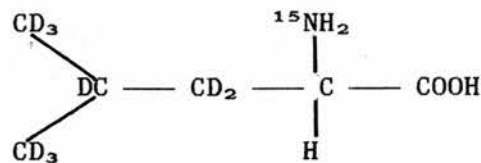
They found a high isotopic concentrations in the dicarboxylic amino acids. This has subsequently been clarified. These amino acids undergo rapid transamination and ammonia is incorporated into

glutamate via the glutamate dehydrogenase system. At the time, Schoenheimer's results were very significant, since there had been much debate as to whether amino acids exchanged their nitrogen in vivo (Krebs 1933a; Krebs 1933b; Von Euler et al 1938).

The net conclusion from this experiment was that under normal conditions in adult rats a number of biochemical reactions involving both exogenous and endogenous tyrosine occur. The amino acids from both sources can be incorporated into tissue protein and are incorporated to a fairly considerable extent, especially in liver.

A later experiment investigated the nature of the biochemical interactions which take place between exogenous and endogenous amino acids and the extent of amino acid incorporation in various tissues (Schoenheimer et al 1939b). ^{15}N -labelled L-leucine was used. The leucine also contained a deuterium label distributed over the side chain (see figure 1.1.3).

Figure 1.1.3



1.1.3 ^{15}N and deuterium labelled leucine.

The ratio of D: ^{15}N should not change unless some metabolic interconversion has occurred. In fact, it was found that more than one third of the marked nitrogen in the original leucine, i.e. that containing deuterium as a label, was replaced by ordinary nitrogen. It was concluded that this was due to transamination reactions of the type outlined by Braustein and Kritzman (1937), rather than de

novo synthesis.

The authors also showed that the carbon and nitrogen groups of leucine could follow different pathways. Only 32% of the administered deuterium-labelled leucine was reisolated. The remainder was not found. It would appear that the group were concentrating on the heavy nitrogen label and made no real attempt to study the fate of the carbon chain. They suggested that the remaining deuterium-labelled carbon chains were probably degraded or involved in the formation of some other body constituent. They did not look for deuterium in any of the body fluids, not even urine.

The results showed that of the ^{15}N administered, 2.1% was found in the faeces, 27.6% in urine and the bulk was retained in tissue protein (i.e. 57.5%). The authors proposed from this the concept of nitrogen conservation. The nitrogen group of dietary amino acids is preferentially retained in the body because it is a limiting or "essential" dietary constituent on a mixed diet (cf. however Pflüger and Voit's dogs).

Over this series of experiments, the extent of nitrogen retention was shown to be a function of the duration of the experiment. The longer the feeding time the smaller the relative amount of label retained in tissue protein. The authors concluded that part of the ^{15}N originally introduced into protein will be liberated and excreted. The group suggested two mechanisms by which this might occur. One was by the continual synthesis and degradation of protein. However with limited information on the process of synthesis or degradation, this possibility was not favoured. The second involved replacement of amino acids,

individual amino acids being replaced at particular positions in proteins by exogenous amino acids. This would involve breaking and remaking peptide bonds. This second method may have appeared more attractive at the time; however, the group did not provide any theories as to why or how such a particular process might occur.

The distribution of ^{15}N among other amino acids was similar to that found in previous experiments. Glutamate had a high concentration and lysine contained virtually no label at all. This implies that redistribution of nitrogen in amino acids was a widespread but not universal phenomenon.

^{15}N distribution between different tissues and organs, shown in table 1.1.1, provides evidence for differing rates of synthesis in different tissues. Serum proteins showed the highest activity, internal organs (intestinal wall, liver, kidney, heart, spleen and testes) were less active while muscle and skin had a very low activity. Nevertheless muscle and skin play an important role in the total replacement process due to their large size and high total nitrogen. Schoenheimer's group showed that the internal organs had taken up 9% of isotopic nitrogen while 48.5% was taken up by the rest of the body.

Table 1.1.1

<u>organ</u>	<u>^{15}N concentration (atom% excess)</u>
blood plasma	1.108
erythrocytes	0.019
liver	0.061
intestine wall	0.097
kidney	0.089
heart	0.058
spleen	0.072
testes	0.05
skin	0.102
muscle	0.02
carcass	0.03

1.1.1. ^{15}N concentrations in protein-N of blood and organs from 4 rats given isotopic L-leucine (from Schoenheimer et al, 1939b).

The analysis of these results presumes that each organ synthesises all its protein at a similar rate. This is now known not be the case. Even if this were true the authors should have realised that such results do not in themselves measure the rate of synthesis or incorporation. The label would be taken up by fast-incorporating tissues more rapidly, but it might also be released more rapidly, if the total protein of that tissue remained constant. Therefore a tissue with rapid incorporation, and release, may have quite a low level of ^{15}N at a particular point in time towards the late stages of the feeding experiment. Thus analysing tissues at any single time after the administration of the label will not discriminate between a protein or tissue which is turning over rapidly and one which is incorporating amino acids slowly and may still have retained much of its incorporated ^{15}N .

From these experiments Schoenheimer proposed the concept of a continual exchange of substances by cells into and out of a metabolic pool. This he termed "metabolic regeneration". In this

case nitrogenous groupings of tissue proteins are constantly involved in chemical reactions. Amino acids, from whatever source, diet or tissue, are taken up and released by tissue protein. This mixture of free amino acids also takes part in a variety of other reactions, some re-enter proteins, others transfer their nitrogen to deaminated molecules to form new amino acids. These in turn re-enter the same chemical cycles, which renders the source of nitrogen (i.e. diet or tissue) indistinguishable. Thus the excreted nitrogen is taken from a metabolic pool whose source is from both the diet and from the tissue.

Schoenheimer died soon after this series of experiments was completed, but Rittenberg (with Bloche) went on to investigate the synthesis of cholesterol. This was one of the early uses of ^{14}C in metabolic studies and a reminder that at this early stage technical difficulties in measurement were as limiting as conceptual advances.

Perhaps it was because Schoenheimer was in the still unfashionable field of protein turnover that his work is virtually forgotten today. However the idea of the dynamic state of body constituents was rapidly extended to lipid and carbohydrate metabolism, and makes it possible for us today to visualize the transient time for plasma amino acids, plasma glucose and plasma fatty acids (all about 5 minutes) or the replacement time of an entire cell membrane (perhaps a little more than one hour). This is almost entirely due to his work and his vision.

Another advance which was almost as important as the experimental work was the mathematical analysis of the results, which depended, and still almost entirely depends, on the concept of

a metabolic pool. The first mathematical treatment was presented in 1943 by Zilversmit et al. Rittenberg's Harvey Lecture, published in 1949, includes an elementary mathematical analysis of protein turnover, while Shepard and Householder's classical paper on the interpretation of tracer movements in closed steady state systems (i.e. metabolic pools) appeared in 1951. The planning and analysis of the experiments reported in this thesis depends on the concept of a series of metabolic pools, both of free amino acids and of proteins, which may not have been in a steady state (see chapter 4 and appendix).

1.2 Protein metabolism in different tissues

Following the pioneering work of Schoenheimer, many investigators began to use tracer techniques to look at the dynamic state in more detail. Unfortunately, individual values varied considerably between studies, mainly due to their experimental technique and a common inability to estimate the specific activity of a label in the protein precursor free amino acid pool (see section 1.4). Nevertheless more recent workers have produced fairly consistent values for protein synthetic rates over a number of experiments; these are quoted in Table 1.2.1.

Table 1.2.1 (from Waterlow et al 1978a)

Investigators	A	B	C	D	E
mean body weight of rats	190	120	120	117	108
Fractional synthesis rate					
rate %/day					
liver	48.9 ± 8.6	-	49.8 ± 2.8	67.5 ± 6.5	52.9 ± 7.0
kidney	50.8 ± 9.7	0.7 ± 5.0	-	47.9 ± 6.1	55.4 ± 7.1
heart	19.9 ± 6.5	17.0 ± 2.9	16.8 ± 0.9	17.2 ± 1.0	23.7 ± 3.0
muscle	12.1 ± 2.2	15.0 ± 2.7	13.6 ± 1.0	13.0 ± 0.9	18.7 ± 2.0
brain	-	11.8 ± 1.5	-	17.0 ± 1.8	17.0 ± 1.9

1.2.1 Protein synthesis in tissues of growing male albino rats. Each value is the mean ± the standard deviation from 5 - 6 animals. All results were obtained by continuous infusion of [U - ¹⁴C]-tyrosine (from Waterlow et al, 1978).

These results are not directly comparable to those of Schoenheimer, not only because the techniques used only give information on rates of synthesis, but also because the studies were carried out on growing rats. Nevertheless the results are of considerable interest.

The synthesis rates of kidney proteins are high but are less variable than those of liver, while the ranges of heart and skeletal muscle overlap. Synthesis rates in brain are similar to those of muscle. These differences are not due to variations in metabolic rate since Keynes (1975) has estimated (for rats) the relative metabolic rate of heart and kidney to be about twice that of liver and brain, and these latter in turn to be about ten times that of resting muscle. The differences may exist because some tissues need to respond rapidly to environmental changes. Liver, and to a lesser extent kidney, has to react to changes such as those caused by feeding and fasting (Sitprija and Suvanpha 1983; Waterlow et al

1978). In order to adapt these organs require fast turning-over enzymes. This is however a very simplified hypothesis, since as we shall see later, the turnover rates of individual enzymes within one tissue are very different.

1.2.1 Protein metabolism in muscle

Differences in protein synthetic rates between heart and muscle (see table 1.2.1) may be related to their rate of work. In individual muscles there is a relationship between the muscle composition, the extent of involvement in continuous work and the rate of protein synthesis (Preedy and Garlick, 1984).

Waterlow et al (1978) pointed out the non-uniformity of muscle types. Individual muscles differ in structure, composition, function and turnover rates. Heavy and light myosin chain vary in structure depending on the nature of the muscle from which they are isolated (Mannherz and Goody, 1976; Huszar and Elzinga, 1972; Sarker et al, 1971; Hoh et al, 1976; Lowey and Risby, 1971). At present it is still not possible to predict what effects (if any) these differences in composition might have on the overall turnover of myosin.

The rate of work of a particular muscle may be related to myofibrillar turnover. Goldspink (1970, 1972) proposed that during growth individual myofibrils of large diameter may split longitudinally. He suggested that a component of the force generated during contraction acting at right angles to the axis of the myofibril "rips" the z-band. This may separate the myofibril into daughter myofibrils. The completeness of splitting may depend on the rate of development of tension. In fast-acting muscle the

split would be complete along the entire fibre, giving the so-called "Fibrillenstruktur" characteristic of fast muscle. Slow tension development would produce incomplete splitting resulting in the "Felderstruktur" seen in slow muscles. Goldspink suggested that this splitting may explain the increased rate of protein breakdown observed in growing muscles.

Goldspink's model has been criticised by Waterlow et al (1978). The theory does not account for differences in myofibrillar turnover in non-growing muscle, unless the daughter myofibrils are completely degraded. Since no daughter myofibrils have been shown to exist it is not possible, at present, to prove or disprove their breakdown. It has, however, been shown that the rate of force generated is inversely related to the rate of breakdown, i.e. slow muscle has a high breakdown rate, and fast muscle a low rate (Garlick et al, 1973). A high breakdown rate, therefore, is not due to increased splitting caused by rapid contraction. The increased breakdown may be due not to the rate of force generation but to the total work output - slow muscle can remain contracted longer than fast muscle. Cardiac muscle would be a good example of this since it has a high total work output and a high rate of protein turnover.

Many investigators have used isotopic tracer techniques to study protein metabolism in muscle. These workers have tended to concentrate on actin and myosin, since they make up more than half of all the muscle protein (Bates and Millward 1983). In addition some investigators have compared the turnover rates of these myofibrillar proteins to those of the "soluble" proteins located in the sarcoplasm. It has been shown that sarcoplasmic proteins turn

over faster than myofibrillar proteins and that each fraction may be affected differently by effectors like diet (Hagan and Scow, 1957), starvation (Dickerson and McCance, 1960), growth (Wildenthal et al 1976) and growth hormone (Greenbaum and Young, 1953). There has been relatively little work carried out on the synthesis and degradation of individual sarcoplasmic proteins. One of the earliest studies was that of Simpson and Velick (1954). They injected radioactive carbon labelled amino acids (glycine, DL-phenylalanine, DL alanine, DL lysine and DL methionine) into adult rabbits and after 38 hours two enzymes, aldolase and glyceraldehyde phosphate dehydrogenase (GAPDH), were isolated from skeletal muscle. The specific activity of each amino acid in each enzyme was estimated as well as the total specific activity of each enzyme. They found that the ratio of the specific activities of the five amino acids remained constant between the two enzymes while the absolute specific activities varied between the two enzymes. From this they concluded that the two enzymes were being synthesised from a common amino acid pool, but at different rates. Further consideration will show that this latter conclusion at least, cannot be true.

This follows from the fact that the ratio of specific activities between the five amino acids, which were injected at a nominal common specific activity of $100/\mu$ Ci/m mol, varied by a factor of over twenty when they were isolated from the proteins. They must therefore have been metabolised at very different rates, and the decay curves for the specific activities of the five amino acids in plasma must have had very different gradients over the 38 hours

before the proteins were isolated. The effect of this can be most graphically demonstrated with reference to glycine and methionine. Figure 1.2.1 shows a hypothetical decay curve for plasma methionine and glycine. It can be seen that a rapidly synthesised protein might be expected to have a ratio:

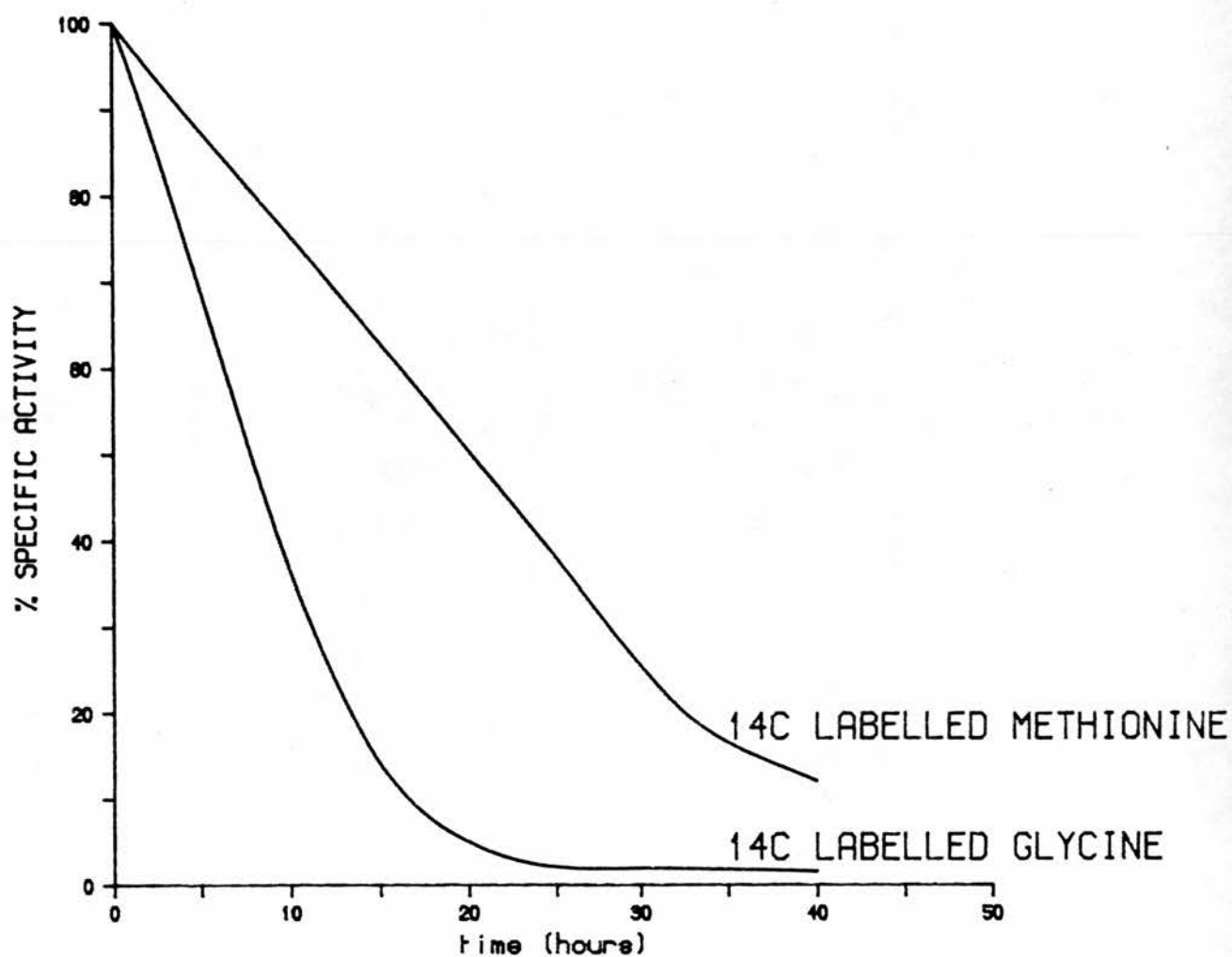
$$\frac{\text{specific activity of glycine}}{\text{specific activity of methionine}} > 1$$

whereas a slowly synthesised one would have a ratio < 1 . There should be similar, but less pronounced, differences expected for the variation with time of the $\frac{\text{specific activity of amino acid}}{\text{specific activity of methionine}}$ ratios for the other three amino acids.

Thus the fact that the ratios were constant for the five amino acids, far from justifying Simpson and Velick's conclusion, strongly suggests that the two enzymes were being synthesized at the same rate, but that different fractions of the enzymes were labelled.

There is one piece of evidence that suggests that the pool from which the amino acids were drawn for the synthesis of aldolase and GAPDH in this experiment was intracellular. The specific activity of glutamate was detectably above zero, while that of aspartate was not. Since all the ^{14}C -labelled amino acids were labelled in the 1-position the only way in which the label could have reached glutamate would be by incorporation of ^{14}C into oxaloacetate, through the actions of pyruvate carboxylase or phosphoenol pyruvate carboxykinase. It would be very unlikely that aspartate (in liver) should not become labelled in this process. However Mowbray (1973) and Hogg and Ottaway (1977) have both shown that in heart muscle,

FIGURE 1.2.1.



1.2.1. HYPOTHETICAL DECAY CURVE

Possible decay of ^{14}C labelled methionine and glycine in rabbit plasma indicated by data from Simpson and Velick (1954). This may suggest that the tissue protein ratio of the specific activity glycine:specific activity of methionine from a rapidly synthesised protein would be >1 while a slowly synthesised protein would have a ratio <1 .

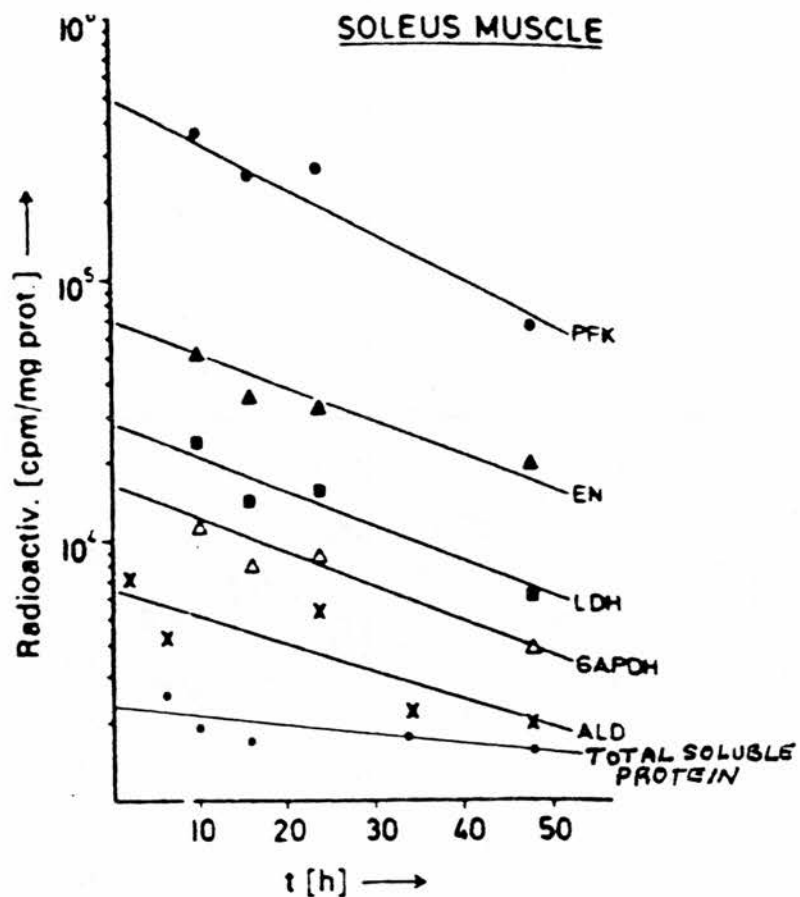
aspartate does not become labelled when the heart is provided with a source of labelled acetyl-CoA, while glutamate does. This piece of evidence suggests that the source of aspartate for synthesis of labelled aldolase and GAPDH in this experiment was exclusively intracellular.

Other than Simpson and Velick's most of the work on individual sarcoplasmic proteins turnover of muscle has looked at specific proteins (Fritz et al, 1969; Schapira et al, 1960; Brinkworth and Masters, 1978; Matsuda and Fischer, 1975) rather than comparing rates of turnover between different proteins. However there are two notable exceptions: the first is the work carried out by Pette's group in the 1970's. This group looked at the turnover rates of many glycolytic enzymes and creatine kinase in rabbit heart, liver and muscle (Dölken and Pette, 1974; Pette and Dölken, 1975; Illge and Pette, 1979). In one of the experiments they injected ^{14}C -labelled leucine into adult rabbits and isolated various enzymes from each of the three organs.

Although Pette worked on rabbit soleus muscle (red slow twitch) and this thesis concerns chicken breast muscle (white fast twitch) some of the results are of interest. Figure 1.2.2 shows the loss of radioactivity from individual glycolytic enzymes from soleus muscle found by Pette's group.

Unfortunately Pette's group isolated the enzymes at time intervals which may not have been optimal for detecting any variation in the decay curve. In addition it appears that each data point represents only one animal, so any "bumps" in the curve may be assumed to be due to variation between animals, and each decay curve

FIGURE 1.2.2.



1.2.2. Loss of radioactivity from individual glycolytic enzymes from rabbit soleus muscle.

Each data point represents the specific radioactivity from one animal. (Taken from Dolken and Pette (1974))

was plotted as a straight line. Pette's group suggested from these results that most of the enzymes studied have a similar rate of degradation and since each enzyme is present, in muscle, in different amounts, it must be the rate of synthesis which is the main controlling factor in the regulation of enzyme levels in vivo.

In contrast to Pette's work the experiments presented in this thesis used carefully-chosen sampling time intervals after an initial "trial run" and used three animals at every time interval. It is hoped to provide evidence to indicate that the decay curve of radioactivity cannot be assumed to be linear and that differences in the shape of the curve may exist between enzymes.

H.G. Lebherz

Herbert G. Lebherz led a group investigating protein turnover in various muscle types in the chicken (white leghorn). In one experiment the group looked at the turnover of some glycolytic enzymes plus creatine kinase in slow twitch fibers (lateral adductor) and fast twitch fiber (breast) (Lebherz et al, 1982). The fast twitch breast muscle, which is the subject of this thesis, is particularly suitable for studying glycolytic enzymes, since apart from actin and myosin, and the sarcolemma and sarcoplasmic reticular membrane proteins, the enzymes of glycolysis plus creatine kinase make up almost all the protein present in any quantity. To try to isolate all the glycolytic enzymes would be a very time-consuming task. Lebherz therefore concentrated on the four most abundant soluble proteins, enolase, aldolase, GAPDH and creatine kinase. These were shown to make up around 60% of the total soluble protein. Lebherz pointed out that this was apparently in excess of the amount

required purely for catalysis and suggested these enzymes might have some other structural or physiological role in addition to their well-defined role in catalysis. This theory is supported by the possibility that some glycolytic enzymes and creatine kinase may be associated in specific ways to the myofibrillar apparatus (for review see Ottaway and Mowbray, 1977).

Lebherz showed that major glycolytic enzymes turned over at considerably different rates in muscle. This conclusion was contrary to an earlier suggestion by Pette and Dölken (1975), but in agreement with Velick's early work. Lebherz also showed that the relative rates of synthesis of the three glycolytic enzymes in slow twitch muscle fibers were about four-fold lower than in fast twitch fiber types. The differences in synthesis rates were similar to the differences in the steady state concentrations of these enzymes in the two fiber types. From this it was concluded that regulation of the differing levels of enzyme in muscle is mainly controlled by protein synthesis, rather than protein degradation.

Lebherz's conclusions were based mainly on single-pulse labelling techniques with an incubation time not longer than three hours. This thesis aims to show that such short-term incubations may not give accurate estimations of enzyme turnover rates.

Lebherz's group went on to analyse the effect of denervation and muscular dystrophy on the turnover of various muscle enzymes (Shackelford and Lebherz, 1983a; 1983b; 1985).

1.3 Protein degradation

In the past much work has been directed toward elucidating the process of protein synthesis and its role in regulating the

concentration of proteins within the cell (for a review see Boyer, 1974).

In contrast, protein degradation has largely been ignored until relatively recently, perhaps because of the general belief that breakdown was a random process (Schimke and Bradley, 1975). Consequently very little is known of the mechanism of protein degradation or how it is regulated. Nevertheless breakdown has been shown to be a key component in controlling the levels of proteins in tissues since more protein is broken down per day by tissue protein turnover than by the digestive system (Barret, 1984). Moreover greater than fifty percent of this is carried out in the lysosomal system thus making them the major source of tissue free amino acids (Barret, 1984).

Lysosomes contain all the enzymes necessary for degrading intracellular proteins (Coffey and de Duve 1968), and play a clear role in the degradation of extracellular proteins taken into the cell (La Badie et al, 1975; Jacques, 1969; Morell et al, 1968; Mego et al. 1967) or the degradation of intracellular proteins during cellular autophagy (Deter and de Duve, 1967; Deter, 1971). In addition it has recently been shown that a class of cytoplasmic proteases are also present in the cell (Murachi, 1983; Dahlmann et al, 1983; Kay, 1983; Murachi, 1984; Dahlmann et al, 1985; Kay et al, 1985). The current debate is on the relative contribution of each compartment to the normal breakdown of intracellular proteins.

There is evidence for the lysosome being the main site for intra-cellular proteolysis. Wildenthal and Griffin, (1976) demonstrated a direct relationship between activities of cathepsin D

and other lysosomal hydrolases, and the rate of intracellular protein degradation in cultured foetal mouse heart after treatment with cyclohexamide. Moreover, Neely and Mortimore, (1974) demonstrated the presence of labelled products of proteolysis in the lysosome under conditions where endogenous proteins were the primary source of label. In addition there appears to be a direct correlation between degradation rates of proteins in vivo and their susceptibility to lysosomal proteolysis (Segal et al, 1974; Dean, 1975; Glaumann et al, 1985). In muscle there is an abundance of lysosomes during periods of rapid breakdown such as during starvation when dense (lysosome-like) bodies appear in the Z-line region of muscle beneath the sarcoplasmic reticulum (Bird, 1975). These are not usually visible although with new staining techniques muscle lysosomes have been shown to be fairly abundant (Roisen et al, 1983; Wood et al, 1985).

There are known to be a number of factors which influence protein degradation rates: these include the concentration of calcium (Millward et al, 1980), triiodothyronine (Rannels, 1982), glucocorticoids (Rannels, 1982; Millward et al, 1981), insulin (Rannels et al, 1975; Jefferson et al, 1974; Jefferson et al, 1977), leucine (Buse and Reid, 1975; Fulks et al, 1975; Mitch and Clark, 1984), ATP (i.e. energy) (Goldberg et al, 1975; Goldberg and Dice, 1974; Goldberg et al, 1980), and other more general environmental factors such as temperature (Munro and Pelham, 1985), diet (Millward, 1970), growth (Waterlow and Jackson, 1981), age (Waterlow and Stephen, 1968) and exercise (Rennie et al, 1981). The means by which any of these regulatory factors influence the protein

degradation system cannot be assessed until the mechanism of degradation has been elucidated.

The main question to be answered in the field of protein breakdown is not how are proteins degraded, but why are they degraded at different rates. Selectivity may be influenced by the conformation of the protein substrate; in general glycoproteins (Dice and Walker, 1980), acidic proteins and proteins with a large subunit size are more susceptible to breakdown than other proteins (Goldberg, 1975). Abnormal or denatured proteins tend to be degraded more rapidly than normal proteins (Goldberg, 1975). Thus there appear to be specific groups of protein conformations which have a higher susceptibility to attack by proteases. This has led to the proposal that enzyme degradation is preceded by a post-translational modification reaction, i.e. a "marking step" making the protein more prone to proteolytic attack. A number of such "marking steps" have been identified: the carbamylation of ϵNH_2 groups of lysine residues (Hood et al 1977), the phosphorylation of serine and threonine residues (Bergström et al, 1978; Hall et al, 1979; Hemmings, 1980; Müller and Holzer, 1981; Arebalo et al, 1981; Bernlohr and Switzer, 1981; Mazon et al, 1982; Parker et al, 1984), the oxidation of non-haem iron sulphur centres (Bernlohr and Switzer, 1981; the formation of mixed disulphide derivatives of cysteine residues (Offermann et al, 1984); the conjugation of ϵNH_2 groups of lysine residues with ubiquitin (Ciechanover et al, 1981); the deamidation of glutamine and asparagine residues (Payne and Tuffnell, 1981); the oxidation of methionine to its sulphone (Levine et al, 1981); modification of constituent amino acids through

exposure to free radicals (Wolff and Dean, 1986); the oxidation of amino acid residues by mixed-function oxidase systems (Levine et al, 1981; Oliver et al, 1982; Fucci et al, 1983). The mixed function oxidase system oxidises enzymes to catalytically inactive forms (Levine et al, 1981; Oliver et al, 1982; Fucci et al, 1983, Stadtmann and Wittenberger, 1985), which are readily degraded by a number of proteases (Oliver et al, 1982; Rivett 1985). Calcium-activated neutral proteinases may also act as marking agents. It has been suggested that these proteinases attack certain substrates causing only partial degradation at specific sites to "nick" the polypeptide altering the conformation sufficiently to increase its susceptibility to attack (Brook et al, 1983; Rivett, 1985) by other cytoplasmic proteases or by lysosomes.

Whatever factor is responsible for making particular proteins more susceptible to degradation the most important question is why the process discriminates between proteins. It is most likely that complete degradation occurs in the lysosome. It may be that selectivity occurs at the level of uptake into the lysosome. The various preferred substrates for proteolysis and the number of structural modifications may simply increase the protein's rate of uptake into the lysosome. Lysosomal degradation is perhaps dependent on factors such as the degree of exposure of the normally shielded hydrophobic regions of a protein producing different degrees of affinity for lysosomal membranes (Bohley, 1968). Dean (1975) showed that at physiological pH and ionic strength cytosolic proteins with higher turnover rates preferentially adsorb to partially purified lysosomes. However other workers could find no

evidence for uptake of short- or long-lived cytosolic proteins by intact lysosomes (Huisman et al 1974). The impermeability of lysosomes to substances with molecular weights above 230 daltons indicates transport of some kind must be involved (Gordon 1973), but the means of entry of proteins into lysosomes remains obscure.

Muscle protein degradation is even more difficult to study, not only because there are fewer lysosomes, but also because of the complex structure of the myofibrillar apparatus, which makes it difficult to envisage parts of the contractile machinery being selectively broken down whilst maintaining a functional muscle. Goldspink (1970; 1972) suggested that the myofibrillar protein may have a lifetime of thirty days. His theory was that during muscle growth large diameter myofibrils split longitudinally to form daughter myofibrils. If splitting is not completely conservative some portions or individual myofibrils may become unattached and broken down (see page 24 for critique).

Another theory is that calcium-activated neutral proteinases (calpains) initiate myofibrillar protein turnover (Brook et al 1983; Rivett 1985). These enzymes may selectively degrade α actinin of muscle z-lines. This would cause the myofibril to break into sarcomeres of approximately 2 μ m in length. Fusion of sarcoplasmic reticular tubules surrounding the sarcomere (as with smooth sarcoplasmic reticulum in other cell types) would form a reasonable sized autophagic vesicle for the compartmentalised degradation of myofibrillar proteins (Busch et al 1972). This is because the sarcoplasmic reticulum is homologous to the smooth endoplasmic reticulum and has no connections to the sarcoplasmic matrix, extra-

cellular space or the T-system (Price 1973). By this means segments of myofibril could be degraded without degrading the complete muscle fiber.

1.4 The intracellular protein precursor pool

Even before the mechanism of protein synthesis had been worked out a number of studies were carried out which tried to ascertain the sources of the amino acids used for protein synthesis. In 1956 Borsook suggested that proteins might be synthesised from pre-existing peptides or proteins without prior hydrolysis to amino acids. This idea was in line with earlier work by Yuile and co-workers (1951). In time it became apparent that proteins were synthesised de-novo from free amino acids and this is the accepted view today. Among the evidence three studies are of note. One is that of Simpson and Velick (1954), in which it was concluded that the two proteins investigated, aldolase and GAPDH, had become radioactive by de-novo synthesis and not from peptide fragments. Simpson and Velick also ruled out the idea of exchange between free amino acids and the completed chain, a view suggested much earlier by Schoenheimer's group (1942).

Askonas et al (1955) added weight to the argument by disproving the theory of Yuile et al that plasma proteins could serve as precursors for tissue protein synthesis, without the need for complete hydrolysis to amino acids. They investigated casein and β -lactoglobulin synthesis in lactating goats. ^{14}C -labelled glycine, valine and lysine were injected and after 3 - 4 hours the proteins were isolated and hydrolysed to produce a number of peptide fragments. They found that the specific activity of a given amino

acid was the same in each of the peptides from a single protein. Moreover the specific activity was higher than that of the plasma proteins. However the group did not measure the specific activity of individual plasma proteins. This left the possibility that tissue protein might have been synthesised from an individual or specific group of plasma proteins, which might have had similar specific activities to those of casein and β -lactoglobulin, but might have been diluted by the bulk of plasma protein. The group did not consider this possibility.

Another investigation used a modification of the continuous infusion technique (see page 39). The authors infused rats for periods of up to 3 days with labelled leucine, isoleucine or valine. It was considered that during this time the specific activity of the tissue (liver) free amino acids had reached a constant value. They then compared this specific activity with that of isolated ferritin. This protein was chosen because synthesis can be induced by an injection of iron at the start of the infusion. Under these conditions the specific activity of the induced ferritin should be the same as that of the precursor pool. The results showed this to be the case. From this it was suggested not only that ferritin was synthesised from a pool of free amino acids, but also that this was the tissue free amino acid pool (Loftfield and Harris 1956). This idea has been accepted although the possibility of intracellular compartmentation of the protein-precursor pool cannot be ruled out (see sections 1.4.3 and 1.4.4) and indeed at present there is debate not only about the existence of a separate protein precursor pool but also about its possible size and composition. This question is

an important one since an estimate of the specific activity of the precursor pool is essential when calculating the rate of protein synthesis from labelled amino acids.

1.4.1 Tracer methods for measuring protein synthesis and degradation

When an isotopically-labelled amino acid is given to an animal it becomes rapidly distributed throughout the body fluids and is subsequently incorporated into proteins. Protein labelling can be described by the equation shown below:

$$\frac{dS_B}{dt} = K_S (S_A - S_B)$$

where: S_B = Sp.Act. of amino acids in protein

K_S = fractional rate of protein synthesis

S_A = Sp.Act. of free amino acids at the site of protein synthesis

t = time

An increase in the specific activity of the protein requires that $S_A > S_B$ and therefore measurement of S_A is very important. Some studies have assumed that S_A is equal to the total specific activity of the intracellular pool of the tissue concerned (Loftfield and Harris 1956; Fern and Garlick 1973; Fern and Garlick 1974; Alpers and Their 1972; Li et al 1973; Dölken and Pette 1974).

1.4.2 The Measurement of protein synthesis

There are two main tracer techniques for measuring protein synthesis, the essential difference being the method of administration of the isotope. The label can be introduced in the plasma free amino acid pool as a pulse or it can be infused so as to

reach a plateau. The decision as to which method is chosen will affect both the experimental approach and the calculation of the results.

Pulse labelling

This technique involves a single injection of a massive dose of labelled amino acid. The specific activity of the amino acid in the blood initially reaches a high level but soon declines. In order to measure the rate of protein synthesis it may be necessary to follow the time-course of the specific activity of the protein-bound and free amino acids for some time after the injection. This has not always been done. It has generally been the trend to measure the specific activity of the protein at an arbitrary time, for example the time at which the protein is maximally labelled. The specific activities of the proteins, and precursor pool, are then estimated and the rate of protein synthesis calculated (Lebherz et al, 1982; Illge and Pette, 1979; Wannemacher et al, 1971).

Such an approach ignores any fluctuations which might occur in the precursor pool and assumes that the protein precursor pool is in equilibrium with the tissue free amino acid pool. It is also assumed that each protein follows the same hypothetical labelling curve. The experiments presented in this thesis may indicate such assumptions are erroneous.

Pulse-labelling can also estimate the rate of protein degradation as the label is lost from the protein pool via proteolysis. However amino acid reutilisation may lead to serious over-estimation of protein half lives (Poole 1971).

Continuous infusion

This technique involves the continuous infusion of labelled amino acid, at a fixed concentration, into an animal until all the body's free amino acid pools are in equilibrium with the infusate. This technique assumes the system being studied is in the steady state, i.e. non-growing. In theory this technique should give a more accurate estimation of protein synthesis, since the tissue precursor pool remains constant. The main problem with such a technique is that the largest fraction of the administered isotope is lost from the system, being broken down and excreted as CO₂ and urea. Therefore unless the labelled amino acid is cheap, such experiments are very expensive. In addition in many experiments the length of incubation may not be long enough for some tissues to equilibrate with the plasma. Muscle, for example, has been shown to take up to 25 hours to renew its tissue glycine pool (Henriques et al, 1955). Practical difficulties must also be considered, for example, it is experimentally very difficult to carry out continuous infusion into unanaesthetised birds. For these reasons as well as the fact that protein degradation rates cannot be measured directly by this technique, this thesis reports a study on chicken breast muscle protein turnover using the pulse labelling technique.

Re-utilisation

The problem of labelled amino acids being released from protein via proteolysis, and then reincorporated by de novo synthesis leads to an over-estimation of a protein's half-life. Some investigators have overcome this by using an amino acid which is altered after incorporation into a protein and cannot be re-used following

degradation. An example of this is 3-methyl-histidine, which becomes methylated during the process of protein synthesis and excreted after protein degradation. 3-methyl-histidine was thought to be mainly associated with the myofibrillar proteins and has been used in the past as an index of myofibrillar protein breakdown. It has subsequently been shown that in the rat, for example, 50 - 60% of the 3-methyl-histidine excreted comes from tissues other than muscle, mainly the intestine and skin (Millward et al, 1982).

1.4.3 Source of the protein precursor pool

It has been assumed by most investigators that only two free amino acid pools exist; the plasma pool and the tissue pool. Many calculations of protein synthesis have been based on this assumption. However amino acids used in protein synthesis may be drawn from a number of different sources (Everett and Zak, 1980). If this is so, this compartmentation may explain some of the anomalous behaviour of metabolites in labelling experiments (Shaw and Stadie, 1959; Sims and Landau, 1966; Landau and Sims, 1967; Antony et al, 1969).

Depending on the circumstances, there is evidence that the protein precursor pool is derived either from the intracellular free amino acid pool or from the plasma free amino acid pool. Pain and Manchester, (1970) incubated extensor digitorum muscle for one hour in a medium containing various amino acids at the concentration at which they occur in plasma. In each experiment one of the amino acids was labelled. After incubation, the medium and tissue free amino acid concentrations were determined by chemical analysis and by distribution of radioactivity. They found that for several amino

acids the radioactive species only penetrated a fraction of the total amino acid pool. The extent of penetration was particularly low for glycine and proline. This would suggest that the plasma amino acids are not the main source of the protein precursor pool.

In contrast to these findings Hider et al, (1969) suggested that plasma amino acids are incorporated directly into cell protein without prior mixing with the intracellular pool. This group incubated extensor digitorum muscle in a medium containing either glycine or leucine labelled with ^{14}C . After a suitable time interval (30 minutes), they then transferred it to a medium containing the same amino acid labelled with tritium. Incorporation of the ^{14}C -labelled amino acid ceased virtually at once, before there had been any appreciable efflux of the amino acid from the tissues, whereas incorporation of the tritium label into protein began immediately before much accumulation in the cell pool had occurred. This would seem to suggest that most of the protein precursor pool comes directly from the plasma.

It is apparent from these two studies that the protein precursor pool is more complex than the early theories of a one - or two - pool model. Many other studies with tissues incubated in vitro and with intact animals have produced the same kind of complicated result (van Venrooji et al, 1973; Fern and Garlick, 1973; Fern and Garlick, 1974; Wildenthal and London, 1975; McKee et al, 1978; Ward et al, 1984).

1.4.4 Factors which may influence the free amino acid pool used in muscle protein synthesis

Diet

The input of food has a major effect on the total body amino acid pool (Waterlow and Harpew 1975).% The effect on muscle is most important in starvation. However if the body nitrogen balance is adequate, food intake has its most significant effect on tissues such as the liver and intestine.

De novo synthesis of non-essential amino acids

This is itself influenced by the availability of amino-nitrogen. When protein input is low it is the total nitrogen and not any single amino acid which is limiting (Waterlow et al 1978). Other factors which affect the rate of amino acid biosynthesis are poorly understood.

Transamination

Some amino acids are in rapid equilibrium with their oxo acids via transamination. The oxo acids are important intermediates in carbohydrate metabolism, and this may affect the tissue free amino acid pool composition. In addition the amino acid/oxo acid may be involved in other areas of intermediary metabolism. For example, in liver 2-oxo-glutarate/glutamate play an important role in the transfer of amino nitrogen from other amino acids to the urea cycle. This has the most immediate effect on the plasma free amino acid pool. Therefore changes in the concentration of such important amino acids may reflect changes in the overall pattern of amino acid oxidation or gluconeogenesis.

The K_m for many of tmhse transaminases is greater than the

concentration of amino acids in vivo. Therefore an increase in amino acid concentration will result in enhanced rates of transamination and hence oxidation. This may act as a fine control operating more rapidly than the slower process of enzyme induction (Krebs, 1972).

Transport across the cell membrane

Amino acid uptake into cells could be a step which limits the rate of incorporation into proteins and the rate of exchange of amino acids between tissues. There are a number of amino acid transport systems, (for review see Waterlow et al, 1978), which have different specificities. Some are capable of transporting a wide range of amino acids but have a low degree of specificity, while others are completely specific for a particular amino acid. Therefore studies which use particular labelled amino acids may only be analysing one specific component in the process of protein synthesis, namely the ability of the transport system to channel that amino acid into the protein precursor pool, which may of course be separate from the general tissue pool which is the only entity than can be directly measured.

Little is known about the amino acid transport mechanism in muscle. Hider et al (1969) suggested that the rate-limiting step for protein synthesis is in the membrane. They did not rule out the idea of a pool of amino acids close to the membrane not in rapid equilibrium with most of the tissue pool. Such a possibility has been suggested for potassium movement in heart (Schreiber, 1956). Manchester (1965) also predicted that amino acids might be dissolved in the water associated with the myofibrils, the rate of exchange in

the fluid between fibrils and membrane being low. It has also been suggested on the basis of steady state release of amino acids from muscle under normal conditions, that extrahepatic amino acid pools may actually constitute a means of storing amino acids (London et al 1965).

Location of ribosomes

If the rate of movement of amino acids within the cell is slow, the spatial location of ribosomes in muscle may affect the rate of uptake of amino acids for protein synthesis. Some ribosomes may be attached to membranes (London et al, 1965; Muscatemlo et al, 1962); Ezermann and Ishikawa, 1967), although it has been more commonly observed that on homogenisation much of the RNA remains with the muscle debris and can be detached by high ionic strength buffer (Breur et al, 1964; Heywood et al, 1967; Heywood et al, 1968; Chen and Young, 1968). This would indicate the association of ribosomes with myofibrils (Mihalyi et al, 1957; Perry and Zydowo, 1959; Zak et al, 1967; Anderson-Cedergren and Karlson 1967). Electron micrographs show polysomes to be either in open sarcoplasm or along the fibrils. (Heywood and Rich, 1968; Ishikawa et al, 1968; Staley and Benson, 1968; Schiaffino and Margreth, 1969). Therefore ribosomes, even if within the cell interior, may come into contact with entering labelled amino acids much sooner than the time it takes to equilibrate the whole cell volume.

One way to get around the problem of amino acid compartmentation would be to measure the specific activity of the ultimate precursor for protein synthesis, namely aminoacyl-tRNA. Some workers have used this approach; however the total tRNA pool is very small. In

heart for example it is less than 0.1% of the total tissue weight (Schcaffino and Margreth, 1969; Earl and Korner, 1966). In addition it has a very fast rate of aminoacylation with a half-life of 1 - 3 seconds (Manchester 1970).

These facts make it difficult to measure the specific activity of the amino acid part of the aminoacyl-tRNA. Nevertheless results from such studies have indicated that the specific activity of the aminoacyl-tRNA may be higher than that of the intracellular free amino acid pool (Khairallah and Mortimore, 1975; Vidrich et al, 1977; Martin et al, 1977). This would suggest compartmentation and that all previous studies may have underestimated the specific activity of the real precursor pool.

Individual amino acids

In addition to the complexities already mentioned when measuring protein synthesis, the rate of incorporation of amino acids themselves may change. Not only is the amino acid precursor pool size important but also its composition. The amino acid pool turns over very quickly; from known values for whole body flux in the rat the free amino acid pool was calculated to renew itself every hour (Waterlow and Fern, 1978). In spite of this the pool maintains a relatively constant composition (Waterlow and Fern, 1978). Any changes in the composition of this pool may affect the rate of protein synthesis, since some amino acids, in particular leucine, may actually directly induce protein synthesis and inhibit protein degradation (Fulks et al, 1975; Hedden and Buse, 1979; Chua et al, 1979; Mitch and Clark, 1984). Branched chain amino acids may also influence the total body nitrogen balance, since they have been

shown to affect the transport of neutral amino acids across the blood brain barrier noticeably through competition with tryptophan (Anderson, 1981). The rate of uptake of tryptophan may influence the animal's appetite for dietary protein. Once within the brain tryptophan levels are rate limiting for serotonin synthesis. Perhaps by an increase in dietary protein tryptophan entry into the brain will decrease because of concomitant rise in branched chain amino acids. Thus branched chain amino acids may be involved in cerebral control of appetite for protein (Munro, 1978).

Other factors

Numerous other factors may influence the tissue free amino acid pool. Slight fluctuations in the rate of protein synthesis and degradation may affect the immediate size of the pool.

Hormones may affect the amino acid pool for example, insulin increases the transport of glycine and proline into the cell and increases the rate of protein synthesis by up to 100% (Litwack, 1970).

Ethanol may alter the rate of protein synthesis by altering the relative contribution of tissue compartments to the precursor pool (Tiernam et al, 1982).

In summary it is apparent that the amino acid pool within the cell is affected by many factors, the relative contribution of which will determine the size and composition of this pool. It is also clear that the tissue pool may not be homogeneous. Therefore the choice of amino acid labelled and the protein isolated may influence the results obtained. Nevertheless the techniques available at present are useful at indicating the rates of synthesis and

degradation for a given protein, although interpretation of results must not ignore the complexities of the system.

Chapter 2

Materials and Methods

2.1 Laboratory Chemicals

All inorganic chemicals used were products of BDH Biochemicals Ltd., Poole, Dorset, England, and were of analytical grade. The following organic chemicals were also obtained from BDH.

Biochemicals: disodium EDTA, acrylamide, polyacrylamide (mol.wt. $>10^6$), sodium dodecylsulphate (specially pure for electrophoresis), N, N'-methylenebisacrylamide, glycerol, methylene, acetic acid, bromophenol blue, glucose, ethanol, trisodium citrate, phenol, perchloric acid.

The following chemicals and enzymes were obtained from the Boehringer Corporation (London) Ltd., Lewes, East Sussex, England: glycerate-2-phosphate, glycerate-3-phosphate, ATP, ADP, creatine phosphate, phosphoenolpyruvate, pyruvate, lactate, phosphoglycerate kinase, (EC2.7.2.3), creatine kinase (EC2.7.3.2), enolase (EC4.2.1.11), aldolase (EC4.1.2.13), glyceraldehyde phosphate dehydrogenase (EC1.2.1.12), phosphoglycerate mutase (EC2.7.5.3), pyruvate kinase (EC2.7.1.40), lactate dehydrogenase (EC1.1.1.27). The source of all enzyme preparations was rabbit muscle.

Sigma Chemical Company Ltd., Poole, Dorset, England, supplied the following: 2-mercaptoethanol, glycine, bovine serum albumin, N-morpholino-ethane sulphonic acid, fructose- 1,6 bisphosphate, NADH, NADPH, NAD⁺, NADP⁺, glutathione.

Carboxymethyl cellulose (CM-cellulose) and phosphocellulose were from Biorad Laboratories, Watford, Hertfordshire, England.

Fisosolve, Fisofluor the scintillation cocktail was purchased from LKB Scintillation Products Fison plc, Loughborough, England.

Heavy nitrogen (¹⁵N) labelled leucine was supplied by Prochem,

BOC Ltd., 24 Deer Park Road, London, SW19 3UF. [$U-^{14}C$]- leucine was purchased from Amersham International, Amersham, HP7 9LL.

Liquid nitrogen and all gases were obtained from the British Oxygen Company (London).

2.2 Animals

Male broiler chickens were obtained at 1 day old from stock produced at the AFRC Institute for Grassland and Animal Production, Poultry Division, Roslin, Midlothian (formerly the Poultry Research Centre, Roslin). The animals were maintained on a standard diet for starting chicks (Bolton and Blair, 1964) with 23 hours light per day in a heated battery brooder. Birds were given free access to food and water at all times. For the tracer experiment birds were placed in perspex cages with removable wire mesh floors located in a laboratory fume cupboard. They were acclimatised to these conditions for 2 days prior to the start of the experiment.

2.3 Metabolic assays

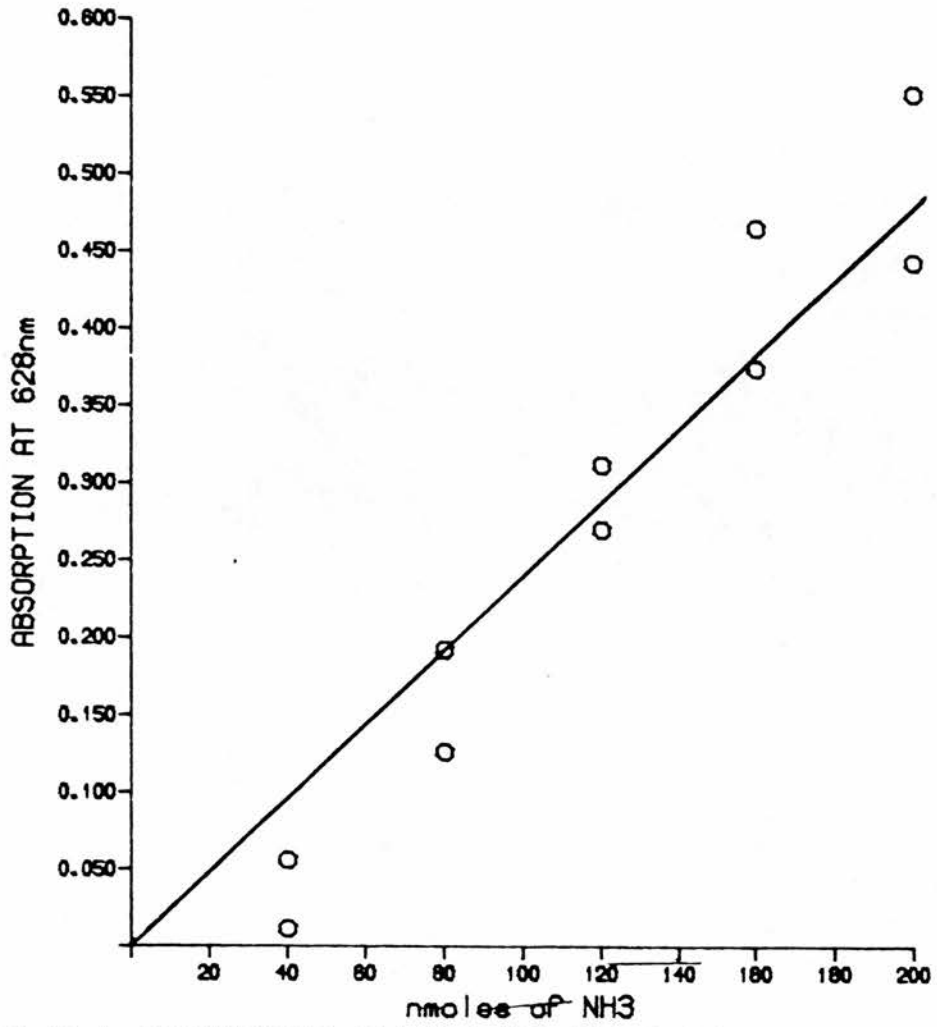
All water used was double distilled.

2.3.1 Ammonia assay

This method is based on the Berthelot reaction as described by Chaney and Marbach, (1962). To an aliquot of sample diluted to 1ml were added 1ml of phenol nitroprusside solution (phenol 50g/l; sodium nitroprusside 0.25g/l) and 1ml of alkaline hypochlorite solution (sodium hydroxide 25g/l; sodium hypochlorite 40ml of 5% w/v solution/l). The absorbance was read at 628nm after incubation for



FIGURE 2.3.1.



2.3.1. STANDARD CURVE OF AMMONIA

Each point represents the mean of duplicate samples of standard solutions of NH_4Cl .

5 minutes at 37° without further dilution. Under these conditions absorbance was linearly related to concentration over the range 0 to 0.2 μ moles of ammonia, with an absorbance of 3.86 per μ mole of ammonia (figure 2.3.1). This method was not sensitive enough to measure an ammonia concentration of less than 0.02 μ moles of ammonia per assay.

2.3.2 Aldolase assay

Aldolase activity was determined using the method outlined by Lebherz and Rutter (1975), whereby the rate of oxidation of NADH was measured at 340nm in 1ml quartz glass cuvettes.

2.3.3 Enolase assay

Enolase activity was obtained by measuring the rate of phosphoenolpyruvate production at 240nm. The technique was first outlined by Wold (1975).

2.3.4 Glyceraldehyde phosphate dehydrogenase assay

Initially this enzyme was assayed by monitoring the rate of production of NADH from NAD⁺ (Alison and Kaplan, 1964). However, for some unknown reason the reaction appeared to be inhibited, even in the presence of protease inhibitors (benzamidine and phenyl methyl sulphonyl fluoride, 2mm). Therefore the reaction was measured in the opposite direction and this gave much more realistic enzyme concentrations, although not as high as found by other workers (Lebherz *et al*, 1982). The method followed was as described in Bergmeyer, (1974). The turnover number of GAPDH in this second assay system was not in the literature so was estimated from the parameters available. (i.e. tissue activity (Bergmeyer, 1974) and molecular weight (Alison and Kaplan, 1964)).

2.3.5 Creatine phosphokinase

Initially this enzyme was assayed by following the rate of NADPH production at 340nm as outlined by Eppenberger et al (1967). However this gave estimates of approximately 10% of the muscle enzyme content found by Lebherz et al, (1982) in the presence of benzamidine and phenyl methyl sulphonyl fluoride (2mM) and in the absence of heavy metals.

The effects of other enzymes in the homogenate on this system, such as LDH and ATPases, were found to be negligible.

The effect of freezing and thawing and the method of homogenisation were also tested and found to be irrelevant.

Therefore the reaction was assayed in the opposite direction i.e. the rate of production of NAD⁺ is measured (Forster et al 1974). This method gave much higher activities for the enzyme although it did not estimate the amount of enzyme present in muscle as accurately as electrophoresis (see section 3:2).

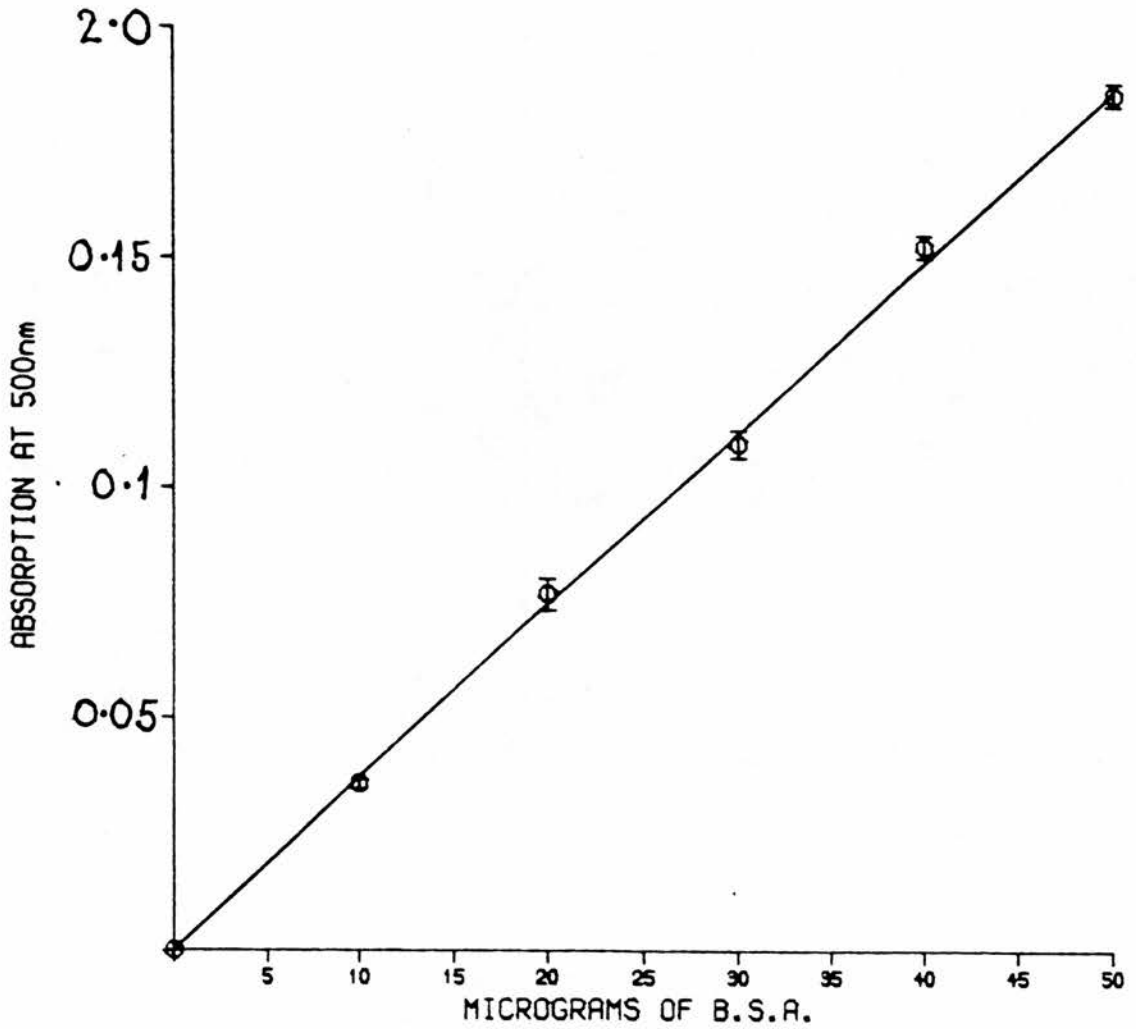
2.3.6 Protein determination

Protein concentration of crude and purified enzymes were estimated in triplicate using the method described by Lowry et al, (1951). A typical standard curve is shown in figure 2.3.2.

2.4 Preparation of muscle homogenates and crude separation of enolase, aldolase and creatine kinase.

The technique used was similar to that given by Lebherz et al, (1982). Muscle samples were homogenised in 10 - 20 volumes of 50mM potassium pH7.5 containing 1mM EDTA, 1mM magnesium acetate, 1mM 2-mercaptoethanol using the Ultra Turrax bench homogeniser (Janke

FIGURE 2.3.2.



2.3.2. STANDARD PROTEIN CURVE

Each point represents the absorption of triplicate samples of BSA at 500nm. The error bars indicate \pm standard deviation.

and Kunkel). Homogenates were centrifuged at 12,000g for 10 minutes (Beckman J2-21 with JA20 rotor). The enzymes were separated as follows; the 12,000g soluble fraction of the muscle homogenates was adjusted to 35% saturation by addition of solid ammonium sulphate (Aristar grade, BDH). After stirring for 30 minutes, precipitated proteins were collected by centrifugation and were discarded. The supernatant fraction was adjusted to pH8.4 by addition of 7N ammonium hydroxide and then ammonium sulphate was added to 75% saturation. After stirring for 30 minutes the precipitated proteins were collected by centrifugation and were dissolved in a small amount of 10mM Tris-HCl, 1mM magnesium acetate, 1mM 2-mercaptoethanol pH7.3. After extensive dialysis against this buffer, at 4°C, the samples were applied to phosphocellulose columns (7cm x 38.5cm²) equilibrated in this buffer. The protein fraction not retained under these conditions contained creatine phosphokinase, which was purified as described later. Enolase, LDH, PGM and GAPDH were eluted from the phosphocellulose column with 100mM Tris-HCl pH7.8 containing 1mM EDTA, 1mM magnesium acetate, 1mM 2-mercaptoethanol. Aldolase was eluted in the same buffer containing 1mM fructose 1, 6-bisphosphate pH7.8.

Final purification of creatine kinase was accomplished as follows: the material not retained by the phosphocellulose columns was dialysed against 1mM MES buffer containing 1mM EDTA, 1mM magnesium acetate, 1mM 2-mercaptoethanol which was titrated to pH6.0 with sodium hydroxide. Then samples were applied to phosphocellulose columns (10cm x 7cm²) equilibrated in the same buffer. Creatine kinase was specifically eluted with this buffer

containing 50mM potassium phosphate pH6.0.

All enzymes isolated by these procedures were tested for homogeneity by electrophoresis followed by scanning of the gel by a gel scanner (chromosocan 3 Joyce Loebel, Gateshead, Tyne and Wear, England).

The resolution on phosphocellulose and cm-cellulose columns was found to be less ideal than that shown by Leberz et al (1982). In addition, after 2 - 3 column runs the resolution deteriorated; for this reason the phosphocellulose was frequently regenerated.

2.4.1 Regeneration of phosphocellulose column

10 - 20 grams of phosphocellulose was suspended in 500ml of 0.25M HCl and stirred for 30 minutes. Vigorous stirring was avoided to prevent the generation of fine particles. After filtering and rinsing with de-ionised water it was suspended in 500ml of 0.25M NaOH and left to stand for 10 minutes, neutralized with HCl, filtered and rinsed with de-ionised water. The HCl wash was repeated and the cellulose rinsed and equilibrated in starting buffer. Equilibration was achieved by washing extensively with starting buffer until the pH of the wash was equal to the pH of the starting buffer.

2.5 Fast protein liquid chromatography

Enolase, aldolase, GAPDH, LDH and PGM were further purified by passage through a FPLC cation exchange column (mono S HR5/5, Pharmacia) using a method adapted from that of Lindblom and Fagerstam, (1984).

2.5.1 Equipment

Controller and accessories

Gradient programmer GP-250

Pump P500 (X2)

Valve and sample applicator V7

Superloop (50ml)

Monitor and accessories

UV monitor, UV1

Absorbance unit (A.U.) range from 0-100 on chart and could be set at between 0- \rightarrow 0.01, up to 0- \rightarrow 2.0

Flow cell, H.R.

Filter kit 280nm

Filters. Sample filter GVWP 013 durapore hydrophillic

Buffer filter GVWP 047 durapore hydrophillic from

Millipore, Harrow, Middlesex.

Recorder REC-48

2.5.2 Separation of enolase, GAPDH, LDH and PGM

Numerous methods and conditions were tried to obtain maximum separation of each enzyme. The method finally chosen is described below. How this was developed will be described in Chapter 3.

Buffers

Buffer A: 25mM MES, 50mM NaCl, 1% w/v betaine, 1mM 2-mercaptoethanol, 1mM magnesium acetate pH6.0. Buffer B: 25mM MES, 350 mM NaCl, 1% betaine, 1mM 2-mercaptoethanol, 1mM magnesium acetate pH6.0.

Operating conditions

Sample volume approximately 25ml

Flow rate 2.0ml per minute

Pressure 2.2 MPa

Chart speed 0.5cm per ml

Absorbance units 2.0

Collection of peaks

- Peak cutting

Sample application peaks cut at 2%

Column run peaks cut at 5%

Collection volumes 1ml

Approximate running time 30 minutes

Samples were dialysed extensively with starting buffer A. The samples and buffers require filtering through a millipore filter under vacuum prior to application on the column. The column was equilibrated with starting buffer (4-5 column volumes). A 50ml superloop was loaded with sample (approximately 20ml, 1-2mg/ml) and connected to the valve leading to the top of the column and to the pumps.

The gradient programmer was programmed to produce a 30ml run in, and then the following gradient.

0 - 40% buffer B in 10ml

40 - 80% buffer B in 40ml

80 - 100% buffer B in 5ml

100% buffer B for 4ml

All proteins were checked for purity by gel electrophoresis followed by scanning on a Chromoscan 3 gel scanner.

2.5.3 Purification of Aldolase

Buffer A: 25mM MES, 50mM NaCl, 1% betaine, 1mM 2-mercaptoethanol, 1mM magnesium acetate pH6.0.

Buffer B: 25mM MES, 350mM NaCl, 1% betaine, 1mM 2-mercaptoethanol, 1mM magnesium acetate pH6.0.

- Operating conditions

Sample volume: approximately 20ml

Flow rate 2.0ml/minute

Pressure 2.2 M.Pa

Chart speed 0.5cm/ml

Absorbance units 2.0

Collection of peaks

- Peak cutting

Sample application and column run peaks were cut at 5%.

Collecting volumes 1ml

Approximate running time 10 minutes

Samples were dialysed and filtered and loaded as specified in section 2.5.2.

The gradient controller was programmed to produce the following profile.

92% buffer B for 20ml

92 - 100% buffer B for 10ml

100% buffer B for 4ml

Aldolase was tested for homogeneity by SDS gel electrophoresis, followed by gel scanning on a Chromoscan 3 gel scanning device.

2.6 SDS polyacrylamide gel electrophoresis

Slab gel technique

2.6.1 Apparatus

- (a) Slab gel stand and buffer reservoir
- (b) Two flat glass plates (4mm), one 18cm x 14cm, the other the same dimensions but with a 13cm x 2cm slot cut out of the top.
- (c) Perspex spacers: two - 2.5cm x 13.2cm x 0.15cm
one - 18cm x 0.7cm x 0.15cm
- (d) Comb 12.6cm x 1.7cm x 0.15cm with a 12.6cm x 0.7cm x 0.3cm ridge with either 13, 16 or 20 teeth
- (e) Spring clamps for holding the cassette together

2.6.2 Assembly of the cassette

- (a) The cassette was assembled with spacers on either side and along the bottom, flush with the vertical spacers
- (b) The two clips were placed on either side with two clips along the bottom
- (c) The spacers were sealed with 3% agar

2.6.3 Pouring the gel

Separating gel solution was slowly added to the cassette up to 1cm below the depth of the comb. Water-saturated butan-2-ol was placed on top of the gel to ensure the gel polymerises evenly. Once the separating gel had polymerised the butanol was removed and replaced with water, until the gel was ready for use.

The cavity was filled with stacking gel solution and the comb was inserted, excluding all the air. The stacking gel was then left to polymerise.

2.6.4 Gel Run

The comb was removed making sure that the stacking gel and separating gel did not separate. Then the clips and bottom spacers were removed. All excess agar was cleaned away and the top of the glass plate, with the slot cut in it, was greased and the cassette was clipped to the tank. Electrode buffer was poured into the lower (300 - 400ml) and upper (400ml) tanks. All bubbles were removed from the bottom of the gel. Electrodes were attached, anode(+) at the bottom.

Runs were usually performed overnight (16 hours) at 35 volts for a 10% separating gel.

2.6.5 Gel solutions

The solutions used were as that of Laemmli (1970) with the following minor modifications

- (a) Stacking gel: 1.5M Tris-HCl; 8mM EDTA; 0.4% SDS pH8.8
- (b) Separating gel: 0.5M Tris-HCl; 8mM EDTA; 0.4% SDS pH6.8
- (c) Polyacrylamide (3%w/v); 1mM NaN₃; 1mM NaF
- (d) Electrode buffer: 0.05M Tris base; 0.38M glycine; 3.5mM SDS; 2.3mM EDTA
- (e) Gel fixer; 10% acetic acid v/v; 20% methanol v/v
- (f) Gel stain ; 0.25% w/v Coomassie blue R methanol 50% v/v; acetic acid 7.5% v/v
- (g) Destaining solution; acetic acid 7% v/v; methanol 10% v/v
- (h) Sample buffer x4 concentrated: 0.5M Tris-HCl pH6.5 - 40ml; SDS (8g); 0.2M EDTA pH 7.0 (4ml); glycerol 40ml; H₂O to 100ml.

Mercaptoethanol and 0.5% bromophenol blue were also added to the sample buffer (about 20 μ l/500 μ l sample). This buffer can be added to samples in solution or used to dissolve precipitated samples, but it must be 1 + 3 volumes diluted in the final solution.

2.6.6 Fixing and staining

The gels were fixed for a least 30 minutes then stained for 10 - 20 minutes at 37°C. The stained gels were washed with H₂O and then destained with an excess of destaining solution and a number of pieces of colourless polyurethane foam (which accelerated the destaining procedure) in a shaking bath at 37°C for 2 - 4 hours.

2.7 Isotopic labelling

A solution of 85ml was prepared containing 1mCi ¹⁴C + 2g ¹⁵N-leucine (this large volume being required to dissolve 2g of ¹⁵N leucine). Birds were weighed and given 0.677ml of solution per 100g body weight (i.e. 7.86 μ Ci ¹⁴C and 15.93mg ¹⁵N per 100g body weight) by intra peritoneal injection, since the volume was too large for intravenous administration. The actual volume administered and time were noted.

Four-week old birds were placed in numbered cages. Eighteen birds were labelled (randomly distributed between 2 fume cupboards). The birds were allowed to become accustomed to the surroundings 2 days prior to injection of label. At 2, 6, 15, 30, 48 and 72 hours after injection of the labelled leucine 3 birds were removed from their cages, randomly, blood was taken by cardiac puncture and plasma prepared. Birds were killed (by dislocation of the neck) and total liver, and breast muscle from one side was removed rapidly, weighed and quickly frozen. The breast muscles from the other side

of the sternum were removed much more carefully and exactly and were then weighed.

Excreta was collected at the time of killing and every morning (i.e. 24 hours) for birds living for 30, 48 or 72 hours after injecting. The table below gives an outline of sampling times:

Table

time incubated (hours)	excreta collected at (hours)
2	2
6	6
15	15
30	24 and 30
48	24 and 48
72	24, 48 and 72

Excreta was collected from the cages, dried at 80° in a fan assisted oven, then ground to a fine powder prior to analysis.

2.8 Analysis of samples for ^{15}N

2.8.1 Sample preparation

(1) Plasma

Blood taken from birds at each time interval was precipitated with 10% TCA and centrifuged for 10 minutes at 5000 rpm. The supernatant plasma was then ready for Kjedahl digestion (section 2.8.2), or scintillation counting (section 2.9.2).

(2) Excreta

Total nitrogen was estimated by NH_3 assay prior to all sample being digested by the Kjedahl technique (see section 2.8.2).

(3) Muscle proteins

Whole muscle protein was prepared by adding 0.2g of muscle tissue to 4ml of 0.6M PCA. The sample was then homogenised and centrifuged for 10 min. at 6000g. The pellet was washed twice with

0.2M PCA and twice with water. The mixture was then freeze dried prior to $^{15}\text{N}:^{14}\text{N}$ ratio determination.

Sarcoplasmic protein was prepared by the addition of 0.2g of muscle tissue to 4ml of 50mM potassium phosphate buffer, containing 1mM EDTA, 1mm magnesium acetate and 1mm 2-mercaptoethanol, pH7.5. The solution was homogenised, and centrifuged at 6000g for 10 min. The pellet was washed twiced with 0.2M PCA and twice with water. The solution was then freeze dried prior to $^{15}\text{N}:^{14}\text{N}$ ratio determination. It was initially assumed that values for myofibrillar protein could be obtained by subtracting the sarcoplasmic data from the whole muscle data. However this indirect method gave unsatisfactory results. It was decided to isolate myofibrillar protein and count this directly (see section 2.9.1).

Individual proteins were isolated from birds at each time interval as described in sections 2.4 and 2.5. All the purified protein samples were extensively dialysed against 1mM potassium phosphate buffer (pH6.5) to remove all nitrogen containing compounds in the FPLC and CK separating buffers (e.g. 1% betaine, 50mM MES, and sodium azide). The samples which were analysed by the Dumas combustion technique were in a volume less than 5ml with a protein concentration of up to 1mg/ml. Samples were concentrated by Centricon 30 micro concentrators (Amicon, Stonehouse, Glos.) or by freeze drying.

2.8.2 Kjehldahl/Rittenberg technique

All plasma and excreta samples were subjected to the Kjehldahl digestion reviewed by Bremner (1965).

Samples containing at least 1mg of nitrogen were added to a

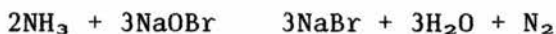
Kjehldahl flask containing 60ml of concentrated H_2SO_4 + 0.8g of catalyst. The catalyst contained K_2SO_4 : $CuSO_4$: SeO_2 in the weight ratio of 25:5:1. The Kjehldahl flask also contained two anti-bump granules. The mixture was heated in heating blocks to a temperature sufficient to boil the sample and allow condensation to occur at the neck of the flask, preventing loss of NH_3 . The solution was left to boil for 6 hours.

The ammonia generated in this reaction could then be separated from the rest of the reactants by steam distillation. This is necessary to prevent any interference in the subsequent hypobromite oxidation or any contamination of the N_2 formed by this oxidation. This separation can be accomplished by distilling the sample with strong alkali in a stream of NH_3 -free air and collecting the NH_3 liberated in 10ml H_3BO_3 indicator solution. The apparatus used in this distillation is designed so that it can be rapidly disassembled and washed to prevent cross contamination. Cross contamination is further cut out by distilling a small volume (15 - 20ml) of ethanol between steam distillations. The apparatus was first used by Rittenberg, (1948) and Springson and Rittenberg, (1948).

Use of H_3BO_3 indicator solution had the advantage that the amount of ammonia in the distillate could be readily determined by titration with standard H_2SO_4 . The reagents present after evaporation of this solution to a small volume do not affect the results obtained by isotope ratio analysis (Bremner 1965).

Conversion of ammonia to nitrogen gas (N_2) for mass spectrometer analysis was accomplished by treatment of the sample with alkaline hypobromite in the complete absence of air. The reaction is

generally represented as follows:



Special apparatus

(1) Gas sample tubes described by Springson and Rittenberg, (1948) were obtained from Eck and Krebs Inc. 27-09 40th Avenue, Long Island City, New York.

(2) The vacuum system for evacuation of gas sample tubes is a modification of the apparatus described by Springson and Rittenberg, (1948). The major modifications are:

(a) an oil diffusion pump is used instead of a mercury diffusion pump;

(b) the trap is cooled with liquid N_2 (instead of dry ice);

(c) the system is evacuated to a pressure $<0.001\text{mmHg}$ (instead of 0.01mmHg).

Procedure

A sample of solution containing about 1mg of ammonia was placed in one arm of the gas sample tube and approximately 2ml of hypobromite solution put in the other arm. The vacuum system was prepared by closing the stop cocks, immersing the trap in liquid nitrogen and evacuating the system until the pirani gauge indicated a pressure of $<0.01\text{mmHg}$. The Y tube neck and the arm of the vacuum system was greased.

The stop cock was opened on the arm and the Y section of the gas sample tube was rotated to evacuate the tube, to less than 0.001mmHg on the pirani gauge. Both sides of the Y tube were then frozen in liquid N_2 and the Y tube was disconnected from the vacuum system by rotating the Y tube sealing the vacuum in the tube. The Y tube was

then attached to the inlet valve of the mass spectrometer. The legs of the tube were thawed and the tube rotated to allow the hypobromite to react with the ammonia. The $^{28}\text{N}_2:^{29}\text{N}_2$ ratio was measured in the mass spectrometer. The operation and specifications of the mass spectrometer were as described by Micromass 602C Instruction Manual, VG Micromass, Windsford, Cheshire.

2.8.3 Dumas Combustion System

All ^{15}N -labelled protein samples had N_2 released by an alternative method to the Kjedahl/Rittenberg technique (see section 2:8:2), called Dumas combustion (Steyermark, 1961). This technique has many advantages over the Kjedahl digestion; not least because Dumas combustion is a single-step process producing nitrogen by the oxidation of organic matter.

(1) Elemental Gas Chromatograph

A Carlo Erba (Milan, Italy) ANA 1400 automatic nitrogen analyser was used throughout this work. The mode of operation is based on the Dumas principle with high temperature "flash" combustion. The carrier gas used was helium (BOC Special Gases, London) at 60ml min^{-1} . The oxygen used was argon free oxygen (BOC) with a 5ml loop. The analyser had a 24 seat automatic sampling manifold capable of accommodating solid or liquid samples. One complete analytic cycle takes 5 minutes. The sample size is between 0.18 and $180\mu\text{mol}$ of N_2 with an accuracy of 0.2% at $35\mu\text{mol}$ of N_2 (Carlo Erba).

Mass Spectrometer

The instrument used was a VG Isogas (Middlewich, Cheshire) MM 622 isotope Mass Spectrometer. Modifications and operating procedure were as described by Preston and Owens (1983) as well as

analysis on resolution, background, precision and memory effects.

2.9 ¹⁴C Counting

2.9.1 Sample preparation

Whole muscle protein was prepared by adding 0.2g of muscle tissue to 4ml of 0.6M PCA. The sample was then homogenised and centrifuged for ten min. at 6000g. The pellet was washed twice with 0.2M PCA and twice with water. The mixture was then added to 1ml of Fisosolve (Fisons, Loughborough, Leics.). The solution was heated for 3 hours at 60°C. After heating, 4.5ml of Fisofluor 3 (scintillation cocktail) was added and the sample was counted.

Preparation of "soluble" and myofibrillar fractions were carried out by adding 0.2g of muscle tissue to 4ml of 50mM potassium phosphate buffer, containing 1mM EDTA, 1mM magnesium acetate and 1mM 2-mercaptoethanol pH7.5. This was homogenised and then centrifuged at 12,000g for 10 min. 4ml of 1.2M PCA was then added to the supernatant which was again centrifuged at 6000g for 10 min. The pellet was washed twice with 0.2M PCA and H₂O. 0.5ml of the redissolved pellet was added to 4.5ml of Fisofluor 3 and counted. This was assumed to be the sarcoplasmic fraction of muscle. The pellet was redissolved in 1ml of Fisosolve and heated for 3 hours at 60°C. 4.5ml of Fisofluor was then added and the solution was counted. This solution was assumed to contain myofibrillar protein.

All the individual muscle protein samples which were counted had a protein concentration of greater than 0.1mg ml⁻¹. In the case of creatine kinase and phosphoglycerate mutase, this meant concentrating the samples in Centricon microcentrators (Amicon).

0.5ml samples were taken to a volume of 5ml by the addition of

Fisofluor 1. After mixing, the samples were placed in a Searle mark III 680 liquid scintillation system and counted for 100 minutes or 2000 counts. Background counts were obtained for 0.5ml of buffer alone + 4.5m of Fisofluor. The efficiency was determined by external standard and was found to be 82%.

2.10 Statistical analysis

Elementary statistical techniques were used to analyse the result obtained. The mean, standard deviation and, in some instances, median values were calculated. It was thought that these were sufficient to present the data in a concise and understandable way.

It was assumed that the mean (\bar{x}) = $\Sigma \frac{x}{N}$.

The standard deviation was equal to $\Sigma \frac{(x-\bar{x})^2}{N-1}$, while the median was considered to be the middlemost or central value of the variable when the values are in order of magnitude (Yule and Kendall (1953)). The majority of the data is present as the mean \pm the standard deviation.

Chapter 3

Experimental

3.1 Introduction

This project set out to measure the synthesis and degradation rates of certain proteins in the sarcoplasmic fraction of chicken, breast muscle, using pulse labelling techniques. Two types of labelling experiment were carried out, the first with heavy nitrogen [^{15}N] and radioactive carbon [$\text{U-}^{14}\text{C}$] labelled glycine, the second [^{15}N] and [$\text{U-}^{14}\text{C}$] labelled leucine. The experiment with labelled glycine was a pilot study aimed at identifying the incubation times (of tracer in the animal) which gave the most information about the pattern of changes in the content of each label in various intracellular pools (e.g. proteins) (see appendix).

In order to calculate the rate of synthesis and degradation of a protein in muscle one must first have some idea of how much of that particular protein is present in the muscle. The first part of this chapter deals then with the determination of the protein pool size. The chapter then goes on to cover the development of protein separation techniques which it was hoped would yield greater than 10mg of protein. This amount of protein was required for the samples containing ^{14}C and ^{15}N labelled glycine in order to produce enough nitrogen, via the Kjeldahl technique, to enable mass spectrometry to be carried out. However by the time the experiment using ^{14}C and ^{15}N labelled leucine was set up a new technique had been introduced, called Dumas Combustion. This made it possible to analyse samples containing as little as 1mg of protein. This allowed a different approach to purification, namely F.P.L.C. The latter part of this chapter is mainly concerned with the development of methods of protein separation using this fast protein liquid

chromatography system.

3.2 The pool size

Since it was clearly impractical to investigate every sarcoplasmic protein it was decided to study the four most abundant; enolase, aldolase, GAPDH and creatine kinase. These four enzymes have been shown to make up about 60% of the total sarcoplasmic protein (Lebherz et al 1982). In addition it was hoped to measure the turnover of lactate dehydrogenase and pyruvate kinase, two less abundant enzymes, for comparison. However pyruvate kinase proved difficult to isolate (see section 3.3.3), Phosphoglycerate mutase was found to be more easily purified and was therefore selected as a representative of the less abundant sarcoplasmic proteins. The fact that enzymes such as alanine aminotransferase, adenylate kinase and hexokinase were not investigated is unfortunate, since they may each have a very interesting labelling pattern, however since enzymes such as these are quantitatively fairly minor, their omission should not greatly affect the labelling pattern of the sarcoplasmic fraction as a whole.

3.2.1 Preparation of sarcoplasmic fraction

Muscle specimens were homogenised in 10 to 20 volumes of 50mM potassium phosphate, 1mM EDTA, 1mM magnesium acetate, 1mM 2-mercaptoethanol pH 7.5 in an Ultra Turrax bench homogeniser. The homogenisation process involved scissor mincing of the muscle samples followed by grinding and shearing of the minced samples by the metal teeth of the bench Ultra Turrax homogeniser. Homogenates were centrifuged at 12,000g for 10 minutes (Beckman, JA20 rotor, 4°C).

3.2.2 Measurement of the selected enzymes by enzymic assay

The activities of aldolase, enolase, GAPDH and creatine kinase were measured spectrophotometrically by the methods described in sections 2.3.2-2.3.5. The values obtained can be converted into mg of enzyme per gram wet weight of muscle, provided the molecular weight of each enzyme and its turnover number is known. To measure the degree of extraction of these four enzymes in the supernatant fraction the pellet was suspended and respun as before. This procedure was repeated until no activity could be detected by enzyme assay. Table 3.2.1 (column 4) shows that all activity was removed after four centrifugations. Column 1 in table 3.1 shows the molecular weights of the 4 enzymes while column 2 gives the measured activity for each enzyme in chicken breast muscle homogenate. The final column in table 3.1 shows the estimated concentration of each of these enzymes in muscle, taking into account that lost in the pellet fraction.

Table 3.2.1

ENZYME	mol.wt.	measured activity $\mu\text{mol}/\text{min}/\text{g}$	% retrieval centrifugations				calculated mg enzyme/g muscle
			1	2	3	4 wet muscle	
Aldolase	15800(a)	229 \pm 148	88.5	10.4	1.1	0	6.18 \pm 1.0
ENO	82000(b)	572 \pm 60	86	13.1	0.9	0	9.8 \pm 0.9
GAPDH	120000(c)	434 \pm 154	87.5	11.2	1.3	0	0.064 \pm 0.0015
Creatine kinase	81000(d)	325 \pm 15.5	86%	13.0	1.0	0	0.015 \pm 0.005

3.2.1 Degree of extraction of 4 sarcoplasmic proteins from chicken breast muscle as determined by enzymatic analysis ($\bar{x} \pm$ standard deviation, N=4)

References

- (a) Kawahara and Tanford (1966)
- (b) Wold (1971)
- (c) Alison and Kaplan (1964)
- (d) Forster et al (1974)

The enolase values are slightly higher than those obtained by Lebherz et al, 1982 (table 3.2) while the values for aldolase were slightly lower; perhaps these results were due to the age or strain of bird used. The values for creatine kinase and GAPDH were much lower than those reported in the literature, even in the presence of protease inhibitors such as benzamidine and phenylmethyl sulphonyl fluoride (PMSF).

It was decided to assay GAPDH and creatine phosphokinase in the direction of glyceraldehyde 3 phosphate and creatine production (as outlined by Bergmeyer (1974) and Forster et al, (1974) respectively). This would determine whether the original values obtained were "real" or whether the initial assays were inefficient at predicting enzyme levels for this system. The latter was found to be the case, assays in the opposite direction producing much higher estimates of enzyme concentration, although still lower than those reported by Lebherz et al (see table 3.2.2).

It was decided to check the reliability of all the values obtained by enzymatic means by measuring the enzyme content using electrophoresis followed by gel scanning (see section 3.2.3). It was hoped that this would support the results obtained by the enzyme assays and prove that the values obtained for GAPDH and creatine kinase, although lower than those found by Lebherz et al, (1982),

were reliable for this system.

Table 3.2.2

	LEBHERZ <i>et al</i> (1982)	duplicate test from 2 separate extractions (enzyme assays)	SDS gel scanning
	mg/g muscle	mg/g muscle	mg/g muscle
Aldolase	10-12	6.18±1.0	7.33±1.06
GAPDH	10-12	4.89±1.5	9.06±1.05
Enolase	7-8	9.8 ±0.9	6.49±1.14
Creatine kinase	5-6	1.87±0.05	4.75±0.78

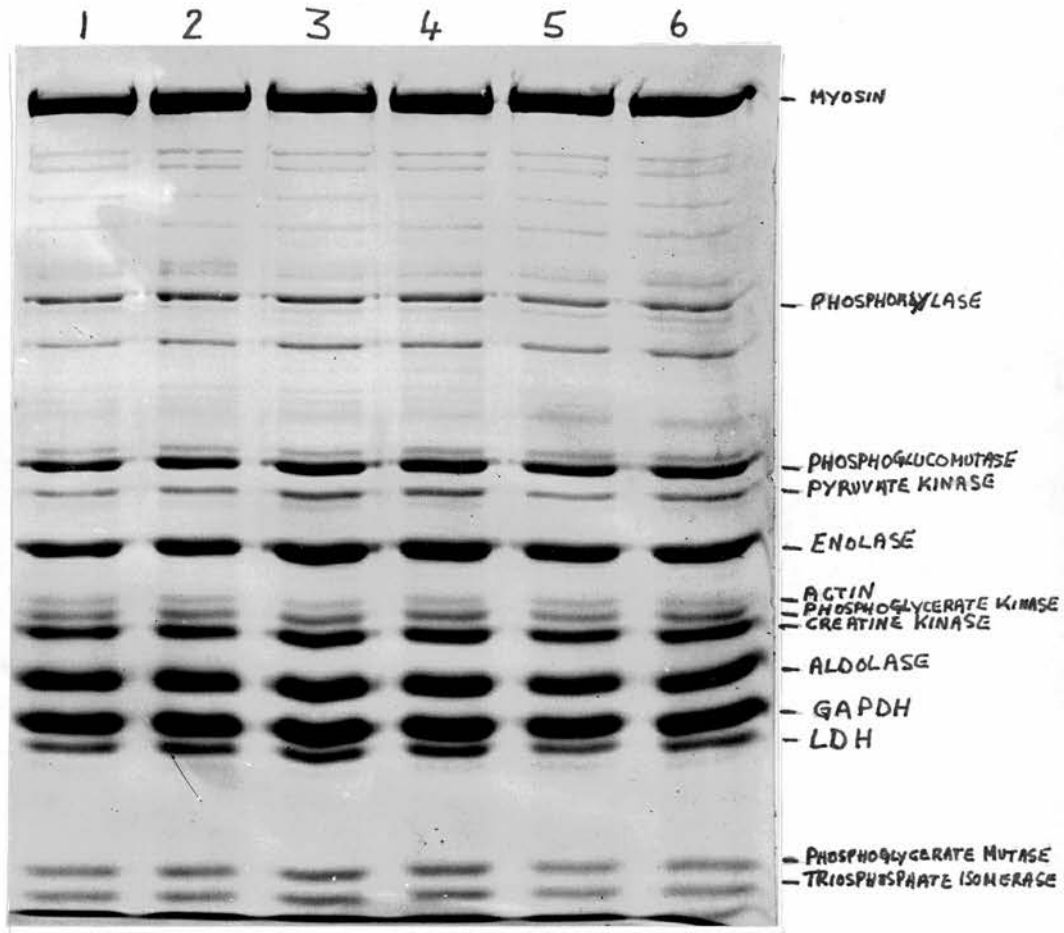
3.2.2 Comparison of data on enzyme content obtained by enzymatic means to that obtained by electrophoresis followed by gel scanning. Column 2 mean ± standard deviation N = 4
Column 3 mean ± standard deviation N = 6

(Lebherz *et al*'s values are based on electrophoretic and not enzymatic work.)

3.2.3 Measurement of the selected enzymes by SDS polyacrylamide gel electrophoresis followed by gel scanning

SDS gels prepared by a modification of the method of Laemmli (1970) (as outlined in section 2.6) were run overnight (15 hours) at ~40 volts; after fixing, the gels were stained with Coomassie blue R and densitometric analysis was carried out using a Gilford model 250 spectrophotometer equipped with a gel scanning attachment. Figure 3.2.1 shows a typical gel pattern from six muscle samples, two taken from each of three birds. All the major enzymes have been identified by their molecular weights and by comparison with the gel pattern obtained by Lebherz *et al* 1982. However as Lebherz did not identify some of the less abundant bands, such as pyruvate kinase and lactate dehydrogenase, these had to be identified by comparison with samples of pure enzyme.

Figure 3.2.1

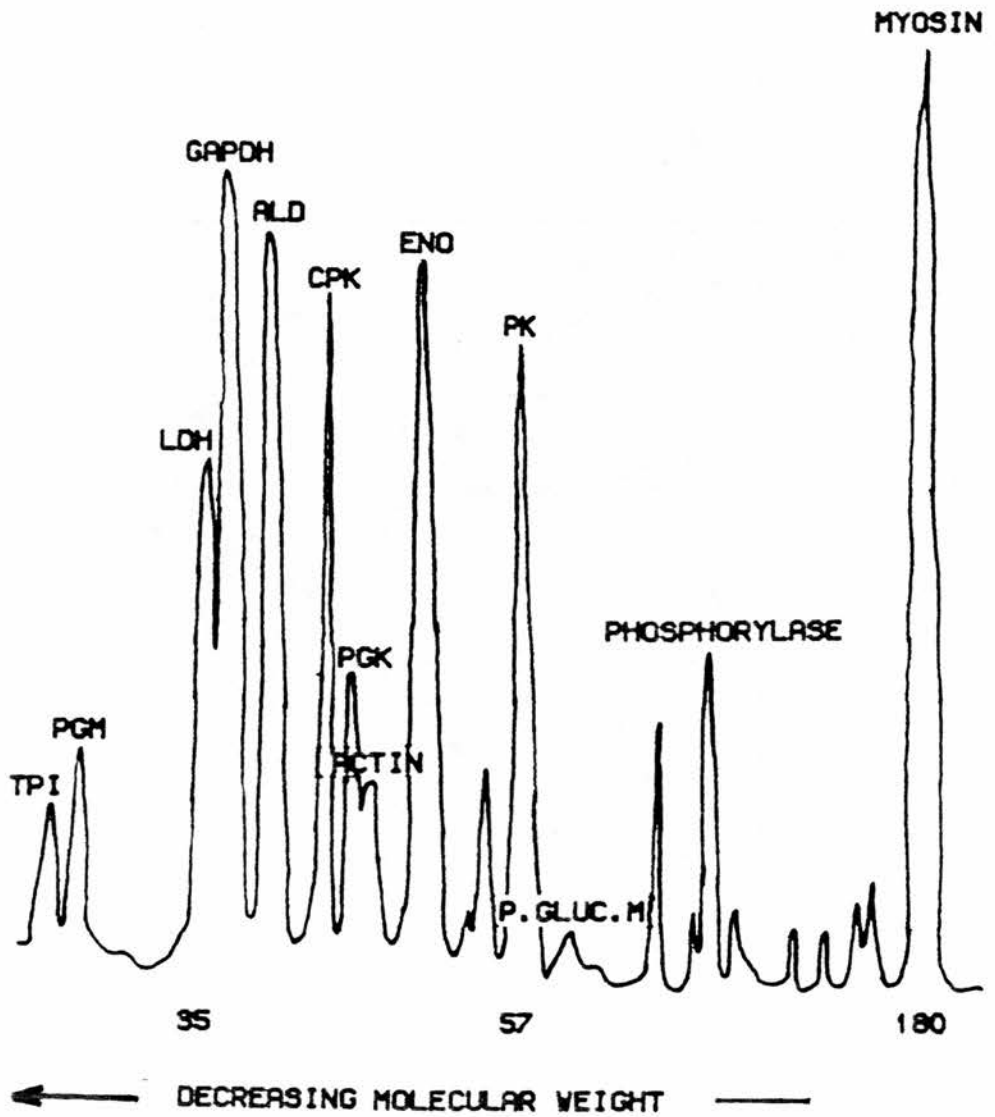


3.2.1 Analysis of the 12,000g soluble proteins of breast muscle by electrophoretic analysis on SDS polyacrylamide gels. The gels were stained for protein with Coomassie blue. 3 birds were tested with 2 muscle samples taken from each, (track 1 and 2 = bird 1; track 3 and 4 = bird 2 and track 5 and 6 = bird 3).

70

The system was made quantitative by densitometric analysis of strips of gel, producing a profile (figure 3.2.2) which can be integrated, and then standardised by using known quantities of pure protein such as bovine serum albumin or enolase. These can be used to produce a reliable standard curve. It was found that by adding only 2 standards a reliable result could be obtained. The results were found to be reproducible on the same soluble fraction, giving similar totals and percentages for all major proteins. Earlier work (Lebherz et al 1982) had shown that each protein band on a one-dimensional SDS gel had no major additional bands when run in two dimensions. That is, each band on a one-dimensional gel was quantitatively homogeneous within the limits of detection. The intensity of staining of the four most abundant enzymes on a one-dimensional gel was found by Lebherz et al (1982) to be similar, using Coomassie blue, amido black or ponceau red as protein stains. It was assumed here that this would also apply to other major abundant proteins. Table 3.2.3 shows the enzyme content and total protein calculated from peak areas of stained SDS gels taking into account the 13% lost in the pellet for 1 to 6 week old birds. It can be seen that the content of actin and myosin varies between samples, perhaps due to variations in the intensity of homogenisation. This will affect the total soluble protein calculations and therefore the percentage of each enzyme in the total. Table 3.2.4 shows the recalculated percentages of the four most abundant enzymes; aldolase, enolase, GAPDH and creatine kinase from 1 - 6 weeks, when the values of actin and myosin were subtracted from the total soluble protein. Together these four make up around 60% of the total soluble protein.

FIGURE 3.2.2.



3.2.2. Analysis of the 12,000g soluble proteins of chicken breast by electrophoretic analysis in SDS-polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm (top of the gel is to the right).

Table 3.2.3

All values are in mg/g muscle wet weight

Protein	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
myosin	0.96	4.18	5.42	9.83	11.61	10.85
unidentified	0.42	0.34	0.33	0.36	0.47	0.46
unidentified	0.24	0.28	0.28	0.34	0.47	0.37
unidentified	0.25	0.27	0.20	0.20	0.37	0.31
unidentified	0.34	0.32	0.29	0.25	0.46	0.31
unidentified	0.52	-	0.28	0.29	1.13	-
phosphorylase	1.92	1.06	0.97	1.24	2.24	1.72
unidentified	0.66	0.36	0.28	0.31	0.45	0.68
unidentified	1.10	0.94	0.87	0.88	1.49	0.83
unidentified	0.28	0.19	0.27	-	-	-
unidentified	0.43	-	0.12	-	-	-
P Gluc M	0.88	0.68	0.58	0.59	1.05	0.45
unidentified	0.54	0.44	0.45	0.64	0.86	0.66
P.K.	2.71	3.16	2.94	4.07	4.58	3.84
unidentified	0.95	0.86	0.84	1.03	1.25	0.95
unidentified	0.34	-	-	-	0.28	-
ENO	5.76	7.46	4.86	6.61	7.99	6.24
actin	0.35	3.16	0.84	0.49	0.69	5.07
PGK	1.06	1.58	1.58	1.13	1.30	1.05
CPK	5.54	4.07	3.95	4.75	5.83	4.34
ALD	7.80	5.99	6.22	7.91	8.77	7.29
GAPDH	8.81	8.93	7.80	9.94	10.61	8.25
LDH	3.16	1.81	1.58	1.58	1.91	2.49
PGM	1.58	1.13	1.36	1.47	2.03	1.24
TPI	1.47	0.96	1.13	1.47	1.85	1.16
Σx	48.07	48.14	43.50	55.50	67.69	53.43
$\Sigma x - AeM$	46.76	40.80	38.92	45.00	55.39	37.51
$\bar{x}_{(6)}$ Lowry	55.54	35.40	51.61	49.00	61.76	59.21

3.2.3 Soluble protein of the sarcoplasmic fraction (calculated from peak areas of stained SDS gels) all peak areas were measured so that several minor components have not been identified. All calculated values include the 13% lost in the pellet after centrifugation. Each value represents the mean from six separate experiments.
(AeM = actin and myosin)

Table 3.2.4

Enzyme	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Aldolase	17.60	14.60	16.70	17.60	15.80	19.40
Enolase	12.90	18.40	13.00	14.70	14.40	16.60
GAPDH	19.70	21.90	20.90	22.00	19.20	22.00
Creatine Kinase	12.40	10.00	10.50	10.64	10.50	11.60
Total	62.60	64.90	61.10	64.90	59.90	69.60

3.2.4 The table shows the percentages of the four major enzymes of the sarcoplasmic protein fraction when the values of actin and myosin are subtrated from the total soluble protein. (Each value is an average from 6 samples - 3 birds 2 samples per bird)

The values estimated from SDS gel electrophoresis were higher for aldolase, GAPDH and creatine kinase than those obtained by enzymic assay and were closer to values obtained by Lebherz et al (1982) for breast muscle. SDS gel analysis of enolase produced similar values to those of Lebherz although the results from the enzymatic assay were slightly higher than that from SDS gel analysis. This may have been because the turnover number of enolase used for the calculation was that for rabbit muscle, and there may be species differences between the enzymes in white muscle from the two species.

From these experiments it was concluded that these enzyme assays were not efficient or accurate enough to be useful as a quantitative method of estimating total enzyme content for the system under investigation. Moreover SDS gel electrophoresis was found to be a quick, efficient and reliable technique for studying the enzyme content of chicken breast muscle. Not only did it give consistent results, but these were similar to those found elsewhere (Lebherz et al 1982). In addition SDS gel electrophoresis followed by gel scanning allowed all of the proteins present in the soluble fraction of muscle to be quantitated. This showed that the concentration of all of these enzymes remained fairly constant relative to each other throughout the first six weeks of growth.

3.3 Isolation of selected enzymes

3.3.1 Introduction

Problems were encountered in separating the proteins, mainly because the Kjedahl digestion technique used for preparation of ^{15}N labelled samples, prior to mass spectroscopy, required a pure sample of about 10mg. The technique described by Lebherz et al

(1982) does not indicate how much of each protein is isolated. Earlier work by this group is quoted by Leberz et al (1982) for the final step in purifying enolase. The column dimensions are given 1.1cm x 10cm. Using 1gm of muscle this final step was tested, (i.e. after phosphocellulose ion exchange chromatography) and it was found that only small amounts of pure enolase was recovered (e.g. 1mg). However increasing the amount of muscle sample only served to increase the degree of contamination of enolase. Therefore the entire separation procedure had to be scaled up at least ten times. When this was done it was found that only creatine kinase was obtained purely. The remaining three enzymes were eluted in specific peaks along with many contaminating proteins. An additional problem found when trying to isolate aldolase was that a large fraction of this enzyme always remained bound to pyruvate kinase. Thus the yield of pure aldolase was reduced.

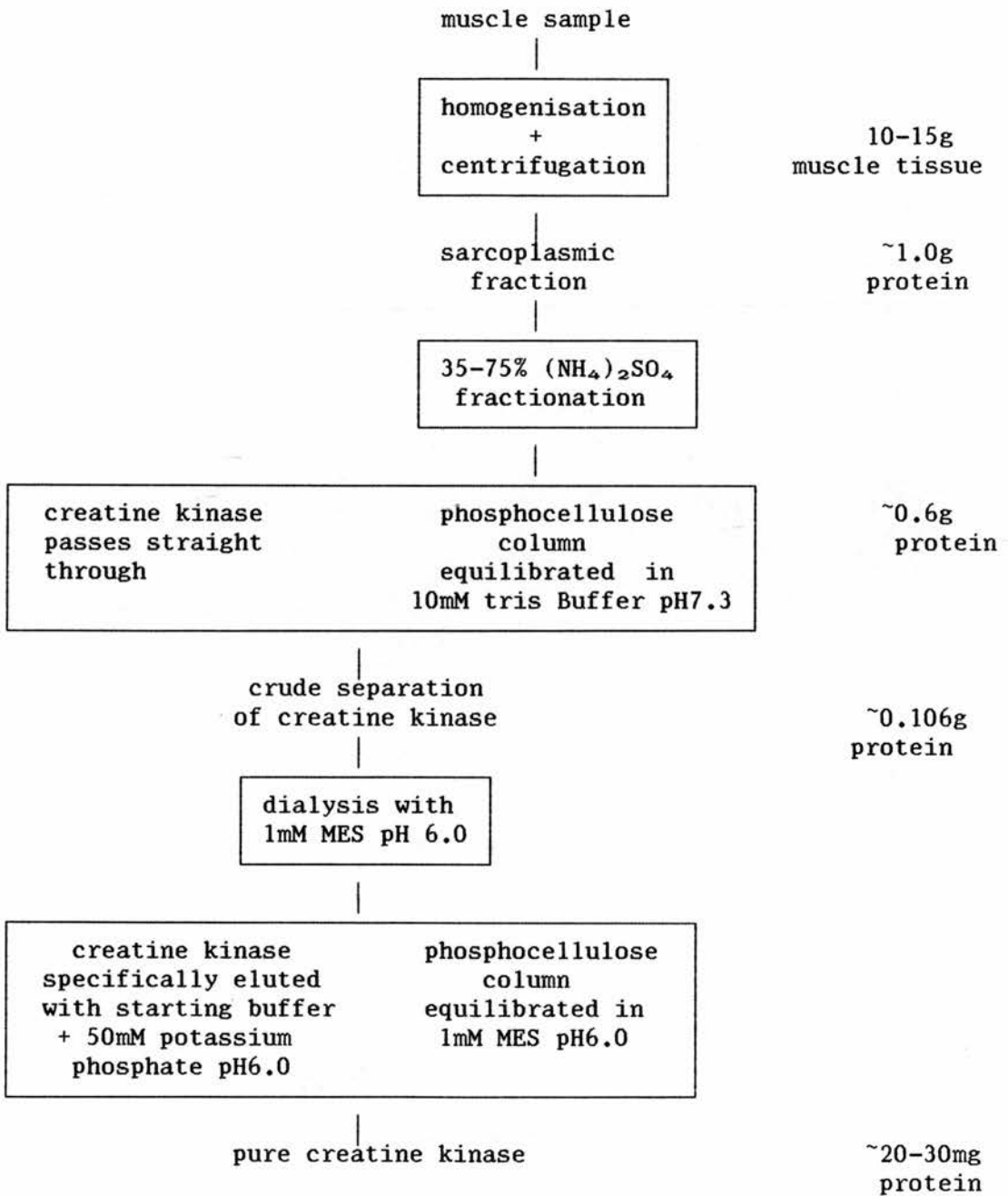
However, by the time the second experiment using [^{15}N][U^{14}C] labelled leucine had been set up, the sample preparation system for the mass spectrometer also had the adaptation of Dumas combustion. This system required only 1mg of each sample. This allowed a different approach to the method of purification, namely fast protein liquid chromatograph (FPLC).

3.3.2 The separation of creatine phosphokinase

Creatine kinase was isolated from muscle sarcoplasmic fraction by ammonium sulphate fractionation collecting that fraction precipitated by 35% - 75% saturation (see section 2.4) followed by dialysis against 10mM tris HCl 1mM magnesium acetate, 1mM 2-mercaptoethanol pH7.3. Samples were then applied to a

phosphocellulose column equilibrated in this buffer. Under these conditions creatine kinase passed straight through the column. Final purification of creatine kinase was by dialysing the sample against 1mM 2(N morpholino) ethane sulphonic acid, 1mM EDTA, 1mM magnesium acetate, 1mM 2-mercaptoethanol pH6.0. The samples were then applied to a phosphocellulose column equilibrated in this buffer. Creatine kinase was specifically eluted from the column with this buffer containing 50mM potassium phosphate pH6.0. All samples were tested for purity by SDS gel electrophoresis followed by gel scanning using a Chromoscan 3 gel scanner. The results of this are shown in table 3.3.1. A summary of creatine kinase purification is shown in figure 3.3.1.

Figure 3.3.1

3.3.1 Separation of creatine kinase

All weight values are for total protein unless otherwise stated.

Table 3.3.1

[¹⁵ N][¹⁴ C] glycine experiment % purity	[¹⁵ N][¹⁴ C] leucine experiment % purity
1	100
2	100
3	100
4	100
5	98
6	98
7	98
8	97=median
9	97
10	90
11	90
12	90
13	89
14	89
15	78
	1
	2
	3
	4
	5
	6
	7
	8
	9
	10
	11
	12
	13
	14
	15
	16
	17
	100
	100
	99
	96
	93
	93
	91
	90
	90=median
	90
	90
	90
	89
	87
	87
	83
	83

3.3.1 Purity of creatine kinase isolated from chicken breast muscle from two separate labelling experiments by the method described in section 3.3.2. Samples were run on SDS polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm. The total area under each peak was calculated and the area under the peak corresponding to creatine kinase expressed as a percentage of the total.

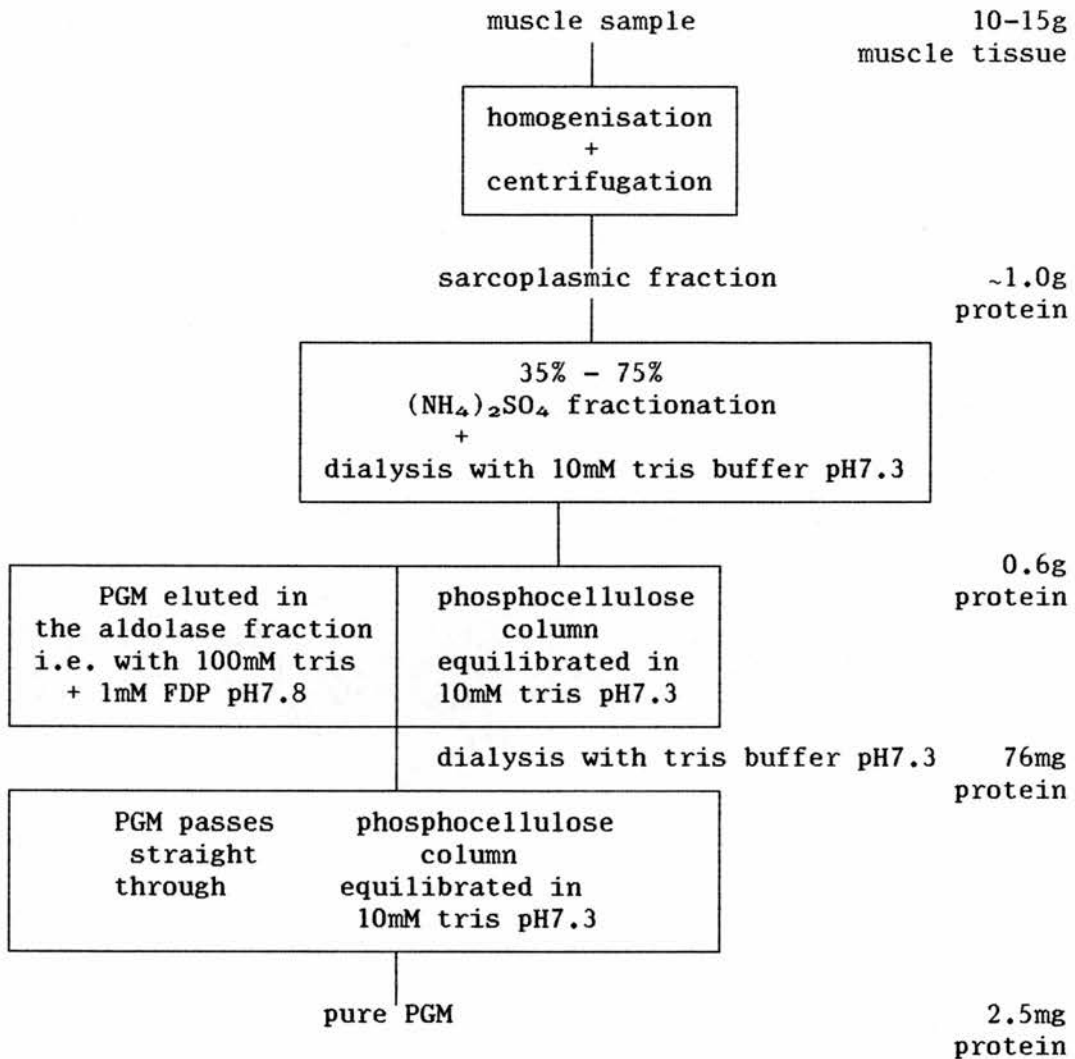
3.3.3 The separation of aldolase

Phosphocellulose ion exchange chromatography

Pyruvate kinase was the main contaminant in the aldolase-containing peak eluted from the phosphocellulose ion exchange column. It was decided to rerun the column using the same eluting buffers. This did not, unfortunately, separate aldolase from pyruvate kinase but it did isolate another contaminating protein, phosphoglycerate mutase (PGM), which passed straight through the column on reapplication and was shown to have a median purity of 100% via electrophoresis and gel scanning (see table 3.3.2 and

figure 3.3.2). It was decided to change the conditions slightly from those of the first column run (see materials and methods). The starting buffer was unchanged (i.e. 10mM Tris pH7.3). However the eluting buffers were 10mM tris + 1mM FDP pH7.3 (instead of 100mM tris pH7.8) and 100mM tris pH7.8 (instead of 100mM tris + 1mM FDP pH7.8). It was hoped that the degree of binding of the proteins to the phospho-cellulose would be greater using the lower ionic strength eluting buffer; this might prevent PK from eluting while aldolase would be eluted by the presence of its substrate - FDP. This method was found to be more successful in that a portion (2-3mg) of the aldolase was eluted pure, although the remainder was eluted together with pyruvate kinase in the 100mM tris pH7.8 buffer. In an attempt to eliminate the pyruvate kinase and possibly purify both enzymes in a single step, the FDP concentration in the first eluting buffer was then increased to 2mM. This, however, eluted both enzymes completely in a single peak. It thus eliminated the separation previously achieved. It was concluded that phospho-cellulose by itself was unlikely to separate these two enzymes completely.

Figure 3.3.2

3.3.2 The separation of PGM

All weight values are for total protein unless otherwise stated.

Table 3.3.2

	% purity
1	100
2	100
3	100
4	100
5	100 = median
6	97
7	88
8	76
9	62

3.3.2 Purity of phosphoglycerate mutase.

Phosphoglycerate mutase was isolated from chicken breast muscle in an experiment using [¹⁵N][¹⁴C] glycine. The enzyme was isolated by the method described in figure 3.3.2. Purified samples were run on SDS polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm. The total area under each peak was calculated and the area under the peak corresponding to phosphoglycerate mutase expressed as a percentage of the total.

Molecular exclusion chromatography

The samples containing aldolase and pyruvate kinase (which had been passed through phosphocellulose twice) were applied to a Sephacryl S300 molecular exclusion gel (Pharmacia Uppsala Sweden). Various column lengths and diameters were tried in attempts to achieve an efficient separation, as well as numerous flow rates and sample volumes. No appreciable separation was found although the molecular weights of the enzymes are very different, namely 158,000 for aldolase and 237,000 for PK. It is presumed that there is a fairly strong intermolecular association between the two proteins, although there is no report of this in the literature.

Ammonium sulphate fractionation

Ammonium sulphate precipitation was then used to try to separate aldolase from pyruvate kinase, using the method of Czok and Bücher, (1960). This gave some separation but aldolase was never totally free from pyruvate kinase.

In spite of the failure to produce completely pure aldolase on the scale required, the procedure summarized in Figure 3.3.3 was used for the routine purification of aldolase for the labelling experiments using ^{15}N and ^{14}C labelled glycine. The aldolase isolated by this method was usually much less than the 10mg needed for mass spectrometric analysis via the Kjehldahl technique. This was partially compensated for by the addition of NH_4Cl to the samples after Kjehldahl digestion, to enable analysis to be carried out. This dilution is not ideal but was the only option available.

SDS gels were run on the aldolase separated and the gels were scanned as described for creatine kinase (section 3.3.2). Table 3.3.3 shows the % purity obtained.

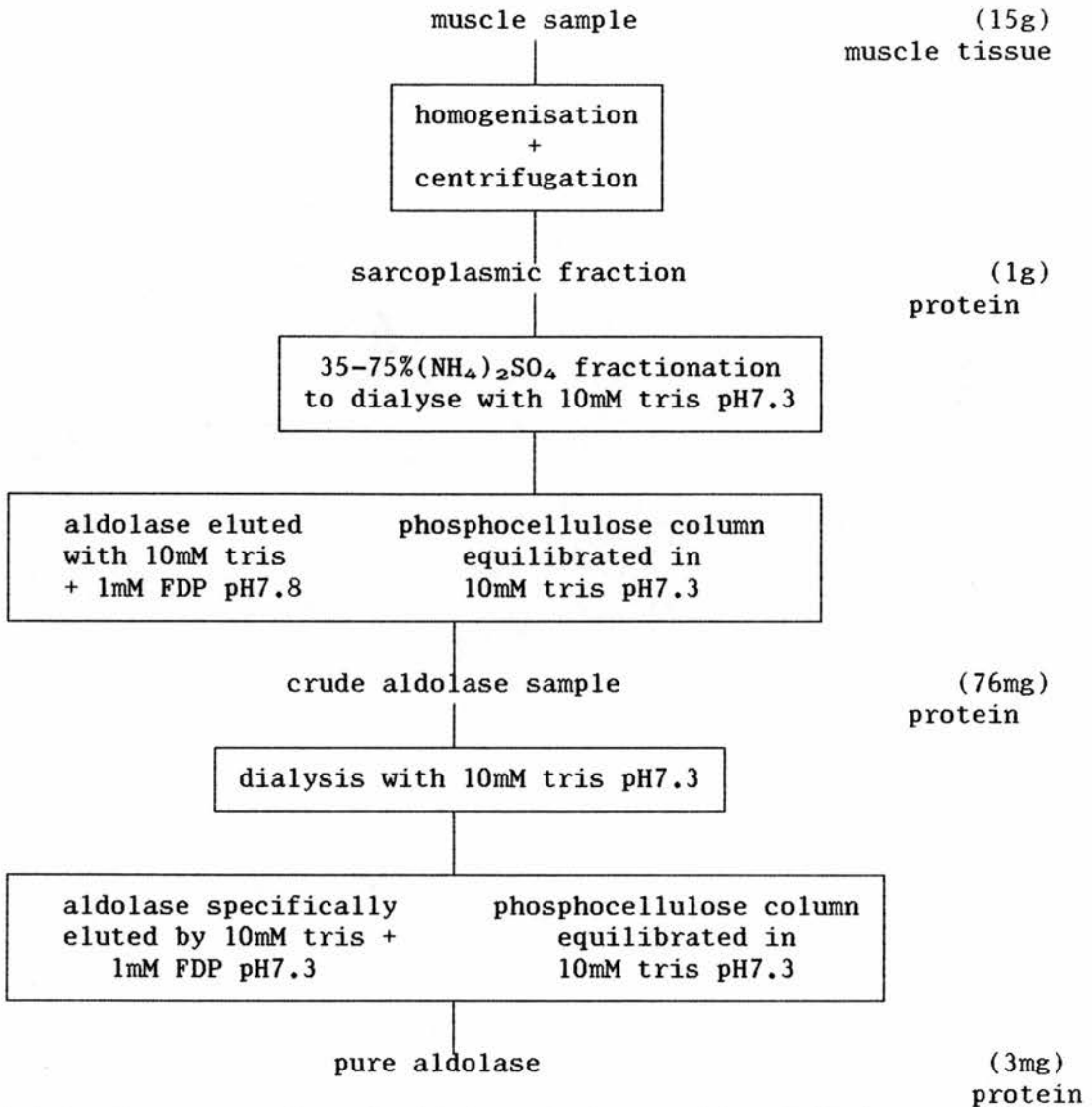
Table 3.3.3

	% purity
1	100
2	100
3	100
4	100 = median
5	100
6	100
7	91

3.3.3 Purity of Aldolase

Aldolase samples isolated from chicken breast muscle in an experiment using [¹⁵N][¹⁴C] labelled glycine were isolated by the method described in figure 3.3.3. Purified samples were run on SDS polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm. The total area under all the peaks found in each sample was calculated and the area under the peak corresponding to aldolase expressed as a percentage.

Figure 3.3.3



3.3.3 The Separation of aldolase Sepharose blue CL6B ion exchange chromatography

All weight values are for total protein unless otherwise stated.

Before the second labelling experiment was carried out, (using labelled leucine), a further attempt was made to increase the efficiency and yield of the aldolase separation. Pure rabbit muscle aldolase and pyruvate kinase (obtained from Sigma) were mixed (10mg:5mg) and applied to a Sepharose blue CL6B column (Pharmacia,

Uppsala Sweden) equilibrated in 10mM tris pH7.5. The eluting buffers were (a) 10mM tris containing 10mM PEP, (b) 10mM FDP, (c) 50mM FDP and finally 2M urea. These eluting buffers were those used by Stellwagen et al (1975). With the exception of 50mM FDP and 2M urea, these were included in order to remove any proteins not eluted by the first two buffers.

It was found that both aldolase and pyruvate kinase were eluted in a single peak with 10mM PEP. The experiment was repeated, with the buffer containing 10mM FDP pH7.5. This time both enzymes were eluted by FDP.

Different conditions were tried using 10mM tris pH7.5 containing either 1mM PEP, 1mM FDP or 1M KCl pH8.5. 1mM PEP was found to specifically elute a fraction of the pyruvate kinase; however the remainder was found with aldolase in a single peak eluted by 1mM FDP. It was therefore decided to find what concentration of PEP would elute all the pyruvate kinase.

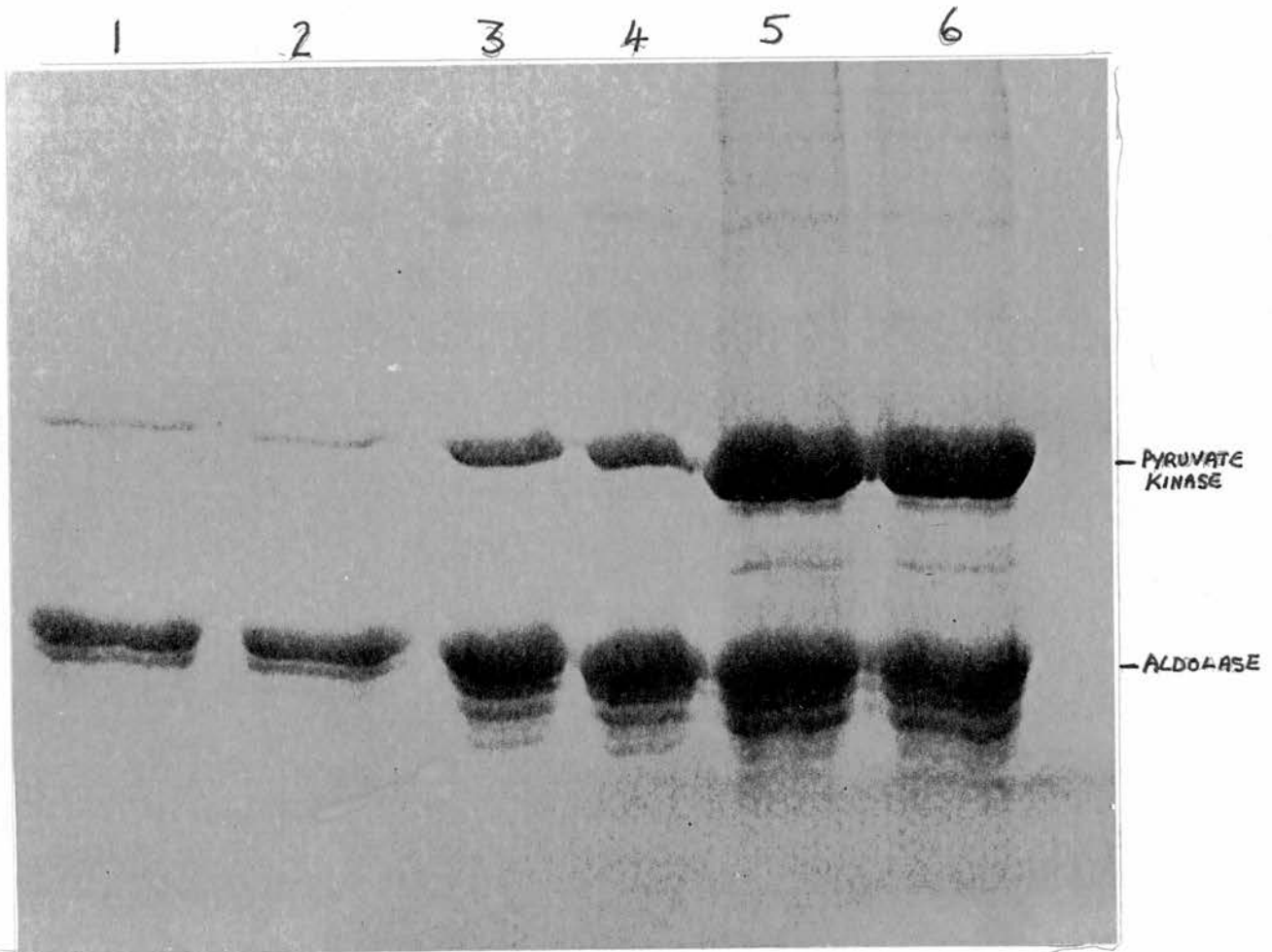
The conditions were kept the same except the eluting buffers contained 2mM PEP, 4mM PEP, 6mM PE, 8mM PEP and 10mM FDP. Figure 3.3.4 shows the subsequent electrophoretic analysis. Thus there appears to be a fraction of PK which is always eluted with aldolase.

SDS polyacrylamide gel electrophoresis

SDS-gels had been shown on numerous occasions to separate aldolase and pyruvate kinase, but the amount of protein that could be separated had been too small to consider gels as a useful preparative method. When ¹⁵N analysis was transferred to the East Kilbride laboratories, where only 1mg protein per sample instead of approximately 10mg would be required, it became sensible to look

at gel separation to see if it could produce samples large enough for ^{15}N analysis.

Figure 3.3.4



3.3.4 Sepharose blue CL6B affinity chromatography. Samples containing aldolase and pyruvate kinase were applied to the column equilibrated in 10mM Tris pH7.5. Peaks were eluted using starting buffer containing various substrates and run on a SDS polyacrylamide gel. The gel was stained for protein with Coomassie blue. The eluting buffers contained 2mM P.E.P. (track 1); 4mM P.E.P. (track 2); 6mM P.E.P. (track 3); 8mM P.E.P. (track 4); 10mM P.E.P. (track 5) and 10mM FDP (track 6) all in 10mM Tris PH7.3.

Two main problems were envisaged: (a) could the gels separate enough protein; and (b) would the SDS gel adversely affect the ^{14}C counting by quenching.

Samples of pure aldolase and PK (Sigma) were used. It was found that the first problem could be overcome by running 2 gels of the proteins mixed together. Each separation produced 0.5mg of pure protein.

The degree of quenching caused by the SDS was tested. Samples of pure aldolase and PK (Sigma) were radiolabelled with ^{14}C -formaldehyde as outlined in figure 3.5 (Dottavio-Martin and Ravel, 1978). $1\mu\text{l}$ of each protein sample was added to $500\mu\text{l}$ of mixed cold protein, $10\mu\text{l}$ of the resulting solution was added to $500\mu\text{l}$ of mixed cold protein. This meant that one solution should have x50 more ^{14}C label than the other. Each was then run on an SDS-gel overnight. The pyruvate kinase and aldolase bands from each gel were cut out and subjected to H_2O_2 digestion. The solution was then mixed with scintillation cocktail (Fisofluor) and counted. The results in table 3.3.4 show the SDS markedly quenches the observed counts. The degree of quenching was so great that the technique was ruled out as a quick preparative method. Moreover the amount of radioactivity used in this experiment was much higher than that expected from enzymes isolated from labelled chicken breast muscle. The amount of labelling per bird was fairly low due to legal restrictions on the level of radiation allowed in one laboratory for one experiment. Therefore with such a high level of quenching, and predicted low levels of radioactivity no attempt was made to develop this technique.

Table 3.3.4

actual radioactivity	observed radioactivity of samples run on SDS-Polyacrylamide gels	
	<u>Ald</u>	<u>PK</u>
10 ⁴ dpm	168dpm	158dpm
200dpm	158dpm	143dpm

3.3.4 The table shows the degree of quenching observed when pure aldolase and pyruvate kinase are labelled with ¹⁴C (see figure 3.3.5 for method of labelling). The proteins were mixed and samples were run on SDS-polyacrylamide gels. The separated bands were cut from the gel and the polyacrylamide digested with H₂O₂. The remaining solutions were counted for 10 min.

Fast protein liquid chromatography (molecular exclusion)

In view of the fact that improvements to the mass ratio isotope spectrometry might lower the requirement of protein per sample from 10mg to 1mg fast protein liquid chromatography was investigated as a general means of separating aldolase and pyruvate kinase.

The columns available (see materials and methods section 2.5) could handle amounts of protein up to 25mg. This, in principle would be sufficient to provide 1mg of pure protein per sample.

Initially pure aldolase and PK (Sigma) were mixed and used to test the FPLC system using a Superose 12 HR 10/30 molecular exclusion column. The column was equilibrated in 100mM tris, 1mM magnesium acetate, 1mM EDTA, 1mM 2 mercaptoethanol and 500mM NaCl, pH7.8. The samples were applied in a similar buffer containing 1mM FDP. The 500mM NaCl was introduced in an attempt to prevent any protein/protein interactions. Both enzymes were eluted together. The buffer was altered to include 1.5M tris + 0.4% SDS pH8.8. It was hoped that these changes would further prevent protein/protein binding and dissociate the enzymes into their respective subunits which differed in their molecular weights (PK = 57,200, aldolase = 50,000); however, no separation was obtained.

Finally a 50mM citrate buffer containing 6.5M urea + 0.1% SDS pH5.0 was used, and once again no appreciable separation was achieved.

FPLC (ion exchange)

An ion exchange column was then used since the molecular exclusion technique was providing little success.

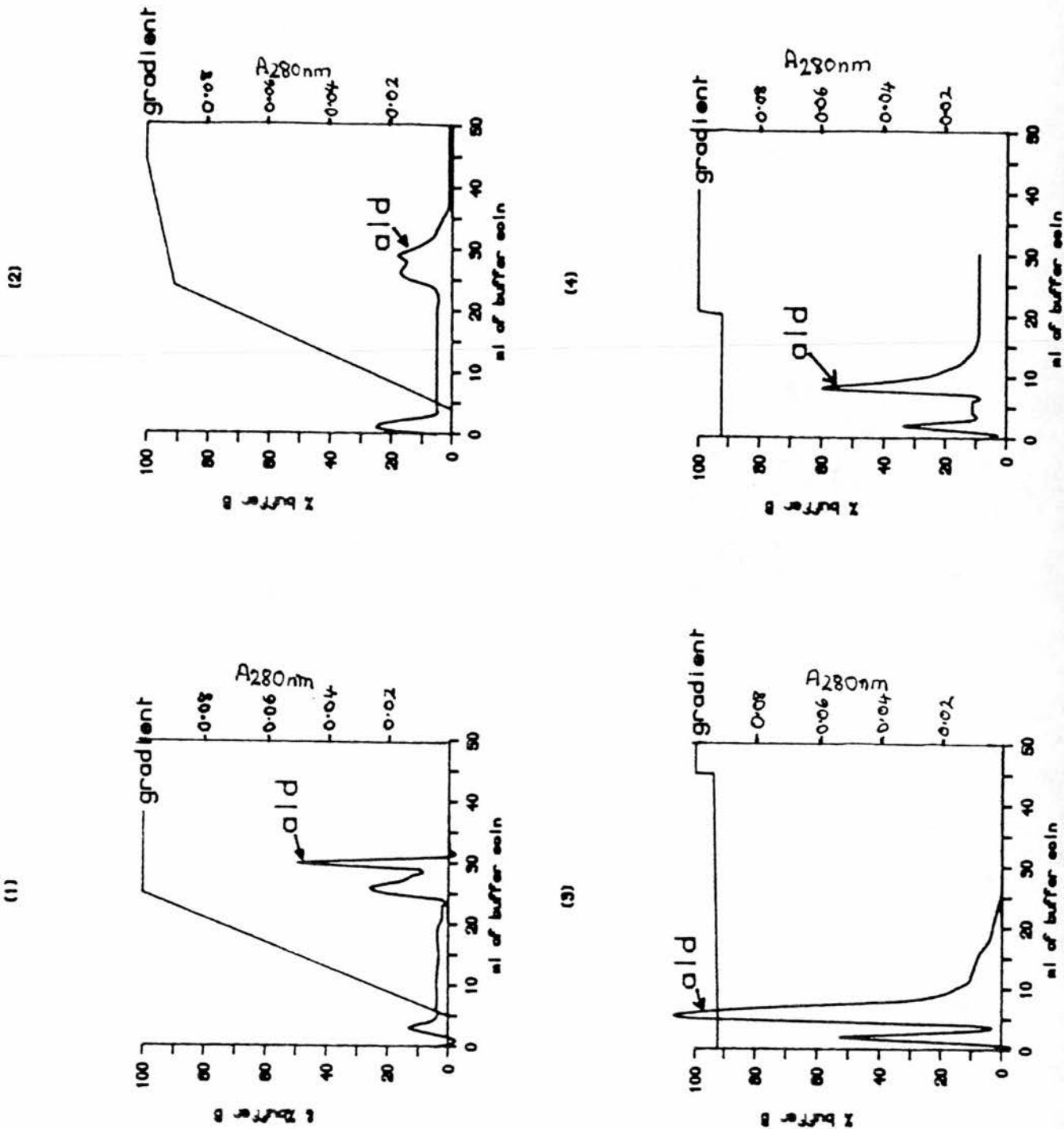
The technique originally followed was that reported by Lindblom and Fägerstamn (1984) because it was recommended by the makers of the FPLC system (i.e. Pharmacia). The system uses a H 5/5 prepacked cation exchange column. Unfortunately the paper by Lindblom and Fägerstamn (1984) does not say how much protein was applied to the column nor how much protein is present in each peak. The investigators also fail to provide electrophoretic evidence of purified proteins. In effect the results merely show a protein profile of chicken breast muscle soluble proteins. Therefore, in

order to find if cation exchange could provide an efficient separation producing enough protein (1mg) for mass spectrometry, a variety of buffers and gradients (based on those of Lindblom and Fägerstamm) were tested. Numerous attempts at separation failed. This was eventually found to be due to omissions from Lindblom and Fägerstamm's paper as well as from the Pharmacia handbook on FPLC, which quotes Lindblom and Fägerstamm's work. In fact the two reports on the same experiment differ in the concentration of buffer (25mM and 50mM) and the order in which the enzymes are eluted. In addition all Lindblom and Fägerstamm's buffers contained 50mM NaCl to prevent the enzymes precipitating and 1% betaine to disperse the charge-rich domains of the enzymes, thereby reducing protein-protein interactions. None of this was actually mentioned in the report of their experiments, and was only discovered by repeated communications with Pharmacia who in turn contacted Lindblom.

Eventually when all this information had been gained, it was possible to begin experiments with various gradients in earnest.

Enough aldolase was present in the phosphocellulose eluent from the ^{14}C and ^{15}N leucine labelling experiment to enable small amounts ($\approx 500\mu\text{l}$) to be used in pilot studies, especially since the final amount required was now only 1mg. When separation was achieved the experiment was scaled up. Figure 3.3.6 shows how the final gradient was selected. The system routinely used $\approx 15\text{ml}$ (15mg) per run. Figure 3.3.7 shows a typical scale up run. Scanning of sample run on P.A.G.E. gave the purity values indicated in table 3.3.5. Aldolase was separated from all major contaminants, in particular

FIGURE 3.3.6.



3.3.6

The 12,000g soluble fraction of chicken breast muscle homogenate was subjected to 35% - 75% ammonium sulphate fractionation, followed by phosphocellulose ion exchange chromatography on a column equilibrated in 10mM Tris pH7.3. The fraction eluted by 10mM Tris + 10mM FDP pH7.8 was then applied to an FPLC cation exchange column equilibrated in 23mM MES, 50mM NaCl, 1% betaine, 1mM magnesium acetate and 1mM mercaptoethanol pH6.0. A peak containing purified aldolase was eluted with the same buffer containing 350mM NaCl using the gradient selection process indicated.

pyruvate kinase. However, pyruvate kinase was unfortunately not eluted in a pure form, but was contaminated with aldolase and some other minor components. A summary of aldolase purification is presented in figure 3.3.8.

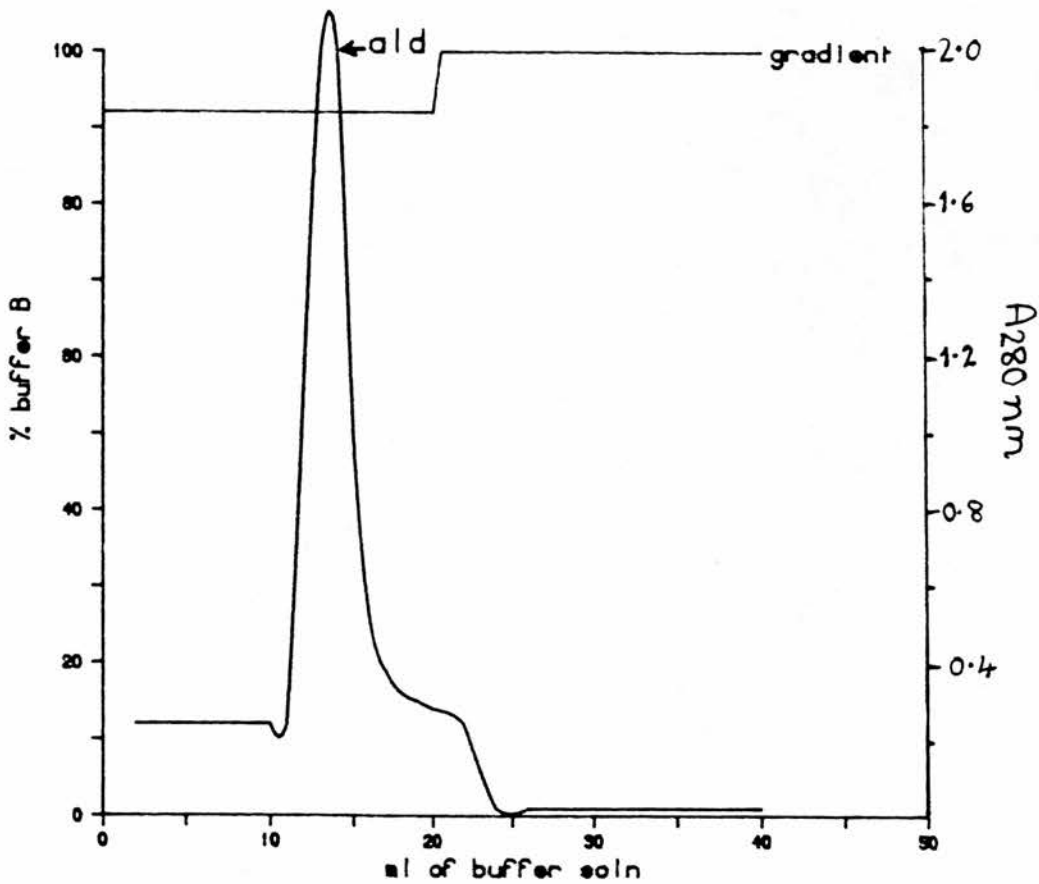
3.3.4 The separation of enolase

Initially enolase separation was aimed at producing at least 10mg of pure protein, which was then subjected to Kjedahl digestion. The first part to this section covers the difficulties encountered in trying to achieve this aim. Eventually a new, small scale sample preparation system was set up and the remainder of this section covers the separation of enolase, GAPDH, LDH and PGM for Dumas combustion.

Phosphocellulose and CM cellulose ion-exchange chromatography

Lebherz et al (1982) noted that enolase was not obtained pure by a single initial passage through their phosphocellulose column. They further purified enolase by passage through a CM cellulose column. This was found to be difficult to repeat, especially when scaled up as the CM cellulose tended to clog up the column even when BDH celite (a 3cm layer at the bottom) was present to increase the porosity. It was decided to try batch separation. The sample and CM cellulose were stirred together in a beaker for one hour and then added to a large-diameter column (7cm x 38.5cm²) with a celite layer at the bottom. This improved the flow rate but the enolase eluted was not pure. The enolase-containing eluate from the phosphocellulose column had contained substantial amounts of phosphorylase, GAPDH, LDH and PGM. CM cellulose did remove these but one unknown contaminant remained. In an attempt to purify

FIGURE 3.3.7.



3.3.7

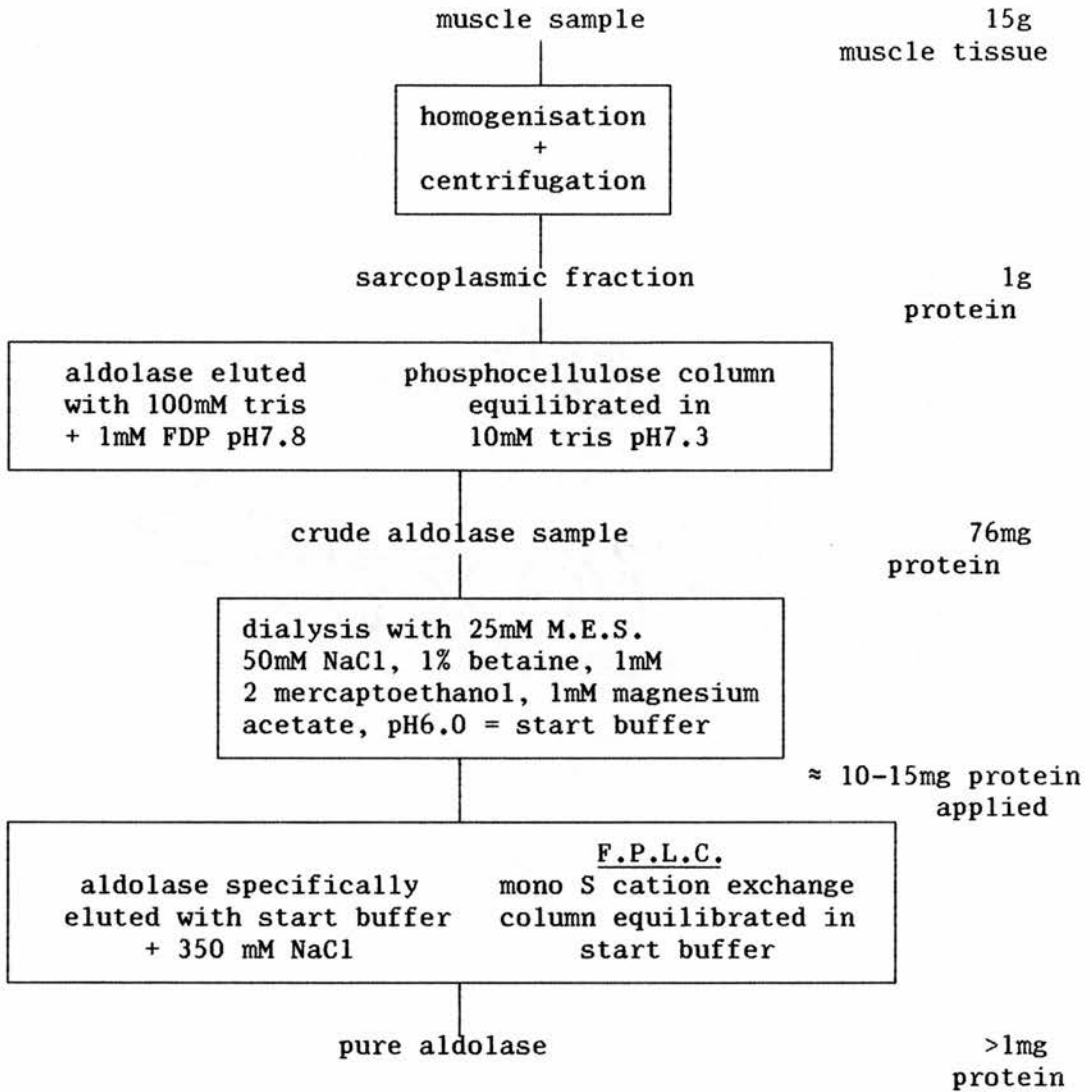
The 12,000g soluble fraction of chicken breast muscle homogenate was subjected to 35% - 75% ammonium sulphate fractionation, followed by phosphocellulose ion exchange chromatography on a column equilibrated in 10mM Tris pH7.3. The fraction eluted by 10mM Tris + 10mM FDP pH7.8 was then applied to an FPLC cation exchange column equilibrated in 23mM MES, 50mM NaCl, 1% betaine, 1mM magnesium acetate and 1mM mercaptoethanol pH6.0. A peak containing purified aldolase was eluted with the same buffer containing 350mM NaCl using the gradient shown.

Table 3.3.5

	% purity
1	100
2	100
3	100
4	100
5	100
6	100
7	100
8	100 - median
9	100
10	100
11	100
12	98
13	97
14	97
15	93
16	90

3.3.5 Purity of aldolase samples isolated from chicken breast muscle from an experiment using [^{15}N][^{14}C] leucine as a tracer. The enzyme was purified by the method described in figure 3.3.8. Isolated samples were run on SDS-polyacrylamide gels. The gels were stained with Coomassie blue and were densitometrically analysed at 600nm. The area under each peak was calculated and the area under the peaks corresponding to aldolase expressed as a percentage of the total.

Figure 3.3.8

3.3.8 Separation of aldolase

enolase completely, another approach was taken.

Water solubility

Enolase is known to be very water soluble (Petell *et al* 1981); it appeared feasible to dialyse the enolase-containing sample from the phosphocellulose elution against water, and to spin out any precipitate, leaving pure enolase. Unexpectedly enolase was also found in the precipitate and the pellet was not readily redissolved probably due to denaturation; because of this it could not even be run on a SDS-gel to check its purity.

Sepharose blue CL6B ion exchange chromatography

The enolase fraction from phosphocellulose chromatography was passed through a column containing sepharose blue CL6B resin equilibrated in 10mM tris pH7.5. This separation was based on work done by Easterday and Easterday (1974) on non-specific protein binding to sepharose blue CL6B. Elutions were made with the same buffer containing 0.1M, 0.2M, 0.5M, 1.0M KCl, and finally 2M urea. Enolase plus a small number of contaminants were eluted by 0.1 M KCl. It was decided to use 0.02M, 0.05M, 0.08M and 0.1M KCl in an attempt to reduce the contamination, but this did not remove any of the contaminating proteins. The column conditions were then changed to include the substrates of enolase. The eluting buffers containing 1mM PGA, 1mM PEP, 0.1M KCl and 1M KCl pH7.5. However this also proved unsuccessful.

Ammonium Sulphate fractionation

Ammonium sulphate fractionation was tested as a means of purifying enolase by the method outlined by Czok and Bucher (1960). The resulting sample contained mainly enolase but still had a number

of contaminants.

Throughout this series of tests the best separation was obtained by passing the sample through a CM-cellulose column as outlined by Lebherz et al (1982). This was therefore adopted as the means of purification. The remaining samples were run on CM-cellulose, using large-diameter columns with 3cm of celite at the base. The eluents were run on SDS gels. The gel was scanned as described previously (3.3.2) and the percentage purity was determined. Table 3.3.6 shows the enolase samples to have a median purity of 91%. A summary of the final separation method is outlined in figure 3.3.9.

Because of the difficulties encountered in isolating aldolase and enolase, it was decided to abandon GAPDH and PK separation for the glycine labelled experiment.

3.3.5 The separation of enolase, PGM, GAPDH and LDH

FPLC (Ion exchange)

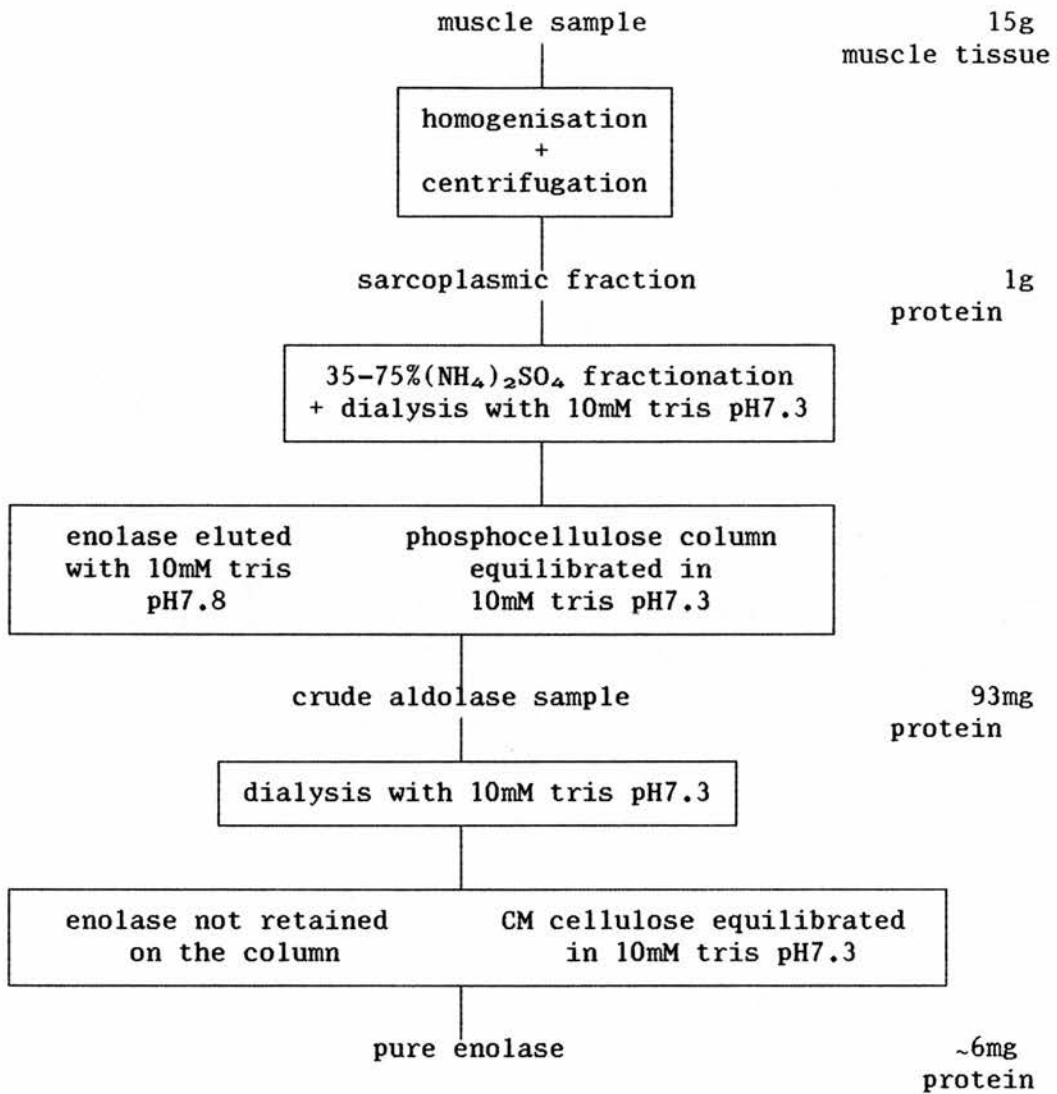
For the labelled leucine experiment it was decided to use FPLC ion exchange chromatography to try to separate enolase, PGM, GAPDH and LDH using the enolase peak from the phosphocellulose column run (see section 3.3.1). The initial problems with this system were the same as those encountered with aldolase (see section 3.3.3). Figure 3.3.10 shows some of the column profiles which eventually led to the separation of enolase, GAPDH, LDH and PGM. This system was set up using 500 μ l samples, and it was subsequently scaled up to run 15ml (see figure 3.3.11). Roughly 15 - 20mg of mixed proteins were run and each purified enzyme peak contained greater than 1mg of protein.

Table 3.3.6

	% purity
1	100
2	94
3	91 = median
4	88
5	79

3.3.6 Purity of enolase isolated from chicken breast muscle from an experiment using [^{15}N][^{14}C] glycine as a tracer. The enzyme was purified by the method outlined in figure 3.3.9. Isolated samples were run on SDS-polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm. The area under each peak was calculated and the area under the peak corresponding to enolase expressed as a percentage of the total.

Figure 3.3.9

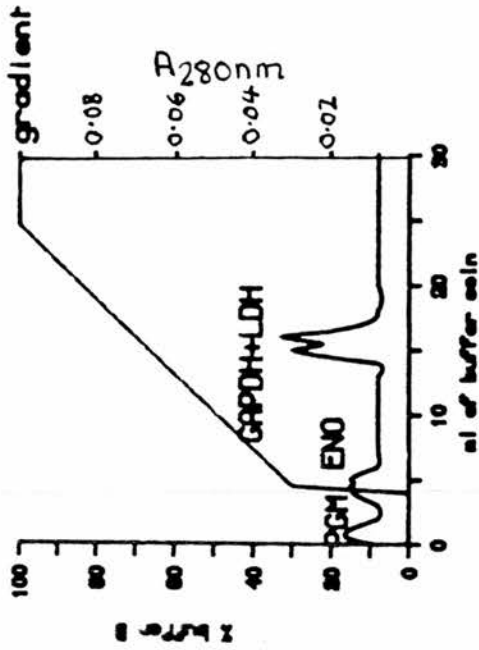


3.3.9 Separation of enolase

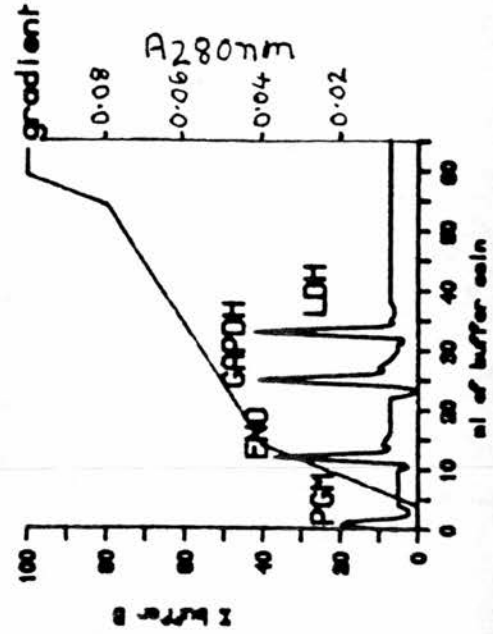
All weight values as for total protein unless otherwise stated.

110
FIGURE 3.3.10.

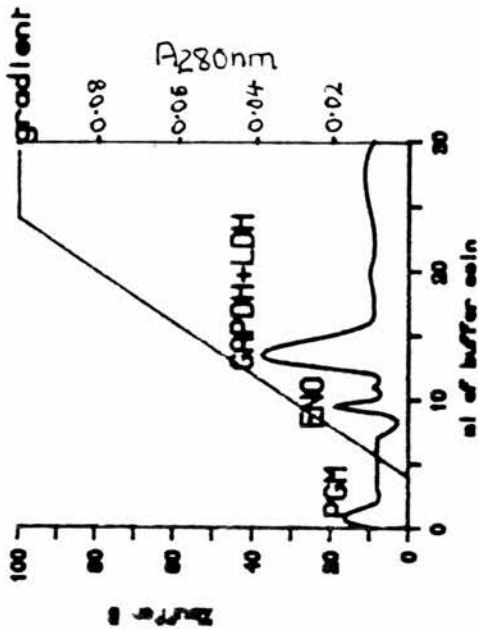
(2)



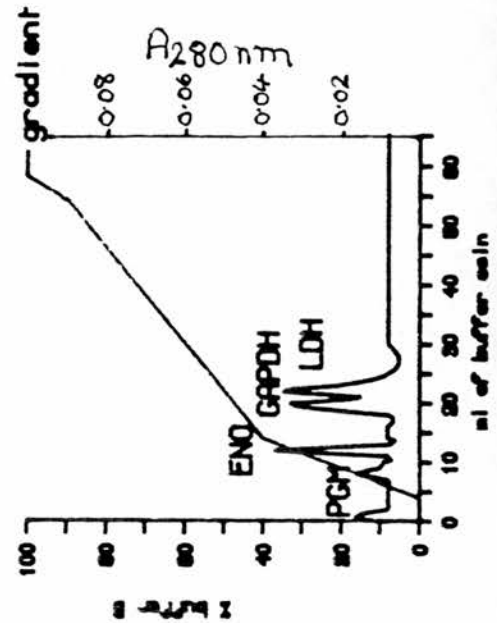
(4)



(1)



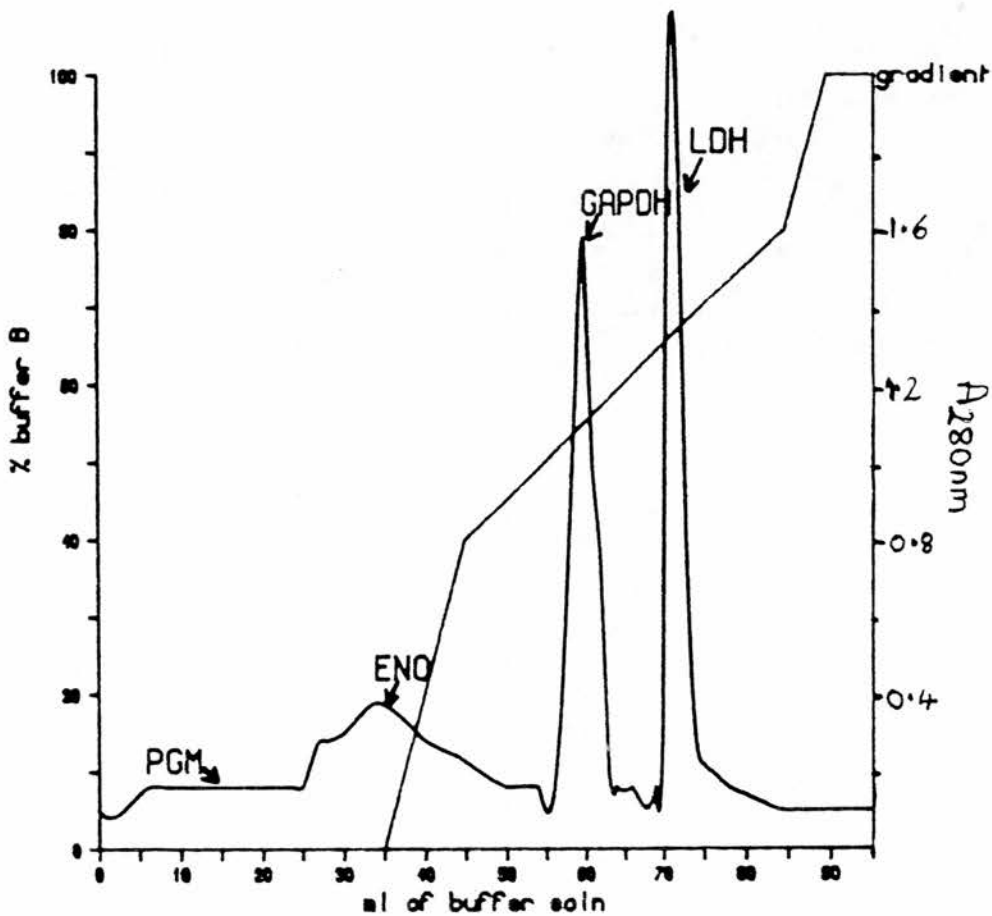
(3)



3.3.10

The 12,000g soluble fraction of chicken breast muscle homogenate was subjected to 35% - 75% ammonium sulphate fractionation, followed by phosphocellulose ion exchange chromatography on a column equilibrated in 10mM Tris pH7.3. The fraction eluted by 10mM Tris pH7.8. The fraction eluted by 10mM Tris pH7.8 was then applied to an FPLC cation exchange column equilibrated in 25mM MES, 50mM NaCl, 1% betaine, 1mM magnesium acetate and 1mM mercaptoethanol pH6.0. Peaks containing purified PGM were eluted with the same buffer containing 350mM NaCl pH6.0 using the gradient selection process indicated.

FIGURE 3.3.11.



3.3.11

The 12,000g soluble fraction of chicken breast muscle homogenate was subjected to 35% - 75% ammonium sulphate fractionation, followed by phosphocellulose ion exchange chromatography on a column equilibrated in 10mM Tris pH7.3. The fraction eluted by 10mM Tris pH7.8. The fraction eluted by 10mM Tris pH7.8 was then applied to an FPLC cation exchange column equilibrated in 25mM MES, 50mM NaCl, 1% betaine, 1mM magnesium acetate and 1mM mercaptoethanol pH6.0. Peaks containing purified PGM were eluted with the same buffer containing 350mM NaCl using the gradient indicated.

Table 3.3.7 shows the percentage purity of each enzyme as calculated by gel scanning. Figure 3.3.12 outlines the separation system.

In summary a great deal of time was taken trying to separate these enzymes, in particular aldolase and enolase. Many of these enzymes appear to have an affinity for other specific proteins. A fraction of aldolase was always found to be isolated with pyruvate kinase. There is some evidence for glycolytic enzymes associating especially in the presence of structural proteins (see 5.4).

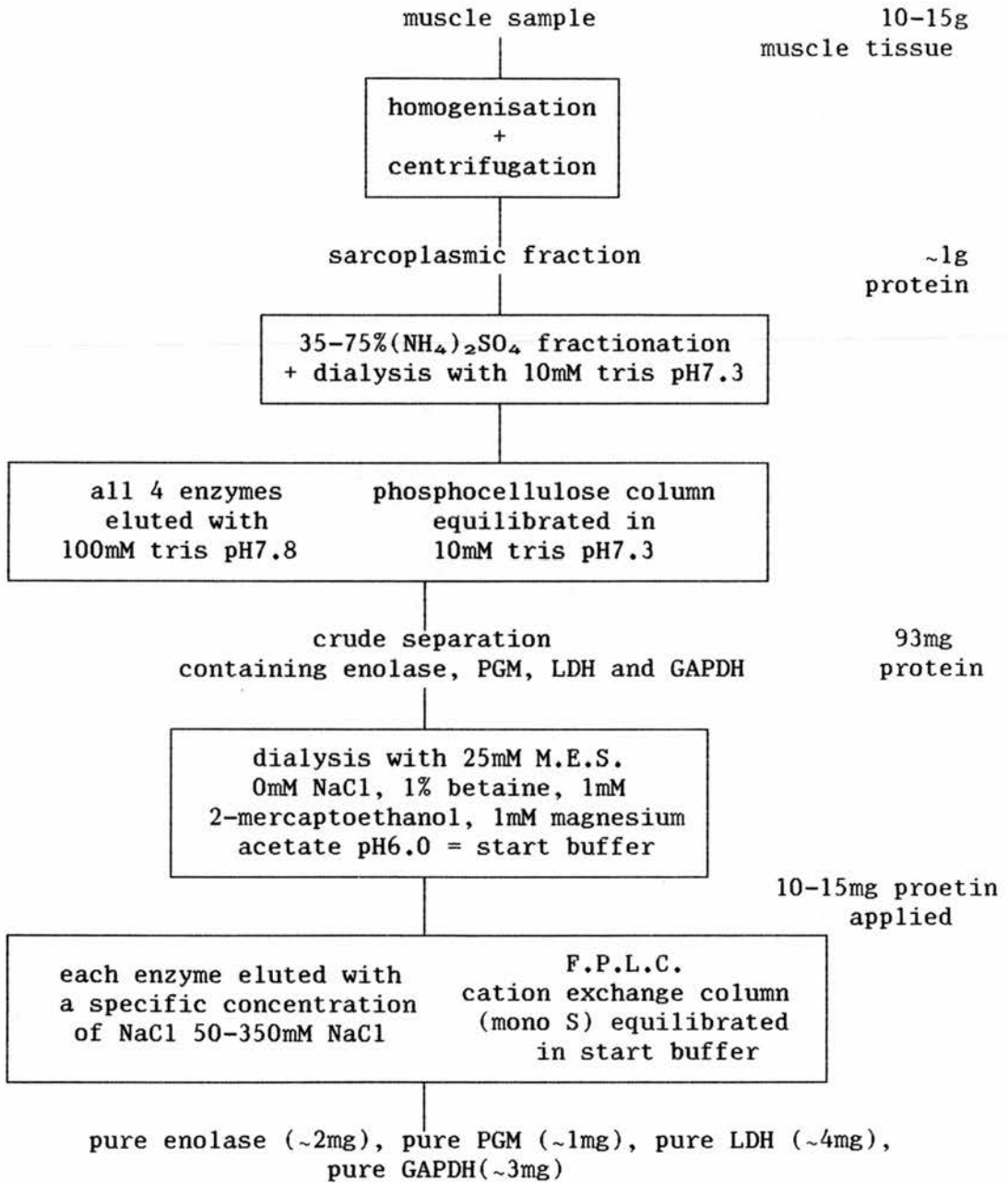
A problem with so much time being taken in preliminary separations was that time became a precious commodity and a multistep separation system was ruled out, thus compounding the problem. The only solution was a system which isolated a large number of enzymes quickly and in quantity (1mg). Phosphocellulose column chromatography followed by FPLC cation exchange chromatography proved to be both quick and capable of providing the required amounts of purified protein.

Table 3.3.7

	Enolase % purity	GAPDH % purity	LDH % purity	PGM % purity
1	100	100	100	100
2	100	100	100	100
3	100	97	100	100
4	100=median	95=median	100	100
5	100	89	100	100=median
6	89	65	100	100
7	83	56	98	100
8	76		93=median	100
9			91	100
10			84	83
11			84	
12			75	
13			71	
14			70	

3.3.7 Purity of enolase, GAPDH, LDH and PGM isolated from chicken breast muscle in an experiment using [¹⁵N][¹⁴C] leucine as a tracer. The enzymes were isolated by the method described in section 3.3.5. The purified enzymes were run on SDS-polyacrylamide gels. The gels were stained for protein with Coomassie blue and were densitometrically analysed at 600nm. The area under each peak was calculated and the area under the peaks corresponding to each enzyme expressed as a percentage of the total.

Figure 3.3.12



3.3.12. Separation of enolase, PGM, LDH and GAPDH.

Chapter Four

Results

This chapter sets out the results from monitoring ^{14}C - and ^{15}N -labelled leucine in breast muscle of four-week old chickens over a 72 hour period.

^{14}C was used as a tracer for leucine. It is assumed that once within the growing muscle there is little or no breakdown of the carbon skeleton of leucine, since most of this essential amino acid would be required for protein synthesis.

It is possible, however, that there are a number of transamination reactions taking place, which may replace the ^{15}N of leucine with ^{14}N (e.g. Harper et al 1983; Rennie and Halliday 1984).

Thus by labelling an amino acid with both ^{14}C and ^{15}N it may be possible to observe differences in the distribution of each in different compartments within the organism.

The method of labelling used was single-dose or pulse labelling. The mathematical framework for this technique is set out in section 4.1. The mathematics is based on the steady state system. In this experiment, however, the birds were growing. The effects of growth upon the system are also discussed in section 4.1, and section 4.2 describes the measurements of growth.

A model for isotope excretion is presented in section 4.3, along with the data from analysis of excreta.

$[^{14}\text{C}]$ -leucine labelling patterns for aldolase, PGM, LDH and CPK are given in section 4.4, as well as those for the summed enzymes, total sarcoplasmic protein, myofibrillar proteins, and whole muscle protein while the final section of the chapter deals with the metabolism of $[^{15}\text{N}]$ -leucine and compares it to that of $[^{14}\text{C}]$ -

leucine by calculating $^{14}\text{C}:^{15}\text{N}$ ratios in various proteins and tissue fractions.

4.1 Pulse labelling

The method used to investigate protein metabolism for this thesis was pulse labelling, or single dose technique (see section 1.4.1).

In order to use this method a number of assumptions were outlined by Zilversmit et al (1943) in a paper which discussed the mathematical basis of the technique. They assumed that the amount of compound present in the tissues must be constant during the experimental period, that is, the rate of its appearance and disappearance are equal and constant. They also assumed that the organism did not distinguish between old and newly-formed molecules.

Zilversmit et al (1943) pointed out that during the early interval after the administration of a labelled substance, the specific activity (or isotope concentration) of a precursor of a compound must be higher than that of the compound itself. They also stated that if the specific activity of the precursor is maintained constant, the specific activity of the compound eventually becomes equal to that of the precursor.

They considered the case in which a single immediate precursor A is converted to a compound B.

Let: p = rate of conversion of A to B (const)

r = the amount of B present in the tissues (const)

x = the amount of radioactive B present in that tissue

$f(t)$ = the specific activity of the immediate precursor A

which as expressed here depends on time.

Then the amount of label converted into B per unit time is $pf(t)$ and the amount of label that is lost from B per unit time is $p\frac{X}{r}$. Therefore the rate of change of amount of label in B in a tissue per unit time =

$$\frac{dx}{dt} = pf(t) - p\frac{X}{r} = p \left[f(t) - \frac{X}{r} \right] \text{ or}$$

$$r \left(\frac{dX}{dt} \right) = p \left[f(t) - \frac{X}{r} \right] \text{ since } r \text{ is constant}$$

$$\text{and } \frac{\left(\frac{dX}{dt} \right)}{f(t) - \frac{X}{r}} = \frac{p}{r} = \text{a constant}$$

$\left(\frac{dX}{dt} \right)$ measures the slope of the specific activity time curve of B. Therefore at any time the slope of the specific activity time curve of B is proportional to the difference between the specific activity of A, i.e. $f(t)$ and the specific activity of B, i.e. $\frac{X}{r}$.

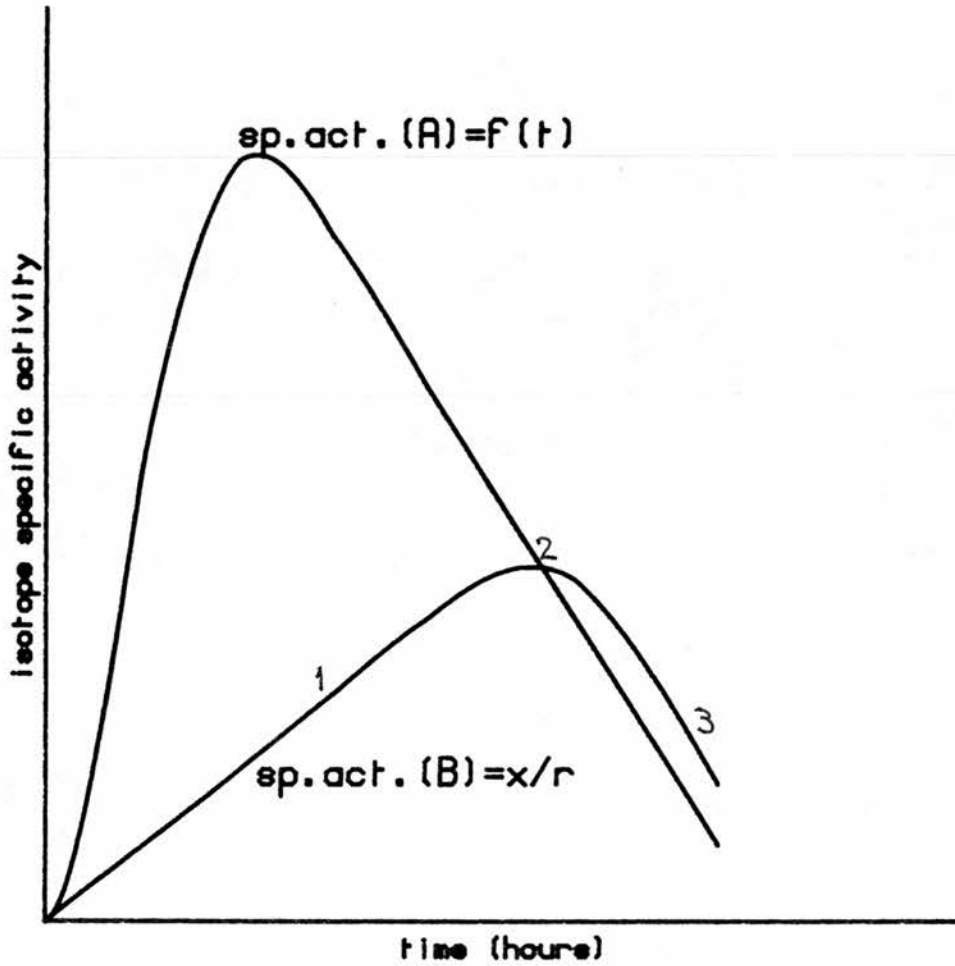
The application of this relationship in which a single dose of labelling agent is administered is illustrated in figure 4.1.1

From this, Zilversmit et al (1943) produced a list of criteria which they suggested would apply in such a situation.

These criteria read as follows:

- (1) If the slope of the specific activity/time curve of B is positive (see figure 4.1.1), that is, before the specific activity of B reaches its maximum, then the function $\left[f(t) - \frac{X}{r} \right]$ must be positive. This means that the specific activity of the immediate precursor A is greater than that of the compound B before the latter reaches a maximum specific activity.

FIGURE 4.1.1.



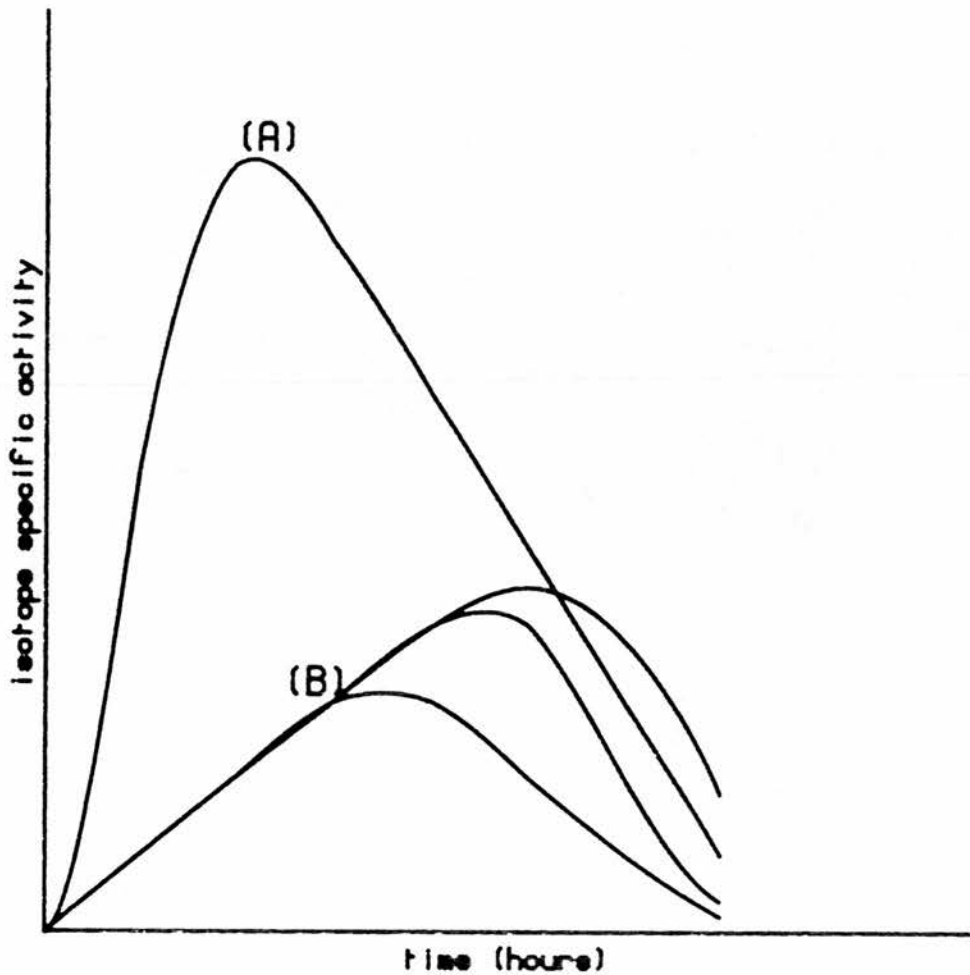
4.1.1. SINGLE DOSE LABELLING:
precursor (A), product (B) steady state relationship.

(2) After B has reached its maximum specific activity the slope of the specific activity/time curve of B is negative and therefore the specific activity of the compound is greater than its precursor.

(3) At the time when the specific activity of B has reached its maximum, the slope of the specific activity/time curve of B is zero and therefore the specific activity of the immediate precursor A equals the specific activity of compound B at that time.

It may be argued that Zilversmit's criteria can also apply to the non-steady or growing system. Such a situation may arise if the rate of synthesis of a compound (protein) from a precursor (amino acid) is constant, but higher than the rate of degradation. In this situation, p would be constant. Moreover, if this were so, then r , the amount of B present in the tissue, would obviously not remain constant, but would increase. The specific activity of B equals $\frac{x}{r}$, so if x , the amount of labelled B in the tissue, remains constant then the specific activity of B would be lower than in a non-growing system. The difference is related to the amount of growth. The same would hold for the specific activity of the precursor A (amino acids), assuming the total amount of amino acids in the plasma increased to the same extent. This would cause the plasma specific activity to apparently decrease faster. However, with a total growth over the experimental period of about 24% for the birds used in this thesis, (see section 4.2), the difference between the specific activities would be fairly small and should allow crossover or near crossover of the two curves (as illustrated in figure 4.1.2).

FIGURE 4.1.2.



4.1.2 THEORETICAL LABELLING PATTERNS possible precursor (A), product (B) relationship in a growing system

If a pulse labelling experiment was carried out on growing muscle the specific radioactivity of (A) and (B) would be observed to reach its maximum point earlier; the maximum value would appear to be lower and the rate of loss of the label would be more rapid due to the diluting effect of growth. The diagram shows that even during growth cross over, or near cross over of curves (A) and (B) should occur.

In the work reported here no such crossovers were found in growing birds (or indeed in "non-growing" birds - see appendix), and the specific activity of plasma amino acids at the predicted crossover point is in fact as much as eleven times higher than that of the labelled protein.

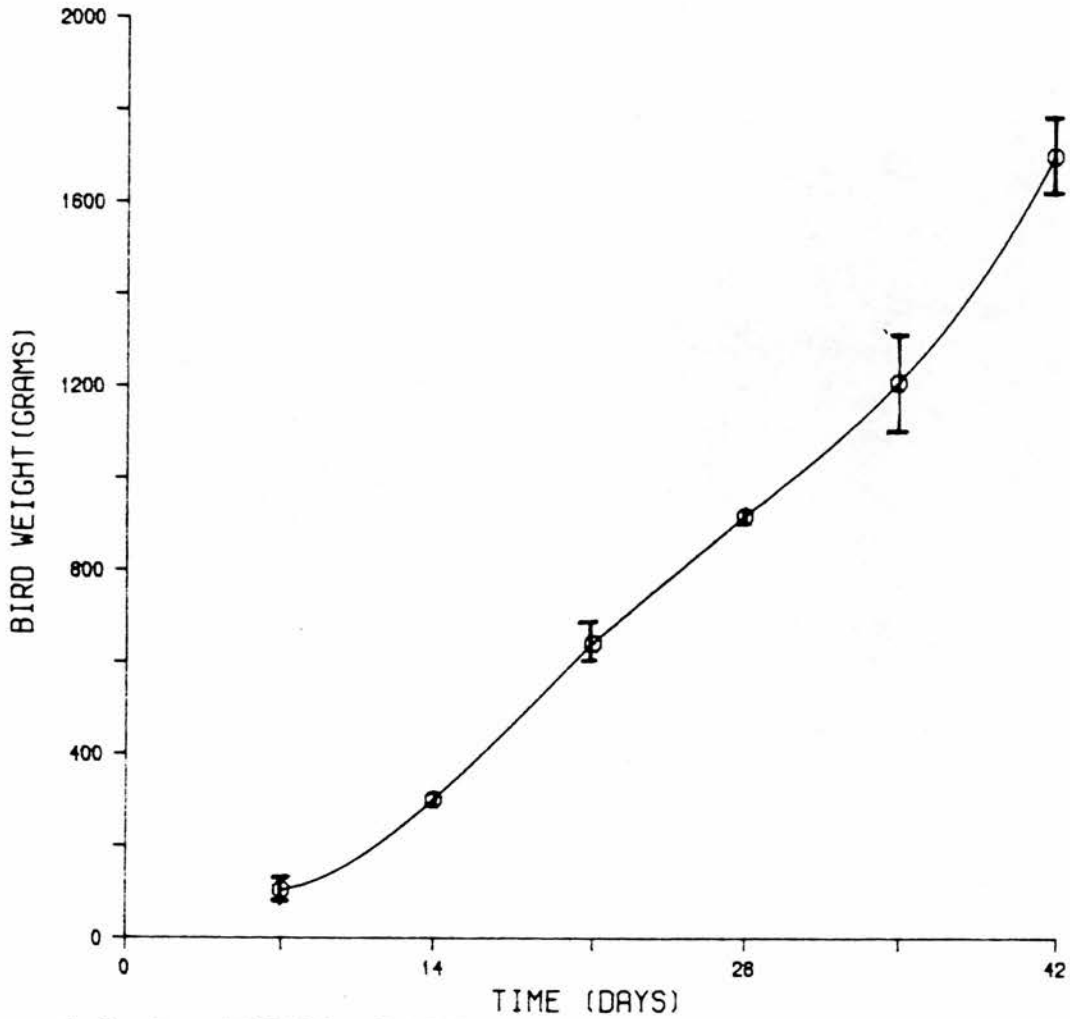
4.2 Growth

An important measurement during the experimental period is that of growth. It is essential to know whether the size of the system is increasing, decreasing or remaining stable. Body weight change has been used as an index of muscle growth, since muscle makes up such a large proportion of the animal's lean body mass (>60% in adult chickens (Hocking et al 1985)). Greater accuracy can be gained by measuring changes in weight of the breast muscle. This is especially so since it is the soluble enzymes of breast muscle that are the principal subjects of study.

The growth in body weight in chicks up to 42 days old is shown in figure 4.2.1.

The growth of the breast muscle for the experimental period is shown in figure 4.2.2 and was calculated to be an increase of 24%.

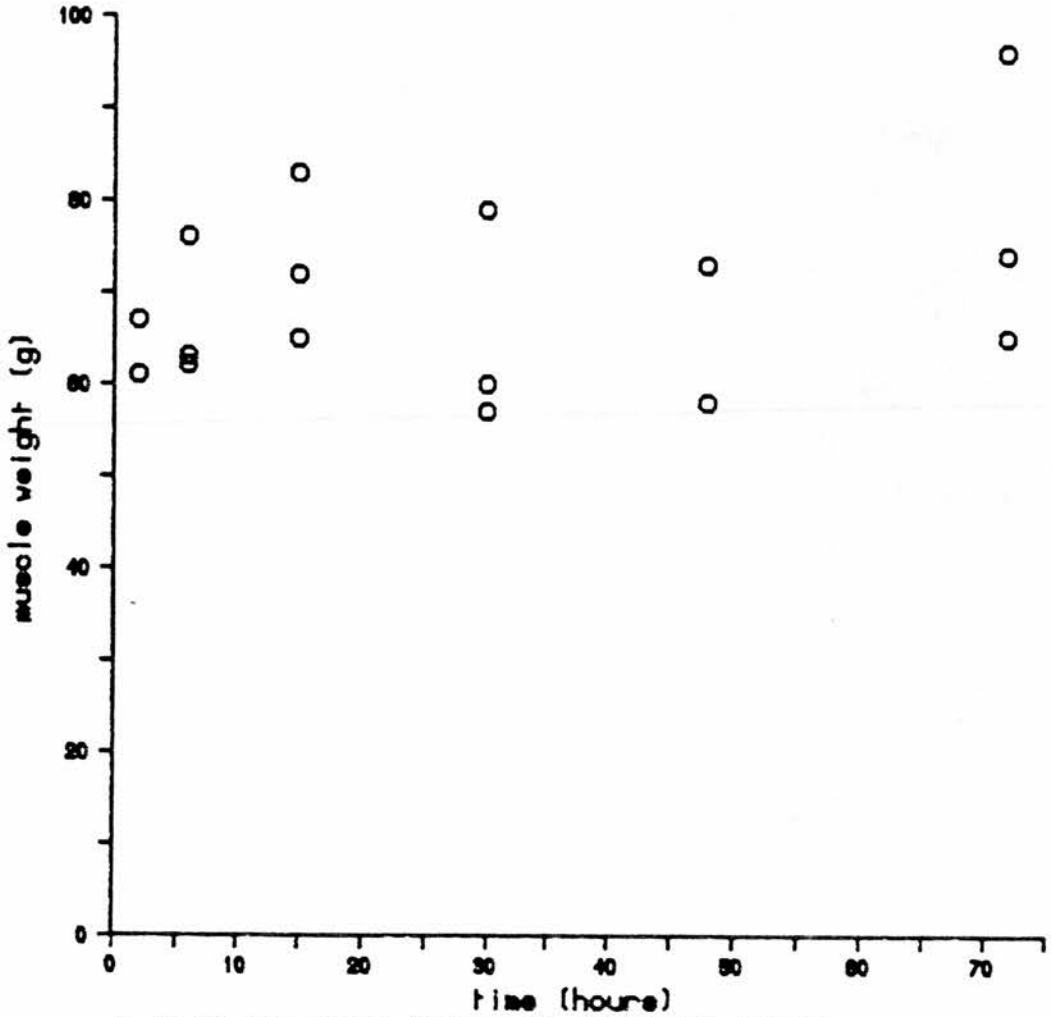
FIGURE 4.2.1.



4.2.1. GROWTH RATE OF BROILER CHICKS ON STARTER DIET

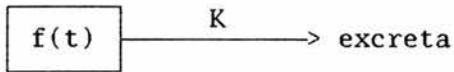
Total body weight of broiler chickens from 7 days to 42 days after hatching. Each point represents the mean. The error bars correspond to \pm standard deviation. Three birds were used for each time interval.

FIGURE 4.2.2.



4.2.2 BREAST MUSCLE GROWTH RATE

Total breast muscle weight over the 72 hour experimental period. Birds were killed by dislocation of the neck and the breast muscle from one side of the sternum was carefully dissected and weighed. Each point represents the breast muscle weight from a single bird. The total growth in weight over the 72 hour period was calculated to be 24%.

4.3 Accumulation of label in excretaRationale

The rationale of analysis is very simple in principle. The rate of change (increase) in the amount of label in excreta $\frac{dF}{dt}$ is set equal to the rate of loss from the carcass, which is presumed to be governed by a single rate constant, which is a multiplier for the function of time which describes the specific activity of the compartment from which excretion takes place.

$$\frac{dF}{dt} = Kf(t) \quad (1)$$

Assuming that $f(t)$ is an exponential function (a single term is used for simplicity)

$$dF_t/dt = K A e^{-\lambda t} \quad (1a)$$

$$\begin{aligned} \text{or } F_t &= \int K A e^{-\lambda t} dt \\ &= \frac{-KA}{\lambda} e^{-\lambda t} + IC \end{aligned} \quad (1b)$$

At $t = 0$ $F_t = 0$ so $IC = \frac{-KA}{\lambda}$ and

$$F_t = \frac{KA}{\lambda} [1 - e^{-\lambda t}] \quad (2)$$

or, more comprehensively,

$$F_t = K \left[\sum \left(\frac{A_i}{\lambda_i} (1 - e^{-\lambda_i t}) \right) \right] \quad (2a)$$

Also,

$f\alpha$ = (by assumption) 100% of the original dose and since $e^{-\lambda t} = 0$

$$f\alpha = \frac{KA}{\lambda}$$

$$\text{and Dose} - F_t = \frac{KA}{\lambda} e^{-\lambda t} \quad (3)$$

or, more comprehensively,

$$y = \text{Dose} - F_t = K \left[\sum \frac{A_i}{\lambda_i} e^{-\lambda_i t} \right] \quad (3a)$$

The advantage of this formulation is that relationships (3) or (3a) but not (2) or (2a) can be plotted on semilog graph paper to give a curve that can be "peeled" into a series of straight lines. It therefore gives an immediate view of whether or not the assumptions fit the data.

If 100% of the dose is not excreted, obviously (3a) as it stands is not strictly true, but if we split the dose into $F_r \times D$, the part excreted, and $(1-F_r) \times D$, the part retained, it is clear that (3a) can be reformulated as:

$$\begin{aligned} y &= F_r \times D - F_e + t(1-F_r) \times D \\ &= K \left[\sum \frac{A_i}{\lambda_i} e^{-\lambda_i t} \right] + C \end{aligned} \quad (4)$$

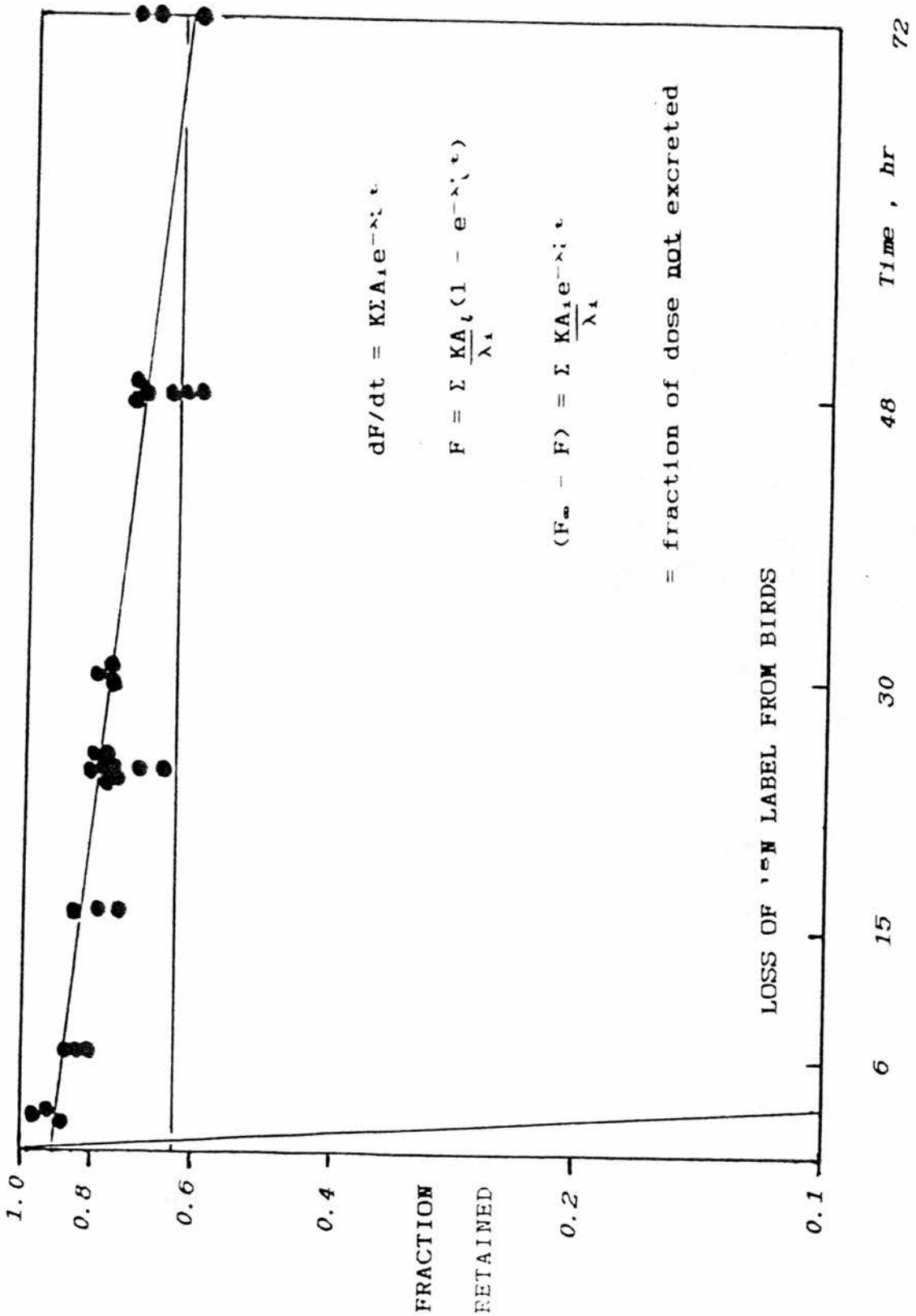
where $C = (1-F_r) \times D$.

Graphical plotting of the data enabled the fraction of the dose retained to be estimated (see figure 4.3.1). From this graph, it can be seen that only 45% of the ^{15}N dose given was excreted and that little or no label was removed from the slow pool.

4.4 Plasma Decay Curve

The rate of loss of ^{15}N and ^{14}C from non-protein nitrogen in plasma is shown in figure 4.4.1. Samples were taken at 2 hours, 6 hours, 15 hours, 30 hours, 48 hours and 72 hours. ^{15}N and ^{14}C specific activities follow an almost identical curve, which by 72 hours is close to zero.

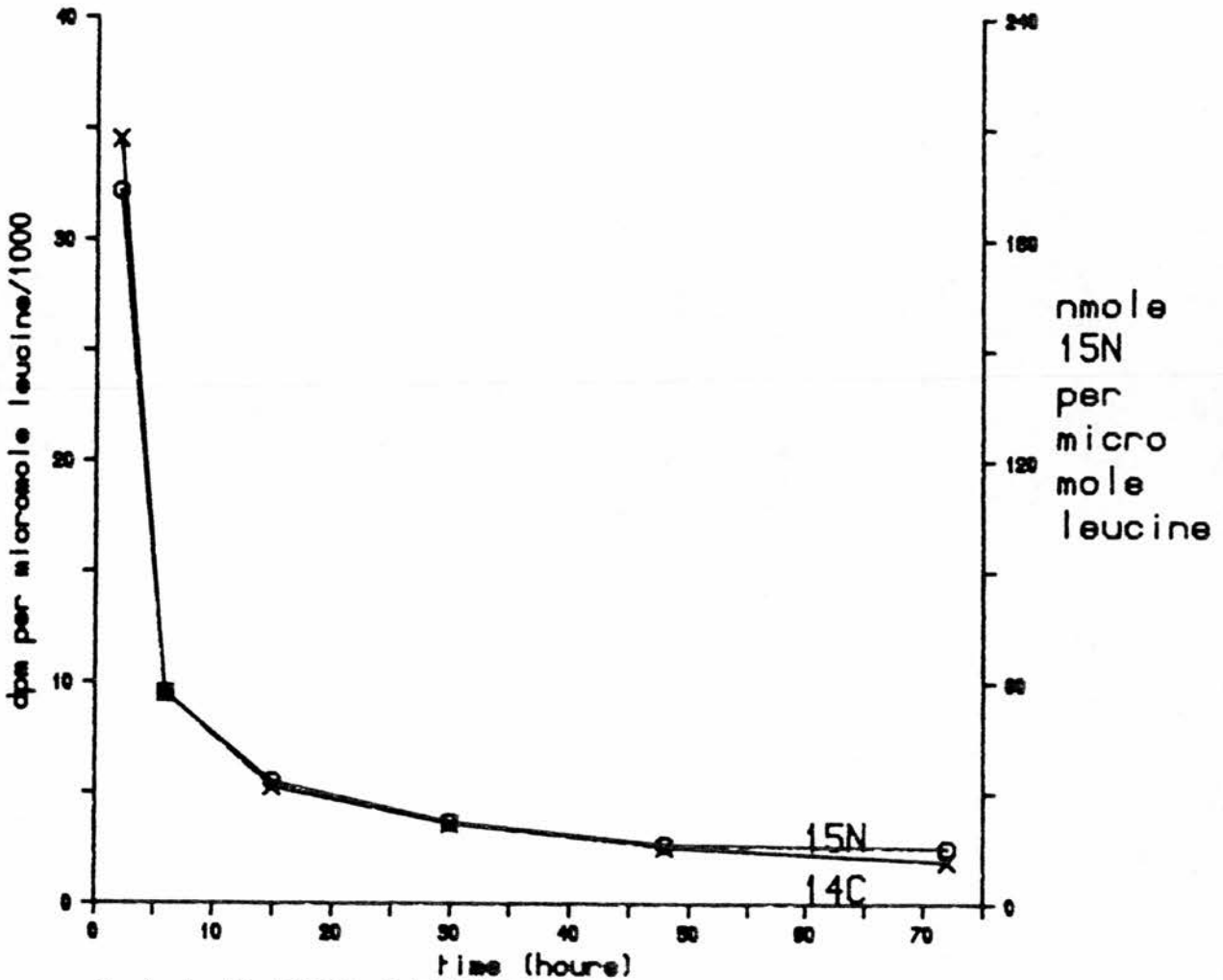
FIGURE 4.3.1.



4.3.1. LOSS OF ¹⁵N FROM BIRDS

The semilogarithmic plotting of data concerning the amount of ¹⁵N found in the excreta of birds injected with a pulse of [¹⁵N] leucine enables the fraction retained to be calculated at 65% and the dose excreted to be 45%.

FIGURE 4.4.1.

4.4.1 PLASMA ^{14}C & ^{15}N DECAY CURVE

The loss of ^{15}N and ^{14}C label from plasma of chickens injected with a pulse of $^{15}\text{N}^{14}\text{C}$ leucine. The dpm per micromole leucine was determined in the 5000g supernatant from blood precipitated by 10% PCA. Each point represents the mean specific radioactivity derived from experiments on 3 separate birds.

4.5 ^{14}C Specific Activities in Individual Enzymes

The ^{14}C specific activity for the enzymes Aldolase, LDH, PGM and CPK are shown in figures 4.5.1, 4.5.3, 4.5.4 and 4.5.5 respectively. Each shows a bimodal peak, with CPK's specific activity curve suggesting the possibility of even a third peak. PGM, LDH and CPK each have a second peak which has a higher ^{14}C specific activity than the first peak.

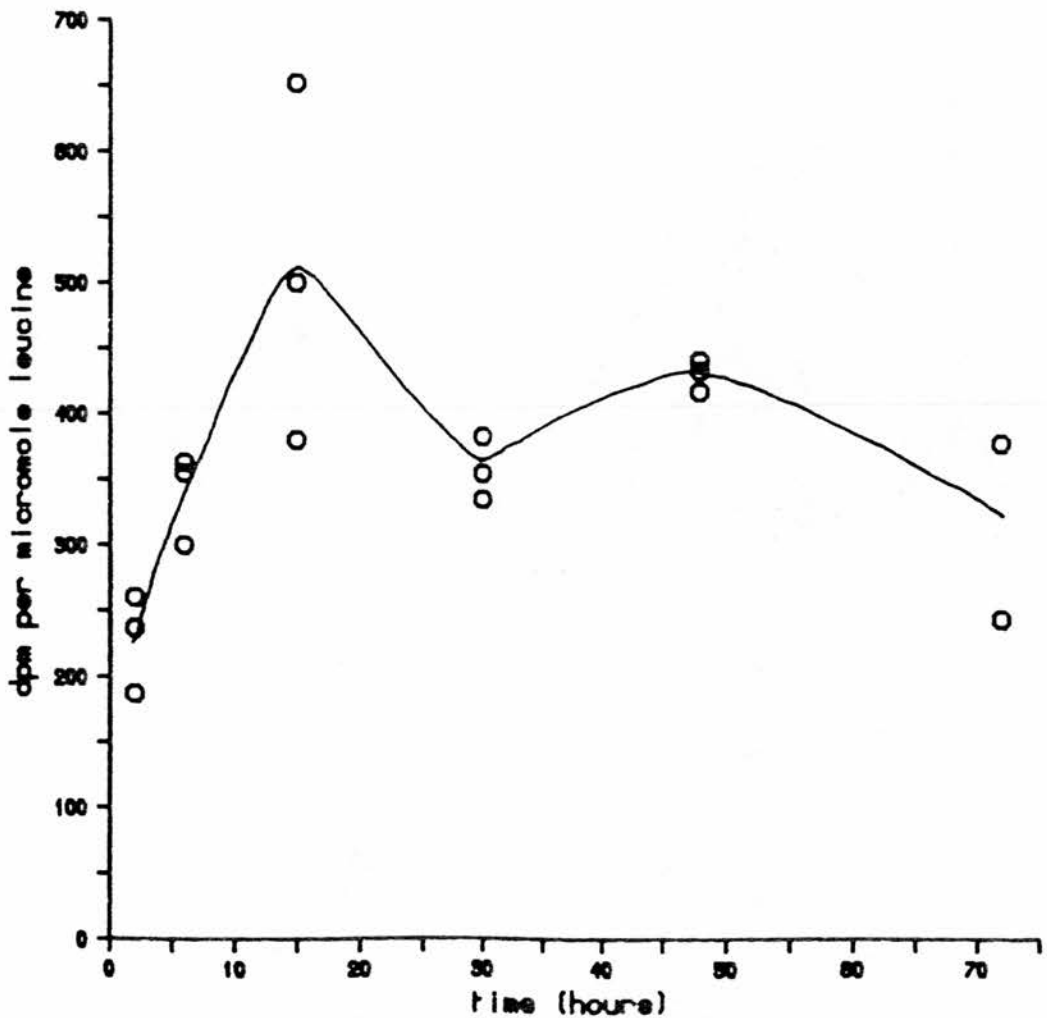
None of the enzyme ^{14}C labelling curves cross the curve of plasma specific activity (figure 4.5.2). It would appear, therefore, that this system cannot be described by the theory of Zilversmit et al (1943).

4.6 Summed Enzymes ^{14}C summed data

The labelling time-courses of PGM, Aldolase, LDH and CPK were combined to determine whether or not their summed data could be used to derive a model which would provide an average of their individual time and rate relationships. It was hoped to compare this model with that of the labelling pattern from the ^{14}C specific activities in sarcoplasmic proteins (see section 4.7).

Since the four enzymes studied, however, are not completely representative of sarcoplasmic protein (~35% - see table 3.2.3), the comparison cannot be absolute. Figure 4.6.1 shows the ^{14}C labelling pattern over the experimental period. The ^{14}C data points appear to have a particularly large scatter, making interpretation difficult. If the average point is taken for each time interval, as in figure 4.6.1, then a fairly smooth, single peak is seen, with its highest point at about 35 hours.

FIGURE 4.5.1.

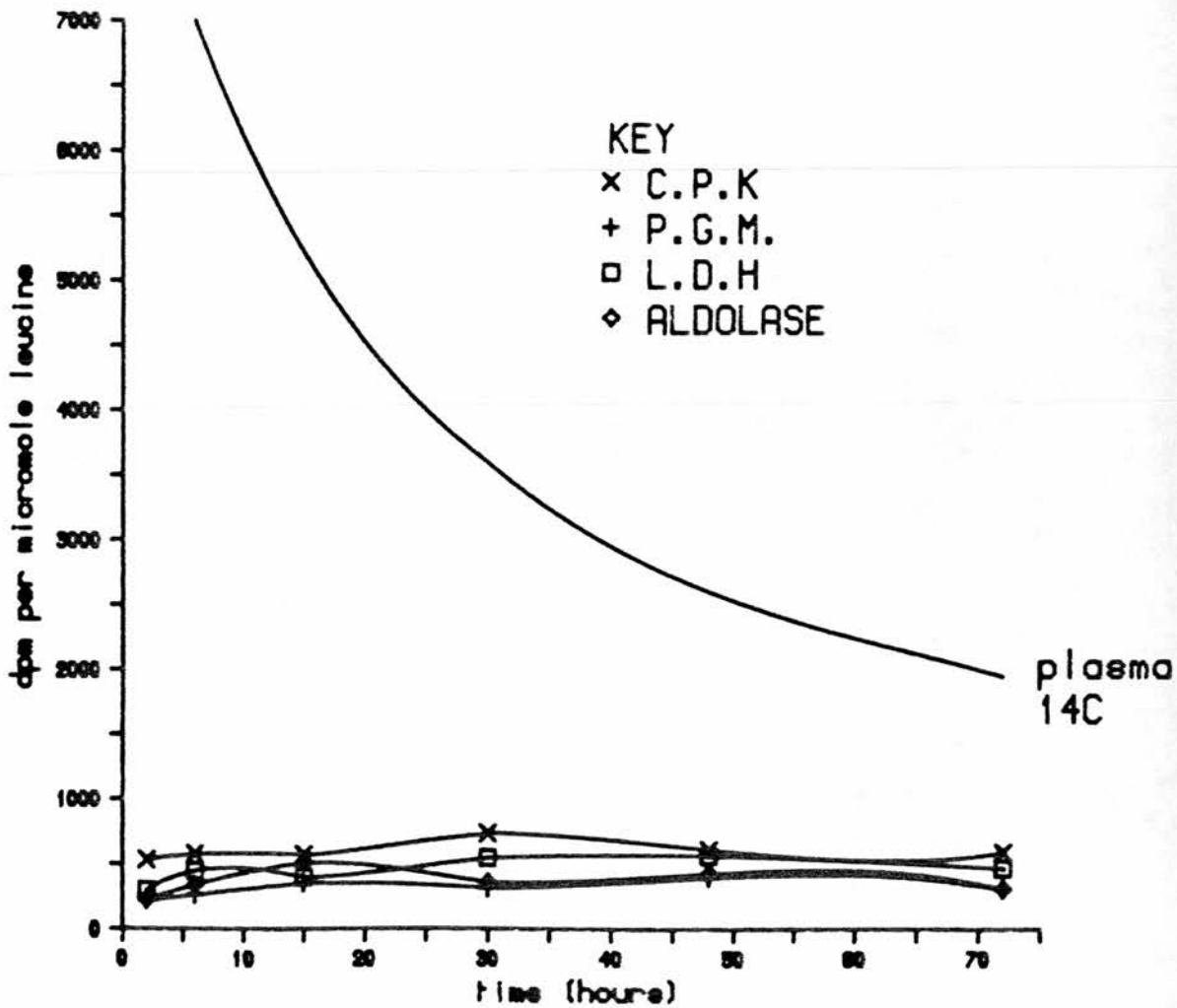


4.5.1. ALDOLASE ^{14}C SPECIFIC ACTIVITY

curve drawn through the mean of the data points

The ^{14}C specific radioactivity of aldolase which was purified from chicken breast muscle after pulse labelling with $[^{15}\text{N}][^{14}\text{C}]$ leucine. Each point represents dpm per micromole leucine from a single bird calculated from the amount of leucine in aldolase and the amount of aldolase in lg of muscle.

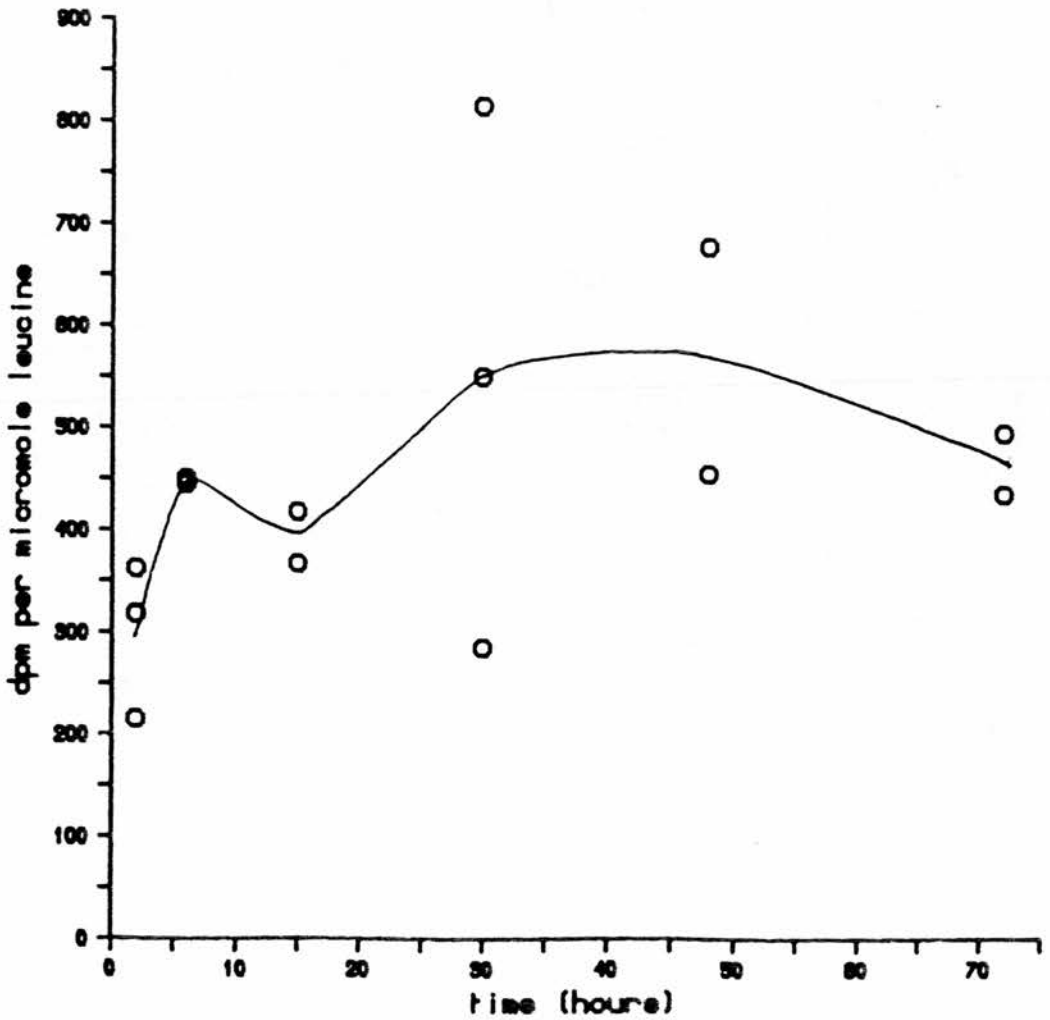
FIGURE 4.5.2



4.5.2. ENZYMES ¹⁴C SPECIFIC ACTIVITY mean values for each enzyme

The ¹⁴C specific radioactivity of the enzymes aldolase, LDH, PGM and CPK which were purified from chicken breast muscle after pulse labelling with [¹⁵N][¹⁴C] leucine. Each point represents dpm per micromole leucine calculated from the data presented in figures 4.5.1, 4.5.3, 4.5.4 and 4.5.5. The plasma ¹⁴C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 4.4.1.

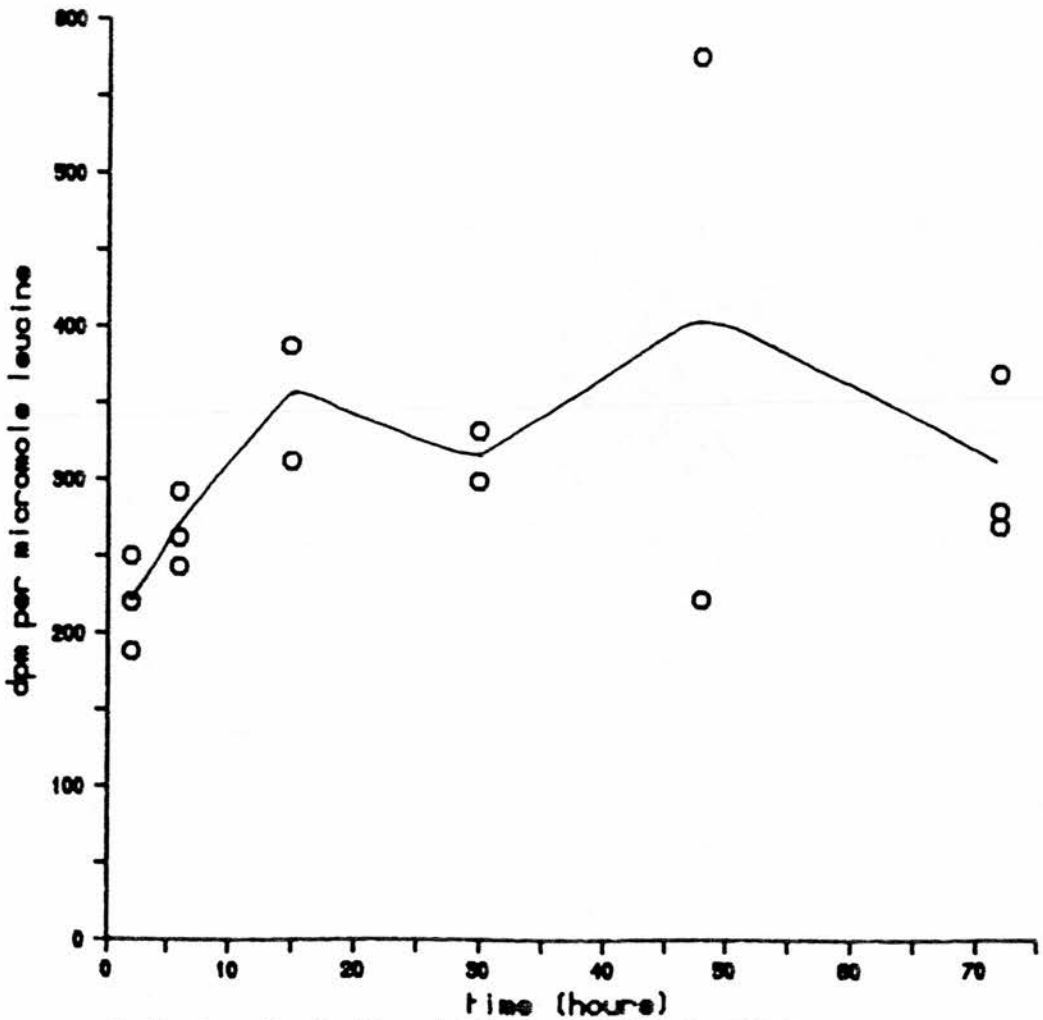
FIGURE 4.5.3.



4.5.3. L.D.H. ^{14}C SPECIFIC ACTIVITY
 curve drawn through mean of data points

The ^{14}C specific radioactivity of LDH which was purified from chicken breast muscle after pulse labelling with ^{15}N ^{14}C leucine. Each point represents dpm per micromole leucine from a single bird calculated from the amount of leucine in LDH and the amount of LDH in lg of muscle.

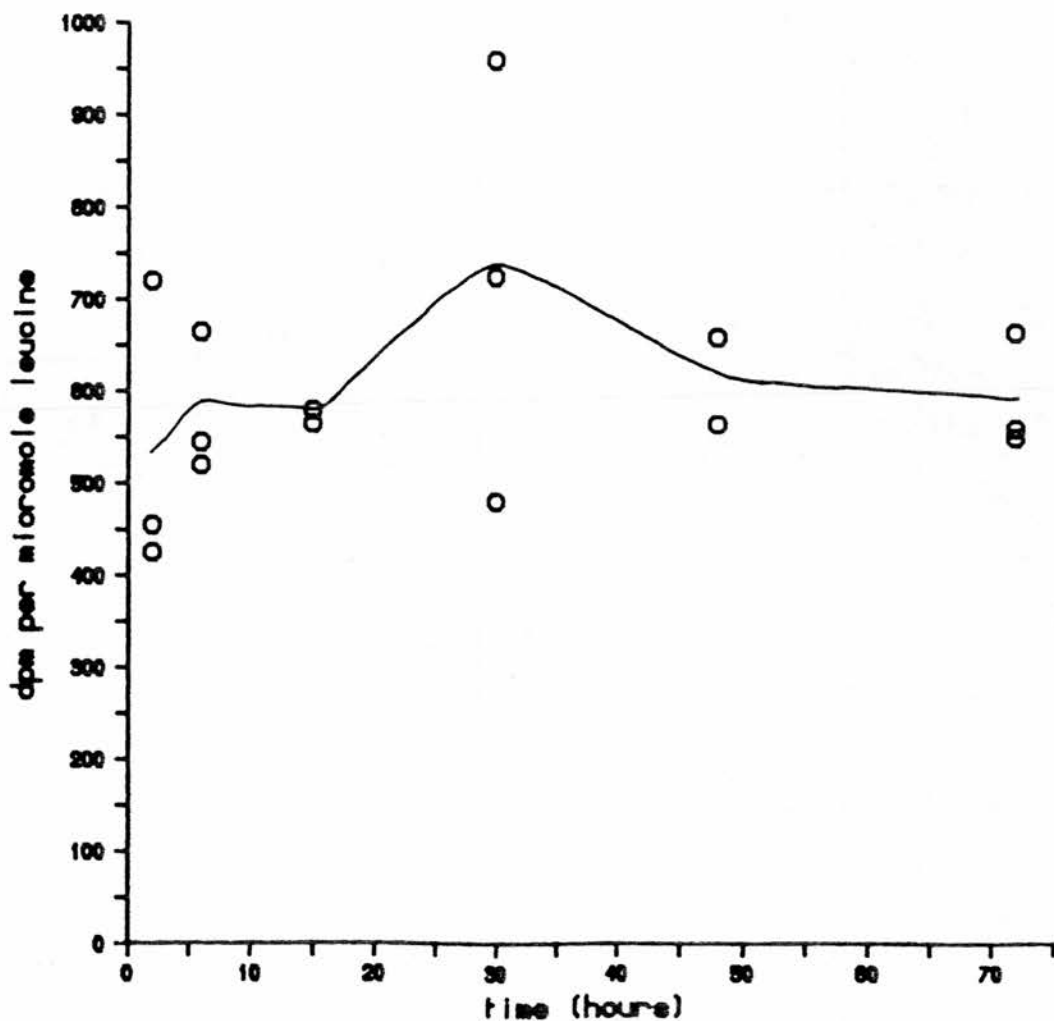
FIGURE 4.5.4.



4.5.4. P.G.M. ^{14}C SPECIFIC ACTIVITY
 curve drawn through mean of data points

The ^{14}C specific radioactivity of PGM which was purified from chicken breast muscle after pulse labelling with ^{15}N ^{14}C leucine. Each point represents dpm per micromole leucine from a single bird calculated from the amount of leucine in PGM and the amount of PGM in 1g of muscle.

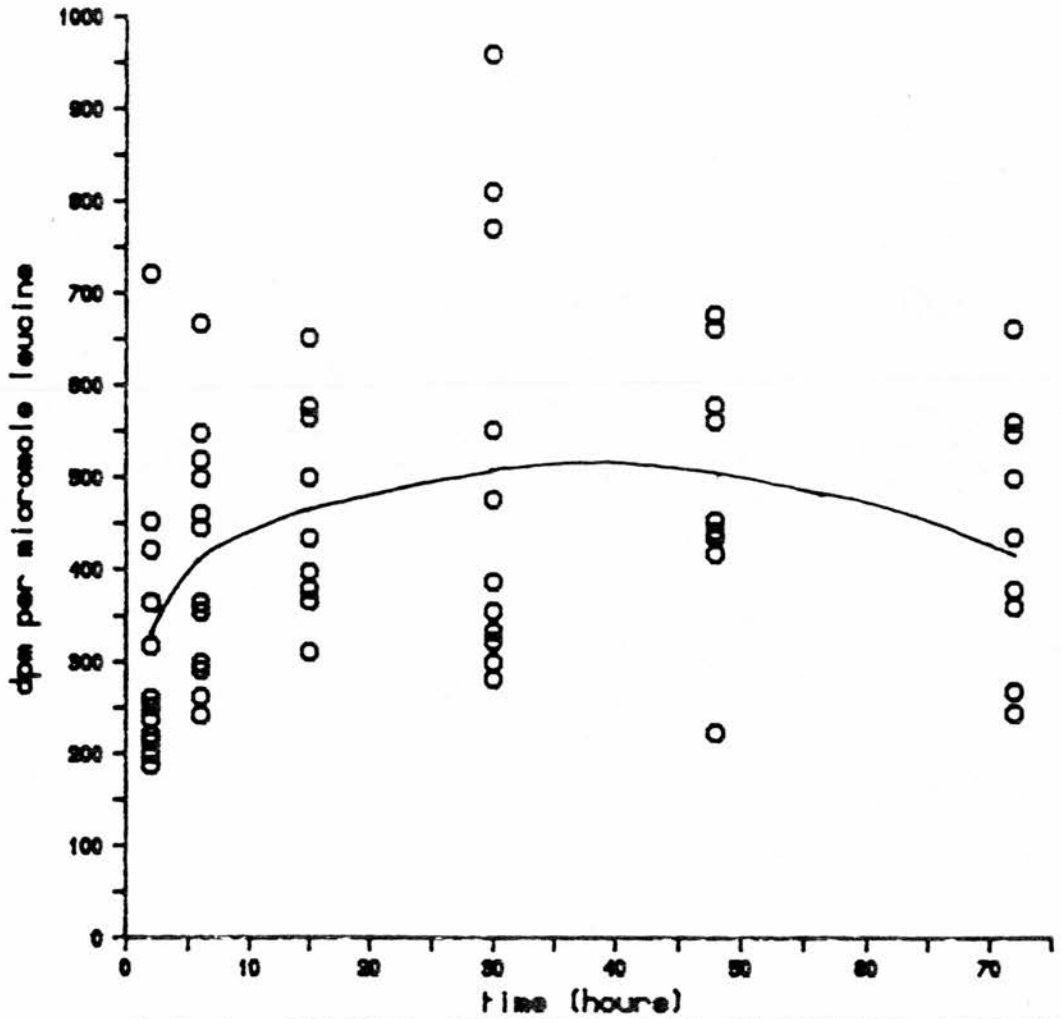
FIGURE 4.5.5.



4.5.5. C.P.K. ^{14}C SPECIFIC ACTIVITY
 curve drawn through mean of data points

The ^{14}C specific radioactivity of CPK which was purified from chicken breast muscle after pulse labelling with ^{15}N ^{14}C leucine. Each point represents dpm per micromole leucine from a single bird calculated from the amount of leucine in CPK and the amount of CPK in 1g of muscle.

FIGURE 4.6.1.



4.6.1. SUMMED ENZYMES ^{14}C SPECIFIC ACTIVITY
curve drawn through mean of data points

The ^{14}C specific radioactivity of aldolase, PGM, LDH and CPK purified from chicken breast muscle after a pulse labelling with $[^{15}\text{N}][^{14}\text{C}]$ leucine. Each point represents dpm per micromole leucine from a single bird calculated from the amount of leucine in each enzyme and the amount of each enzyme in 1g of muscle.

Figure 4.6.2 shows that the ^{14}C specific activity curve never intersects the plasma ^{14}C decay curve.

4.7 Sarcoplasmic ^{14}C specific activity

The labelling pattern of sarcoplasmic protein over the experimental period is shown in figure 4.7.1. It can be seen that at least two peaks are present, one at about 6 hours and the other around 30 hours. At no time does the sarcoplasmic ^{14}C specific activity curve cross that of the plasma ^{14}C decay curve (see figure 4.7.2).

4.8 Myofibrillar ^{14}C specific activity

The specific activity of ^{14}C -myofibrillar protein is shown in figure 4.8.1. The data points suggest a bimodal pattern with no intersection with the plasma ^{14}C decay curve (figure 4.8.2). The curve shows apparent peaks at around 15 hours and 48 hours, although the large scatter at each time point makes the first peak of dubious significance.

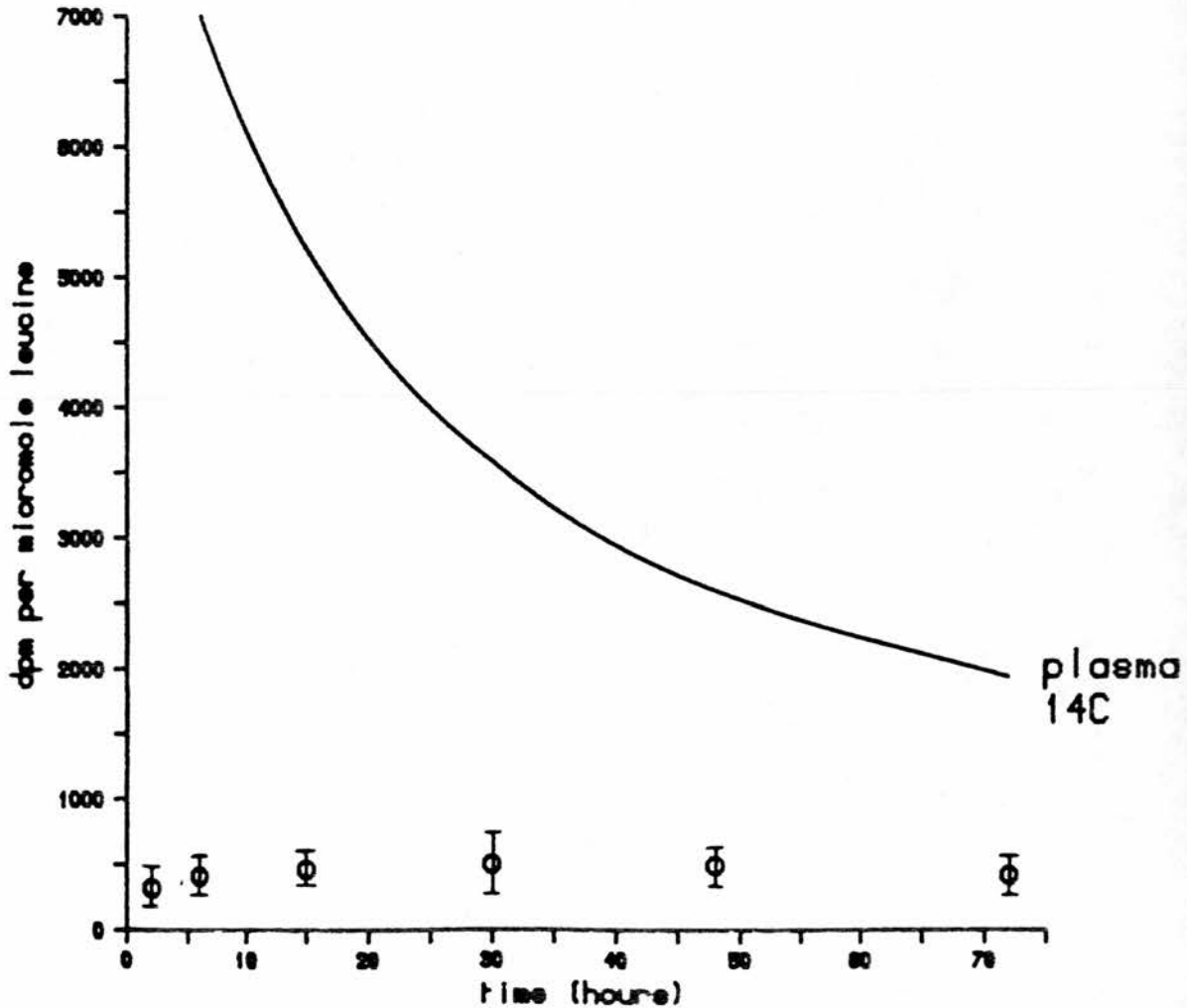
4.9 Whole muscle ^{14}C specific activity

The labelling pattern of whole muscle protein over the experimental period is shown in figure 4.9.1. The data points suggest possibly 3 peaks. This is similar to the sarcoplasmic ^{14}C labelling pattern at around 2 hours, 15 hours and 48 hours. Figure 4.9.2 indicates that at no time does the plasma ^{14}C specific activity curve intersect that of whole muscle.

4.10 $^{14}\text{C}:$ ^{15}N ratio

The ratio of ^{14}C to ^{15}N was calculated for PGM, CPK and LDH as well as for plasma, summed enzymes, sarcoplasmic protein and whole muscle protein (see tables 4.10.1 - 4.10.7 and figure 4.10.1). The ^{14}C

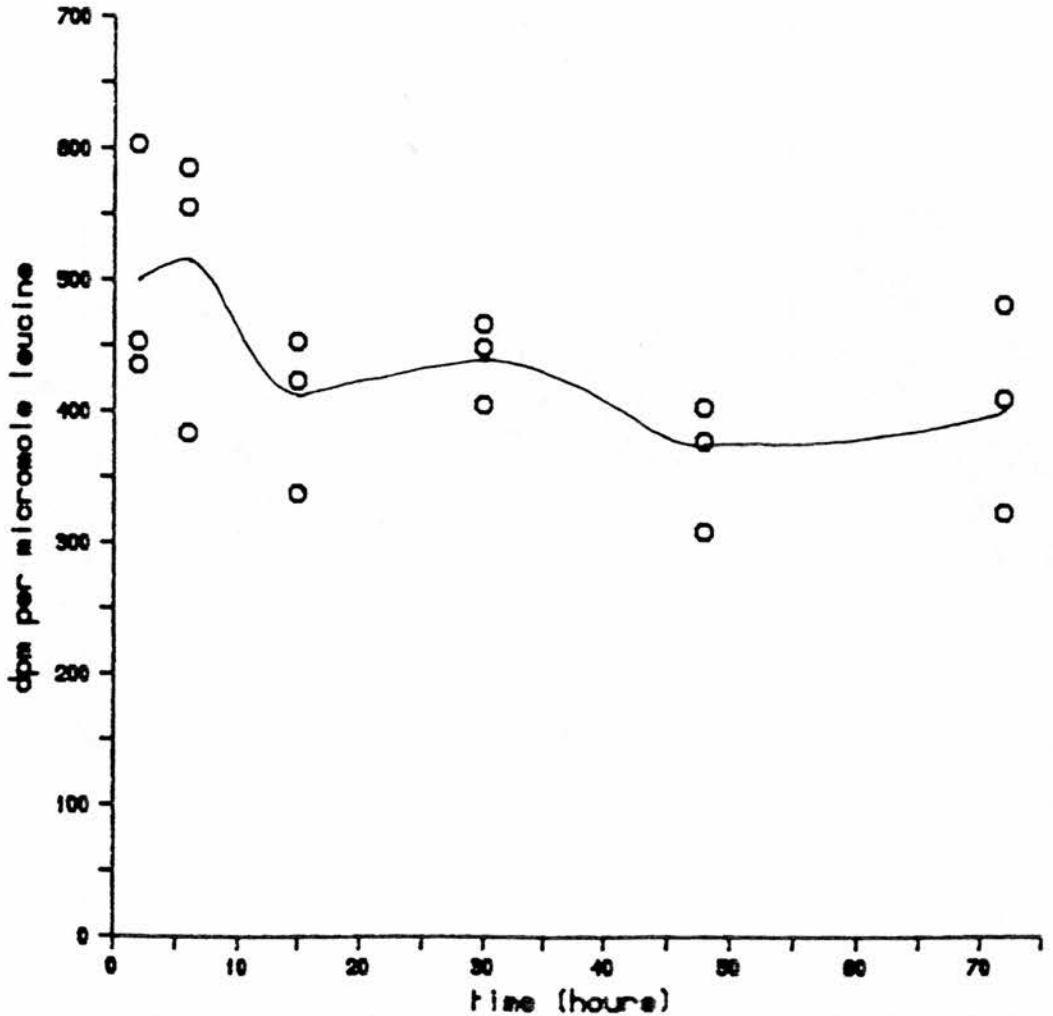
FIGURE 4.6.2.



4.6.2. SUMMED ENZYMES ¹⁴C SPECIFIC ACTIVITY

The ¹⁴C specific radioactivity of aldolase, PGM, LDH and CPK purified from chicken breast muscle after a pulse labelling with [¹⁵N][¹⁴C] labelled leucine. Each point represents the mean of the data points for each time interval presented in figure 4.6.1. The error bars indicate \pm standard deviation. The plasma ¹⁴C specific radioactivity is shown as an idealised curve derived from the data points in figure 4.4.1.

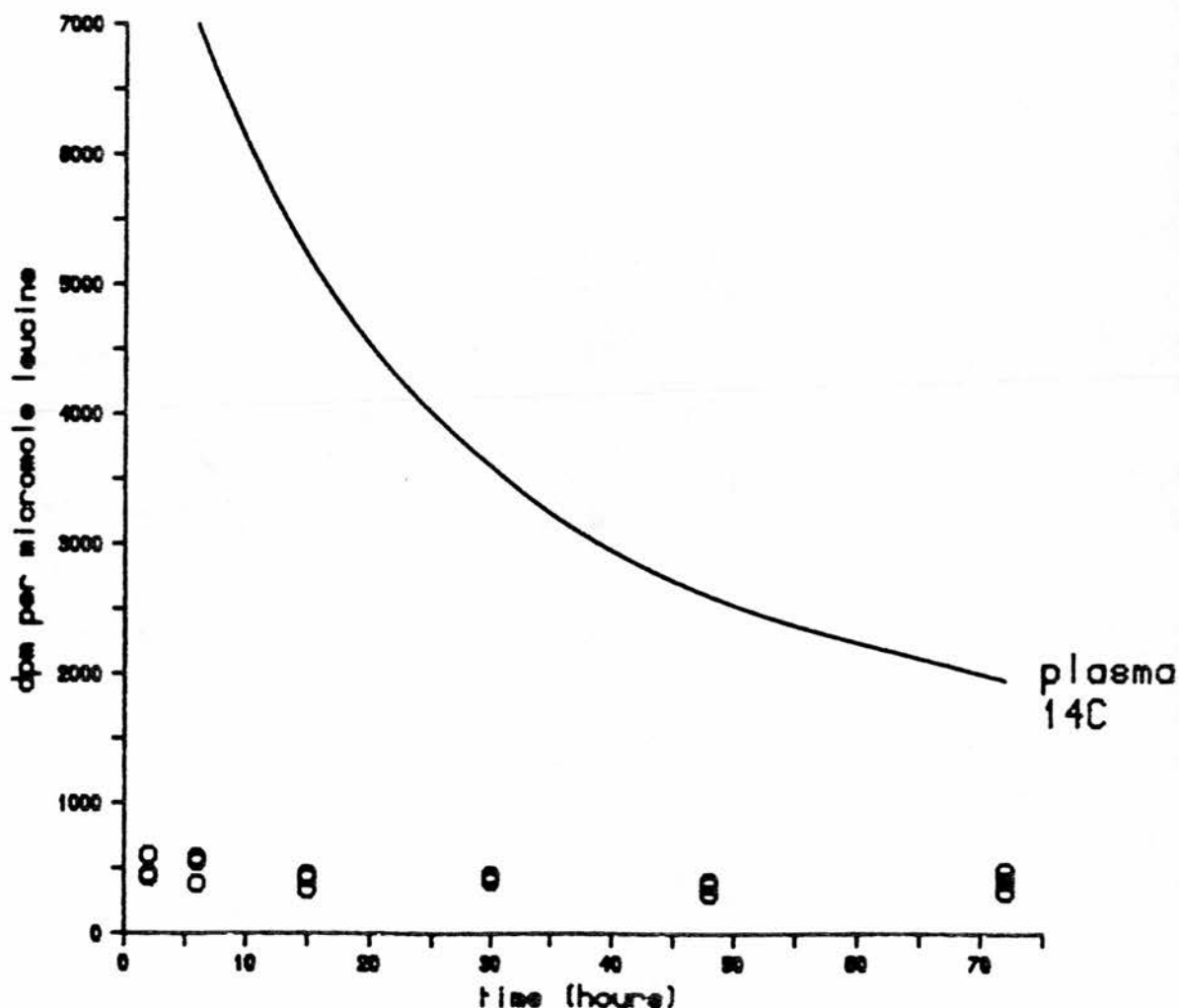
FIGURE 4.7.1.



4.7.1. SARCOPLASMIC ^{14}C SPECIFIC ACTIVITY
 curve drawn through mean of data points

Chicken breast muscle homogenate in 50mM phosphate buffer pH7.5 was centrifuged at 12,000g for 10min followed by precipitation of the supernatant with 1.2M PCA. The pellet produced from centrifugation at 6000g, was counted and assumed to contain sarcoplasmic protein. Each point represents the specific radioactivity derived from a single bird calculated from the amount of leucine in sarcoplasmic protein and the amount of sarcoplasmic protein in 1g of muscle.

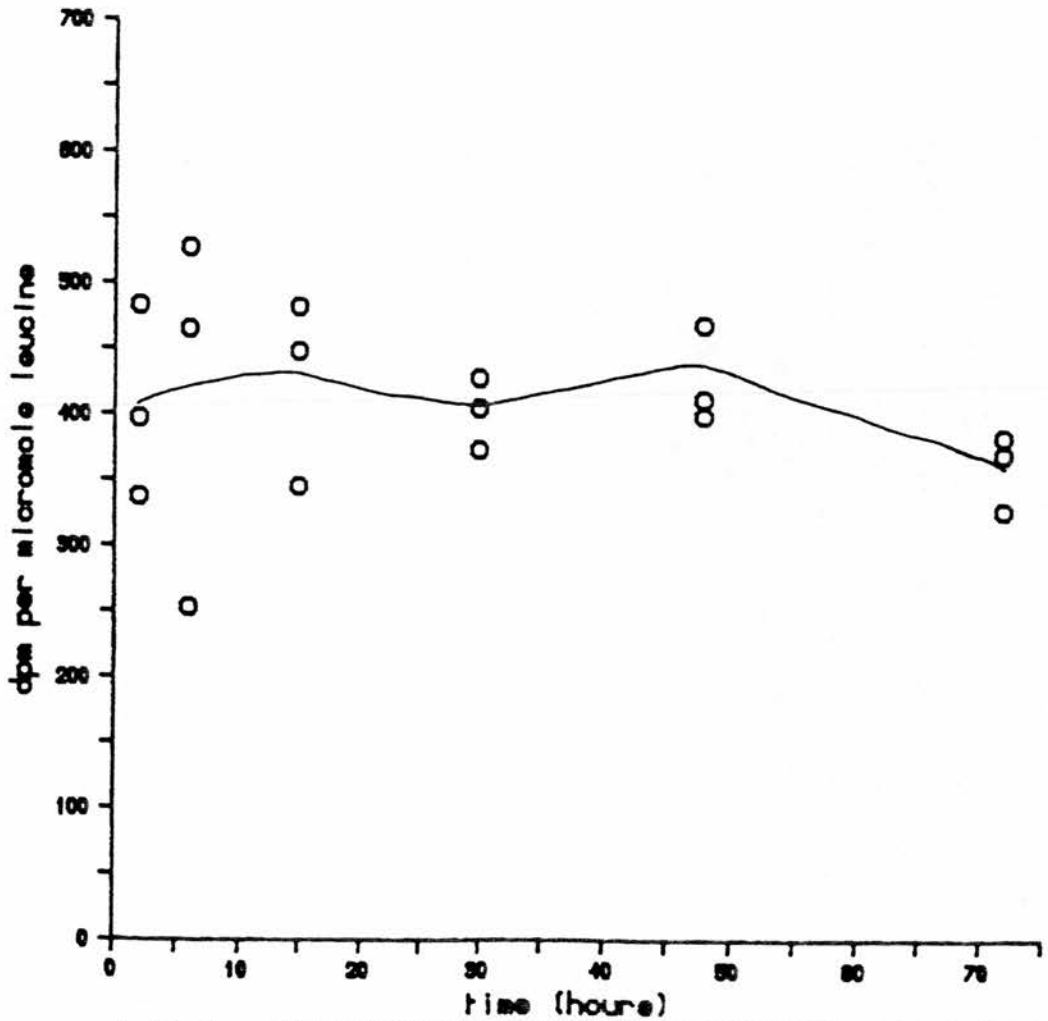
FIGURE 4.7.2.



4.7.2. SARCOPLASMIC ¹⁴C SPECIFIC ACTIVITY

Chicken breast muscle homogenate in 50mM phosphate buffer pH7.5 was centrifuged at 12,000g for 10min followed by precipitation of the supernatant with 1.2M PCA. The pellet produced from centrifugation at 6000g, was counted and assumed to contain sarcoplasmic protein. Each point represents the specific radioactivity derived from a single bird calculated from the amount of leucine in sarcoplasmic protein and the amount of sarcoplasmic protein in 1g of muscle. The plasma ¹⁴C specific radioactivity is presented as an idealised curve derived from the data points presented in figure 4.4.1.

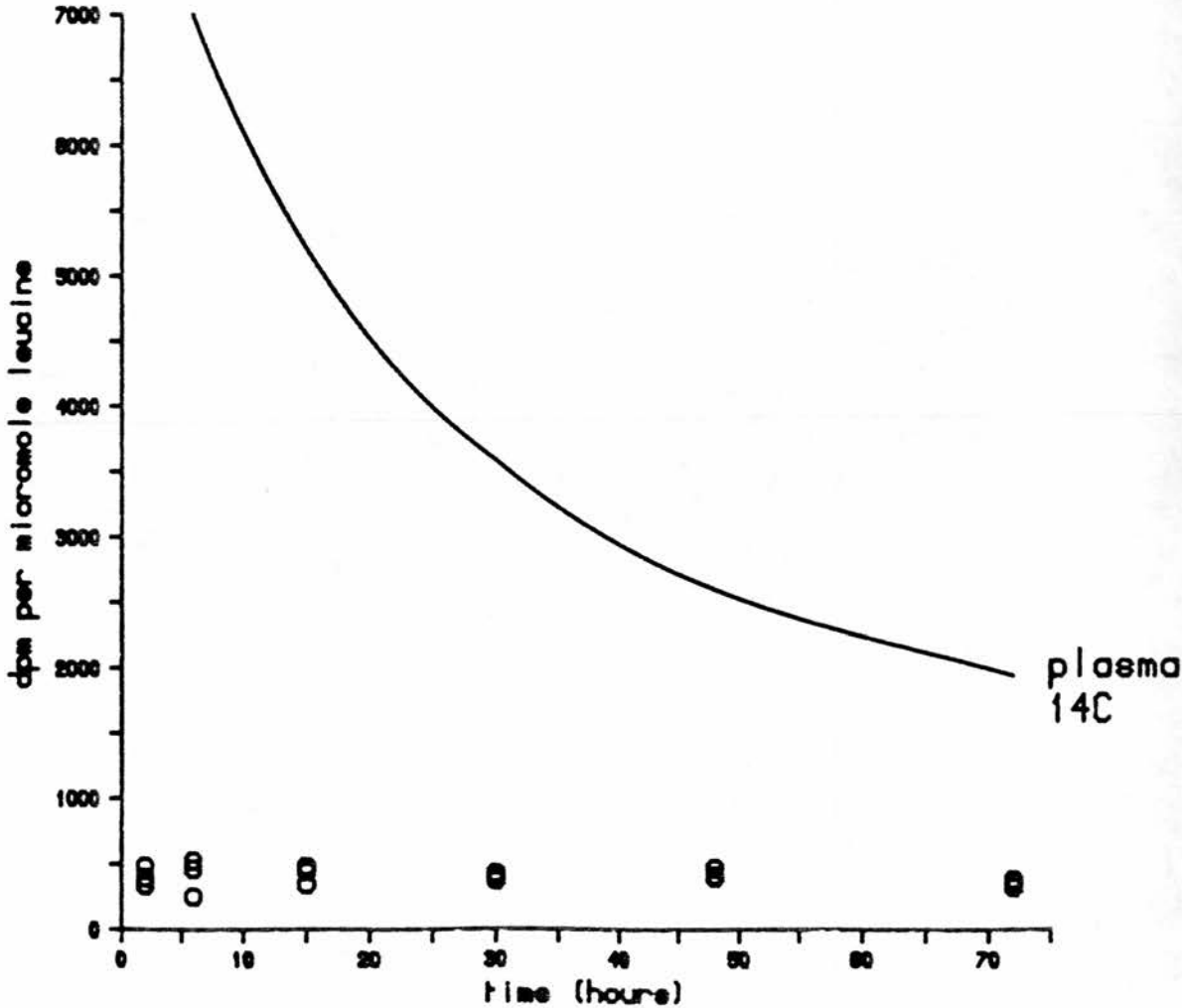
FIGURE 4.8.1.



4.8.1. MYOFIBRILLAR ^{14}C SPECIFIC ACTIVITY
curve drawn through mean of data points

Chicken breast muscle homogenate in 50mM phosphate buffer pH7.5 was centrifuged at 12,000g for 10min. The washed precipitate was counted and assumed to contain myofibrillar protein. Each point represents the specific radioactivity derived from a single bird, calculated from the amount of leucine in myofibrillar protein and the amount of myofibrillar protein in 1g of muscle.

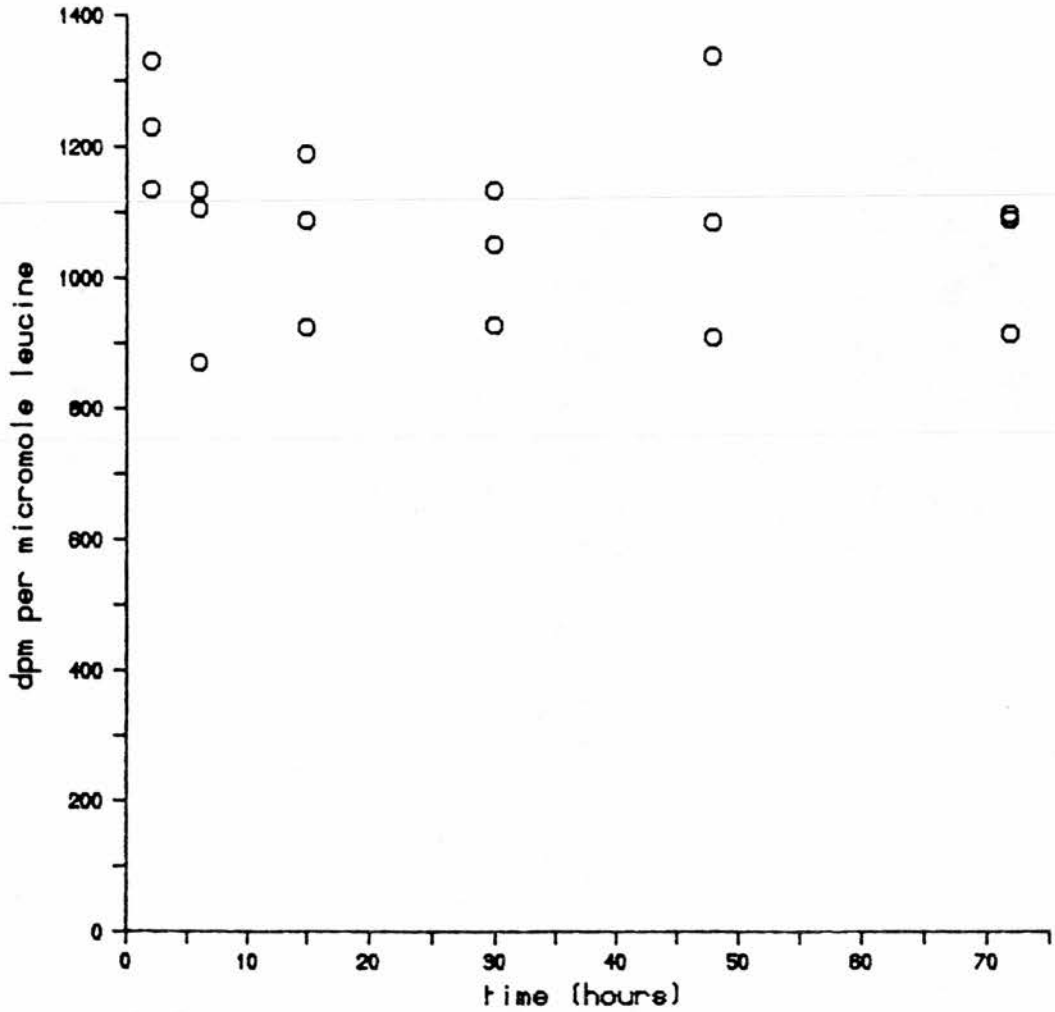
FIGURE 4.8.2.



4.8.2. MYOFIBRILLAR ^{14}C SPECIFIC ACTIVITY

Chicken breast muscle homogenate in 50mM phosphate buffer pH7.5 was centrifuged at 12,000g for 10min. The washed precipitate was counted and assumed to contain myofibrillar protein. Each point represents the specific radioactivity derived from a single bird, calculated from the amount of leucine in myofibrillar protein and the amount of myofibrillar protein in 1g of muscle. The plasma ^{14}C specific radioactivity is presented as an idealised curve derived from the data points presented in figure 4.4.1.

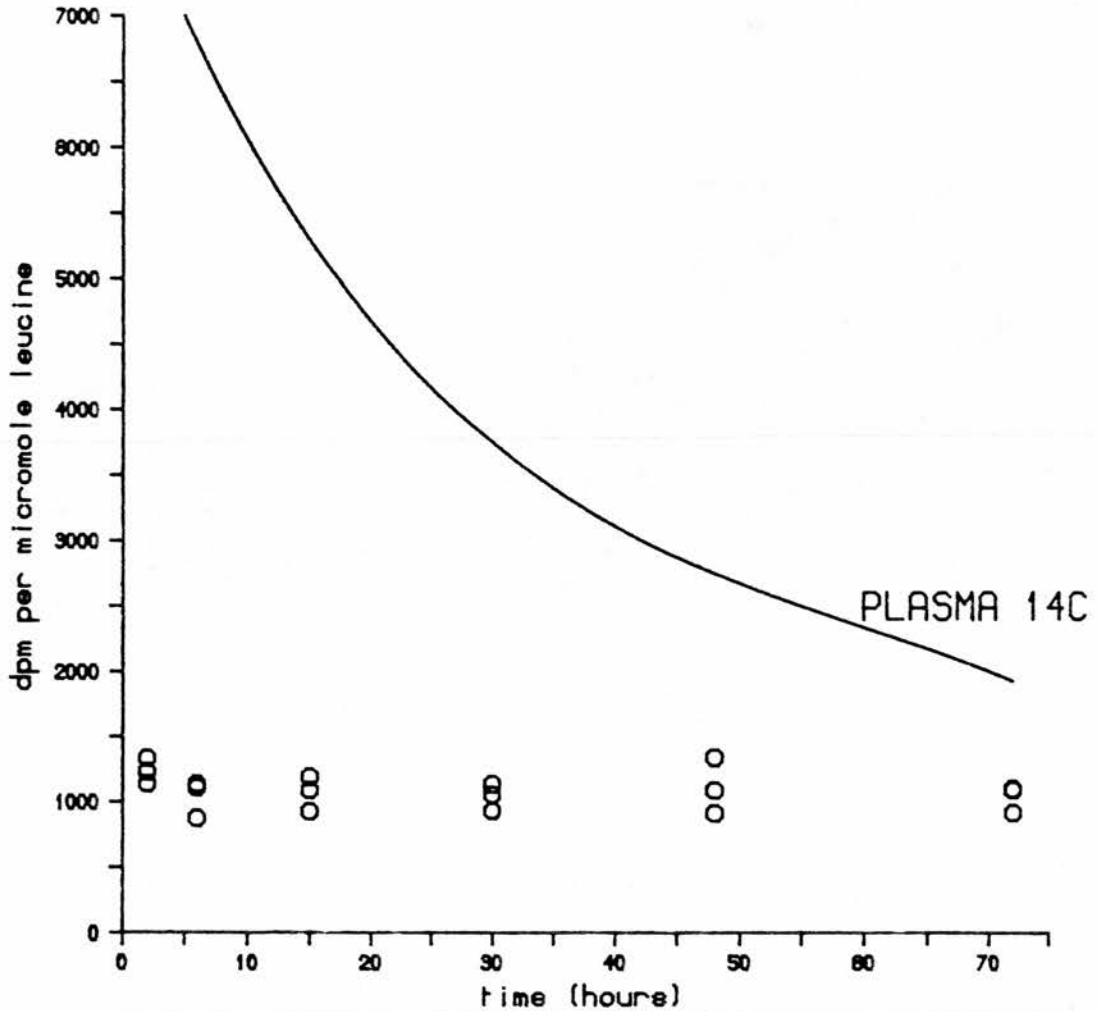
FIGURE 4.9.1.



4.9.1. WHOLE MUSCLE ¹⁴C SPECIFIC ACTIVITY

Chicken breast muscle homogenate in 0.6M PCA was centrifuged at 6,000g for 10min. The pellet was washed prior to counting. Each point represents the specific radioactivity from a single bird, calculated from the amount of leucine in sarcoplasmic and myofibrillar protein and the amount of sarcoplasmic and myofibrillar protein in 1g of muscle.

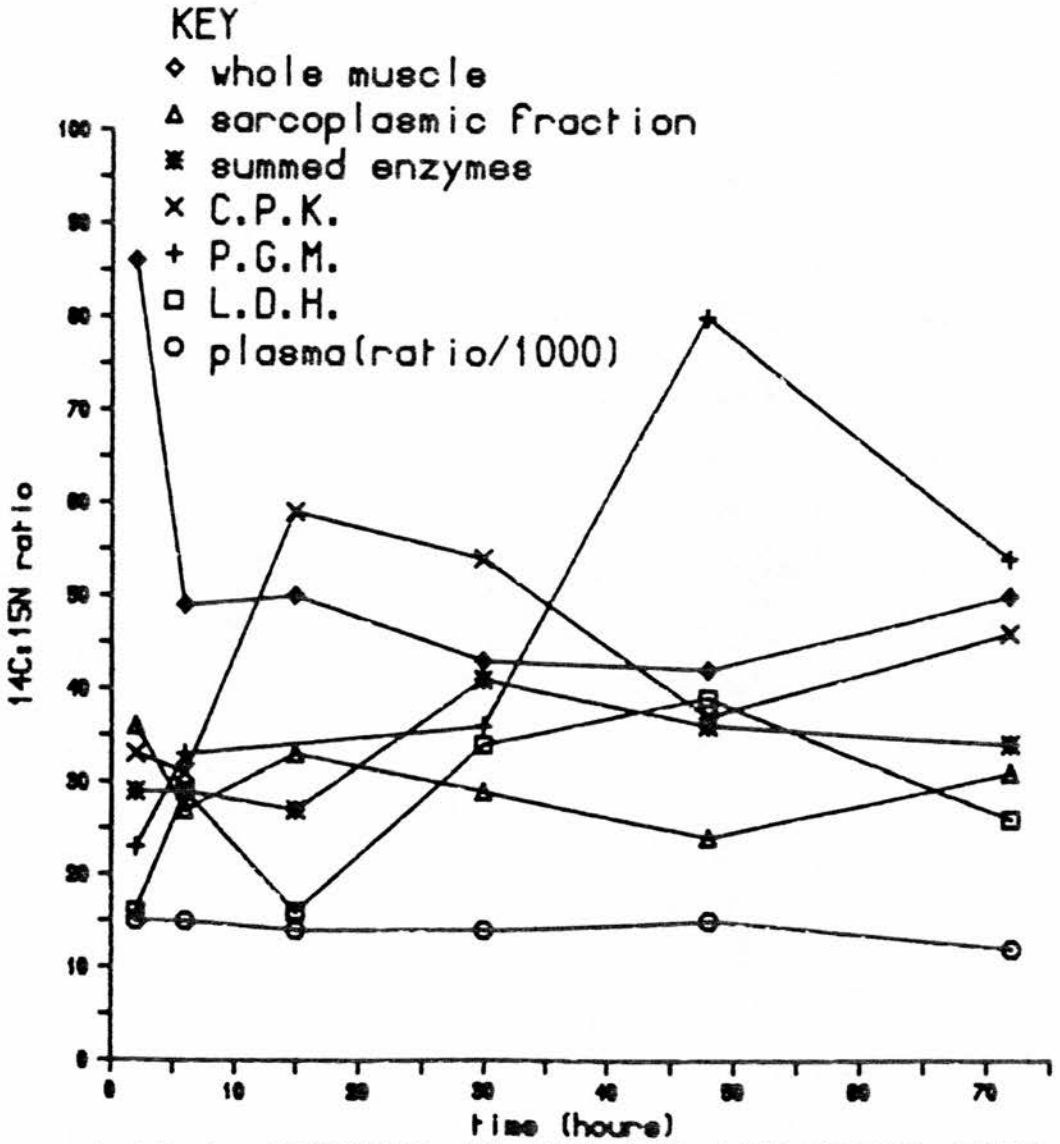
FIGURE 4.9.2.



4.9.2. WHOLE MUSCLE ^{14}C SPECIFIC ACTIVITY

Chicken breast muscle homogenate in 0.6M PCA was centrifuged at 6,000g for 10min. The pellet was washed prior to counting. Each point represents the specific radioactivity from a single bird, calculated from the amount of leucine in sarcoplasmic and myofibrillar protein and the amount of sarcoplasmic and myofibrillar protein in 1g of muscle. The plasma ^{14}C specific radioactivity is presented as an idealised curve derived from the data points presented in figure 4.4.1.

FIGURE 4.10.1.



4.10.1. CHANGES IN THE $^{14}\text{C}:^{15}\text{N}$ RATIO OVER THE EXPERIMENTAL PERIOD

Ratios were calculated from $[^{15}\text{N}]$ per mgN ($\times 10^4$) and ^{14}C dpm per mgN. Each point represents the value obtained by dividing the mean data values from ^{14}C specific radioactivities presented in sections 4.5 - 4.9, with the ^{15}N specific activity shown in tables 4.10.1 - 4.10.7.

and ^{15}N data are expressed in terms of isotope per mg nitrogen rather than per μmol leucine. This is because it is probable that transamination reactions occur throughout the experimental period, thus the ^{15}N is not only a tracer for leucine metabolism but for other amino acids as well. Any future studies should determine which other amino acids are labelled over the experimental period. ^{14}C may be a much better "handle" for leucine, since, once in the muscle, it would appear (from for example figures 4.7.1 and 4.8.1) that most ^{14}C leucine is incorporated into protein with little or no leucine catabolism, that is decarboxylation.

From figure 4.10.1 it can be seen that the plasma $^{14}\text{C}:^{15}\text{N}$ ratio is fairly constant with only a slight fall off at 72 hours. On the other hand, the individual enzymes show variable values of this ratio over the experimental period. LDH shows an early peak at 6 hours and a later peak at around 48 hours. CPK peaks at 15 hours and then again at 72 hours, while PGM peaks at 72 hours although no value was available for the 30 hour time point.

Unfortunately not enough ^{15}N isotope concentration values were available to obtain $^{14}\text{C}:^{15}\text{N}$ ratios for aldolase.

Sarcoplasmic and whole muscle protein show a similar pattern over the 72 hours, while the ratios for the summed enzymes differ from that of the sarcoplasmic protein. These differences are most noticeable at the early time points. The initially high values at 2 hours for the sarcoplasmic and whole muscle protein ratios are due to low ^{15}N concentrations at this time interval (see tables 4.10.5 and 4.10.6).

Table 4.10.1

Plasma ^{14}C and ^{15}N specific activity and $^{14}\text{C}:^{15}\text{N}$ ratios

	1	2	3
Time	$\bar{x}_{(3)}$ dpm/mgN	$\bar{x}_{(3)}$ 15N/mgN($\times 10^{-4}$)	Ratio ($\times 1000$)
2	39099 \pm 2450	2.58 \pm 0.53	15.1
6	11074 \pm 3320	0.74 \pm 0.04	14.9
15	6629 \pm 1920	0.43 \pm 0.1	14.0
30	4095 \pm 914	0.29 \pm 0.1	14.1
48	3057 \pm 524	0.21 \pm 0.05	14.6
72	2240 \pm 426	0.18 \pm 0.06	12.4

Columns 1 and 2 show the specific activity of the plasma PCA supernatant taken from chickens after pulse labelling with [^{15}N][^{14}C] leucine. Each value represents the mean \pm standard deviation calculated with data from three separate animals

Table 4.10.2

LDH ^{14}C and ^{15}N specific activity and $^{14}\text{C}:^{15}\text{N}$ ratios

	1	2	3
time	$\bar{x}_{(3)}$ dpm/mgN	$\bar{x}_{(3)}$ $^{15}\text{N}/\text{mgN}(\times 10^{-4})$	$^{14}\text{C}:^{15}\text{N}$ ratio
2	40.3±10.2	2.56±0.25	15.74
6	61.3±1.0	2.2±0.28	27.86
15	54.5± 6.4	3.45±0.07	15.8
30	87.5±17.6	2.6 ₍₁₎	33.65
48	92 ₍₁₎	2.33±0.45	39.48
72	63.5± 6.4	2.45±0.07	26.0

(1) = only one value measured

Columns 1 and 2 show the specific activity of LDH isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. Unless where indicated, each value represents the mean \pm standard deviation calculated using data from three separate animals. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Table 4.10.3

CPK ^{14}C and ^{15}N specific activity and $^{14}\text{C}:^{15}\text{N}$ ratios

	1	2	3
Time	$\bar{X}_{(3)}$ dpm/mgN	$\bar{X}_{(3)}$ $^{15}\text{n/mgN}(x10^{-4})$	$^{14}\text{C}:^{15}\text{N}$ ratio
2	73.6±23	2.2 ₍₁₎	33.45
6	80±10.6	2.6 ±0.9	30.76
15	79±1.4	1.35±1.2	58.52
30	102±34	1.9 ±1.3	53.68
48	85±9.9	2.3 ±0.5	36.99
72	82±8.7	1.8 ±1.6	45.55

Columns 1 and 2 show the specific activity of CPK isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. Unless where indicated, each value represents the mean \pm standard deviation calculated using data from three separate animals. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Table 4.10.4

PGM ^{14}C and ^{15}N specific activity and $^{14}\text{C}:^{15}\text{N}$ ratios

	1	2	3
Time	\bar{x} dpm/mgN	* \bar{x} $^{15}\text{N}/\text{mgN}(\times 10^{-4})$	$^{14}\text{C}:^{15}\text{N}$ ratio
2	23±17.6	1.0	23
6	40± 3.68	1.2	33
15	53± 8.16	-	-
30	47± 3.36	1.3	36
48	60±37.44	0.75	80
72	46± 8.32	0.85	54

*
(not enough data for \bar{x} values \pm SD ^{15}N)

Columns 1 and 2 show the specific activity of PGM isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. Unless where indicated, each value represents the mean \pm standard deviation calculated using data from three separate animals. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Table 4.10.5

Sarcoplasmic fraction ^{14}C and ^{15}N specific activities

	1	2	3
Time	\bar{x}_3 dpm/mgN	\bar{x} $^{15}\text{N}/\text{mgN}(\times 10^{-4})$	$^{14}\text{C}:^{15}\text{N}$ ratio
2	60 \pm 6.5	1.66 \pm 0.32	36
6	65 \pm 11.5	2.4 \pm 0.4	27
15	71 \pm 8.5	2.14 \pm 0.5	33
30	77 \pm 6.1	2.7 \pm 0.3	29
48	69 \pm 3.7	2.9 \pm 0.35	24
72	75 \pm 9.6	2.4 \pm 0.26	31

Columns 1 and 2 show the specific activity of Sarcoplasmic fraction isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. Unless where indicated, each value represents the mean \pm standard deviation calculated using data from three separate animals. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Table 4.10.6

Whole muscle protein ^{14}C and ^{15}N specific activities
and $^{14}\text{C}:$ ^{15}N ratios

	1	2	3
Time	$\bar{x}_{(s)}$ dpm/mgN	$\bar{x}_{(s)}$ $^{15}\text{N}/\text{mgN}(\times 10^{-4})$	$^{14}\text{C}:$ ^{15}N ratio
2	134 \pm 15	1.56 \pm 0.25	86
6	113 \pm 15.8	2.33 \pm 0.47	49
15	116 \pm 14.6	2.33 \pm 0.15	50
30	113 \pm 11.4	2.6 \pm 0.26	43
48	121 \pm 23.2	2.86 \pm 0.29	42
72	112 \pm 11.2	2.26 \pm 0.29	50

Columns 1 and 2 show the specific activity of whole muscle protein isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. Unless where indicated, each value represents the mean \pm standard deviation calculated using data from three separate animals. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Table 4.10.7

Summed enzymes (Ald, PGM, CPK and LDH) specific activities

and $^{14}\text{C}:^{15}\text{N}$ ratios

Time	and $^{14}\text{C}:^{15}\text{N}$ ratios		3
	1	2	
	$\Sigma\bar{x}$ dpm/mgN	$\Sigma\bar{x}$ $^{15}\text{N}/\text{mgN}(\times 10^{-4})$	$^{14}\text{C}:^{15}\text{N}$ ratio
2	169	7.76	29
6	229	8.0	29
15	258	9.65	27
30	288	7.05	41
48	298	8.18	36
72	236	7.0	34

Columns 1 and 2 show the specific activity for the summed mean values of aldolase, PGM, CPK and LDH for each time interval. Each enzyme was isolated from chicken breast muscle after pulse labelling with [^{15}N][^{14}C] leucine. The values in Column 3 were calculated by dividing the ^{14}C mean value with the ^{15}N mean value for each time interval.

Chapter Five

Discussion and Conclusions

Zilversmit et al (1943) suggested that the specific activity of the precursor pool was the forcing function for the product pool's specific activity labelling patterns. This, the final chapter, therefore begins by looking at the plasma, ^{14}C and ^{15}N specific activity of labelled leucine during the 72 hours of the experiment.

5.1 ^{14}C plasma specific activity

From the plasma specific activity curves, after labelling with ^{14}C and ^{15}N -leucine, it would appear that the labelled leucine is initially rapidly removed from the plasma, but that the rate of removal falls and becomes very low, so that the specific activity eventually levels out.

Analysis of individual proteins and of whole muscle protein (see figures 4.5.2 and 4.9.1) indicates that ^{14}C leucine is retained within the muscle, although some of the ^{15}N may be lost (discussed later). Moreover data from the excreta analysis suggests that a large proportion of the ^{15}N is excreted in the early part of the experiment. One must also assume, that in some tissues (although not perhaps in muscle), the leucine carbon skeleton will be metabolised and excreted into the atmosphere as $^{14}\text{CO}_2$ as well as in ^{14}C -containing compounds in the excreta.

One might therefore have predicted the plasma leucine ^{14}C and ^{15}N specific activity to have fairly rapidly fallen to zero.

This expectation is raised by the fact that the bird is growing during the experimental period, the size of the blood vessels and the volume of blood increasing throughout. Therefore the concentration of label would be seen to decrease, even if it were not being removed. These observations might indicate that the label

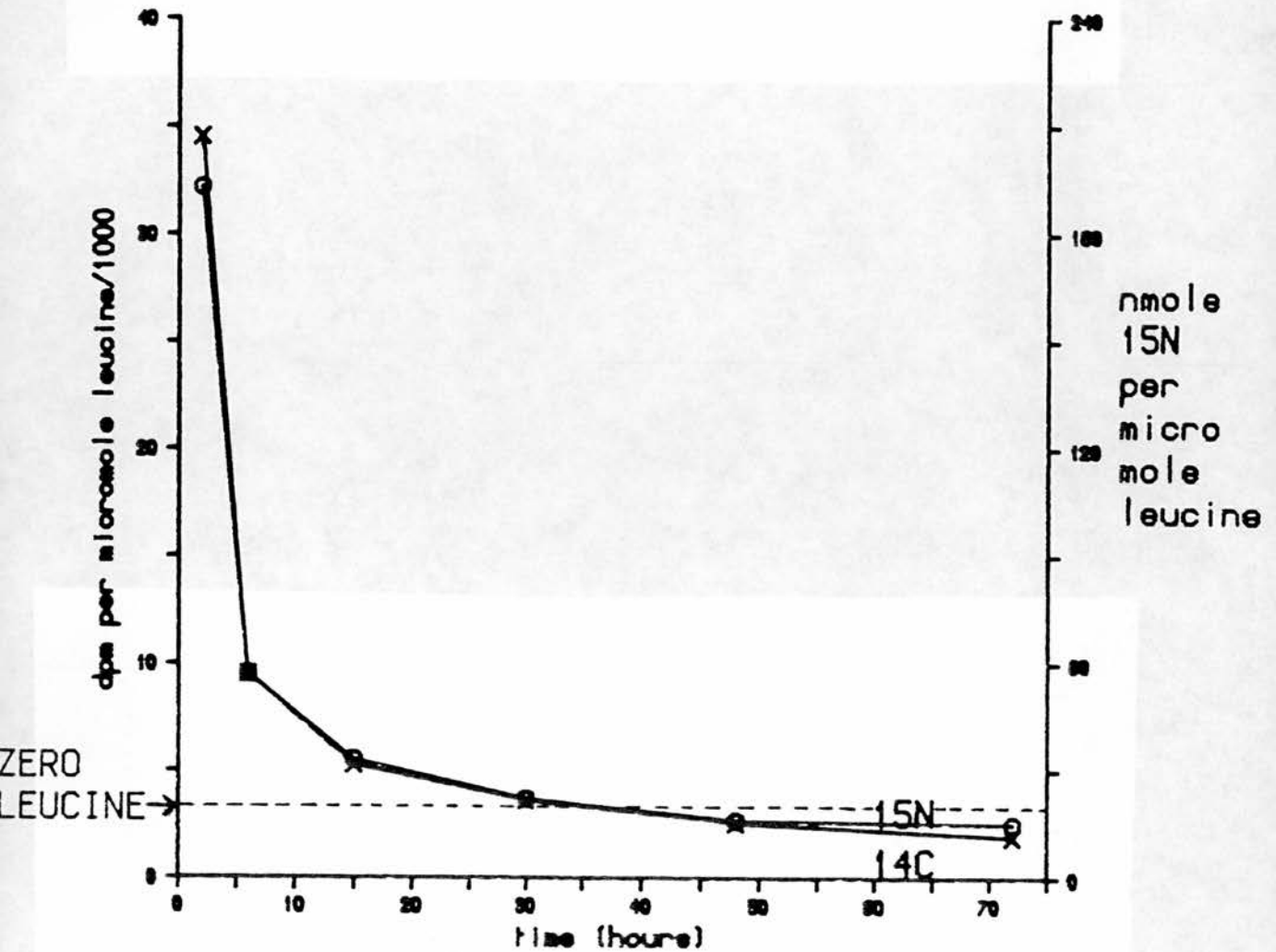
which is present during the plateau stage of the plasma specific activity curve may not be in leucine or any other amino acid, but perhaps some fairly stable, slowly labelled substance (such as creatine or glutathione). The plasma decay curve may then look like fig. 5.1.1. This gives rise to the following question: if at some period before 72 hours all the detectable leucine had left the plasma when did it leave? That is to say, where should the horizontal line in fig. 5.1.1 be drawn?

The ^{15}N excretion data suggests that there is still a little loss of ^{15}N after 48 hours. Although the faecal output calculations do not include ^{15}N from $^{15}\text{NH}_3$ excretion it may be (especially during the early part of labelling) that a large amount of ^{15}N is excreted as $^{15}\text{NH}_3$. This was overlooked at the time, and is a serious error in the experimental design. Any future experiment should include measurement of the $^{15}\text{NH}_3$ excreted, so that a more accurate estimate of the total excretion of ^{15}N can be made.

Some independent evidence can be drawn from the muscle sarcoplasmic ^{14}C specific activity data. If the labelled leucine had completely disappeared from plasma by 48 hours, while the muscle grew by $\sim 8\%$ over the period 48 - 72 hours, the specific activity curve should look like that in fig. 5.1.2 (a).

In fact, it looks like fig. 5.1.2 (b). There must therefore be a slow entry of ^{14}C leucine into the muscle, which ensures that the specific activity remains fairly constant. There is no definite value, then, for the true plasma leucine ^{14}C specific activity at 72 hours, and there is no method to determine this, short of direct measurement (by isolation of the amino acid from plasma). Nor is it

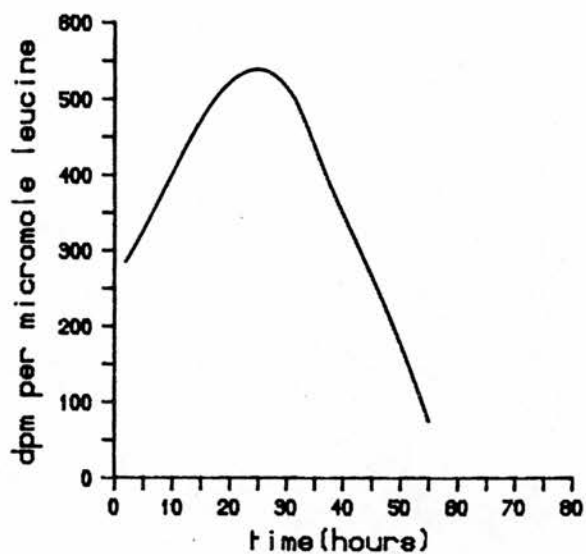
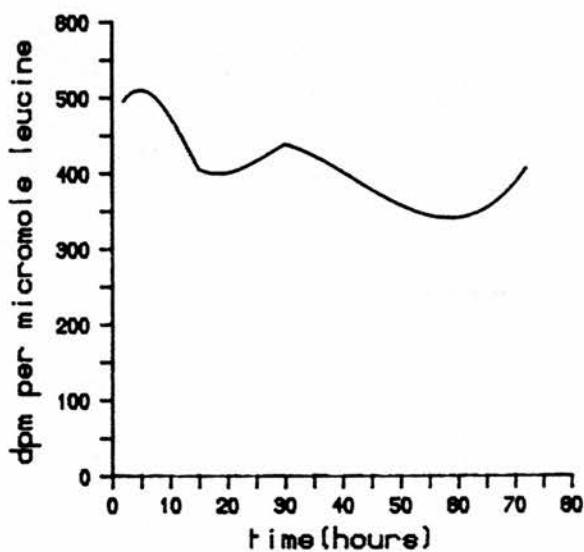
FIGURE 5.1.1.



5.1.1. THEORETICAL ¹⁴C AND ¹⁵N DECAY CURVE

The loss of ¹⁵N and ¹⁴C from plasma of chickens injected with a pulse of [¹⁵N][¹⁴C] leucine. Each point represents the mean specific radioactivity calculated with data from 3 separate birds. The figure shows that there may be a point at which all the free labelled leucine has been removed from the plasma. The observed radioactivity may be from a slowly metabolised substance which is retained in the plasma (e.g. creatine).

FIGURE 5.1.2.

5.1.2. (A) THEORETICAL ¹⁴C
SARCOPLASMIC SPECIFIC ACTIVITY.5.1.2. (B) ACTUAL ¹⁴C SARCOPLASMIC
SPECIFIC ACTIVITY

possible to go back to the ^{15}N data, to combine the N excretion and plasma curve, due to the possible error caused by loss of $^{15}\text{NH}_3$ and the absence of information about transamination rates.

This uncertainty might be said to affect the interpretation of the labelled muscle protein data, since if the ^{14}C specific activity at 72 hours is lower than the muscle protein specific activities, these labelling curves would cross and Zilversmit's criterion of a precursor-product relationship may hold.

However, Zilversmit's theory predicts the intersection to be at the product's highest specific activity value.

For most of the muscle protein, the highest specific activity value occurs considerably earlier than 72 hours, when the plasma specific activity curve is much higher than that of the protein specific activity curve.

Therefore, it is concluded here that Zilversmit's criteria have not been met.

5.2 Implications of results

If Zilversmit's criteria have not been met, how does this affect our thinking?

One might assume that the plasma is not the only amino acid pool used for protein synthesis. This means that there would have to be a reservoir of unlabelled leucine constantly donating ^{12}C and ^{14}N . If this were so, this pool must have to decrease in size. The unresolved problem is then to identify this source of unlabelled amino acid since it has been shown that the muscle is growing overall (figure 4.2.1).

An alternative argument is that a considerable fraction of

protein never gets labelled. This is not easily explained unless one assumes that a large fraction of protein, once synthesised, remains within the muscle and is not broken down. It is possible this situation may occur during periods of rapid growth observed in the early life of the broiler chicken. It has been suggested by Bryan (1985) that the amount of "turnover" in the broiler chicken muscle is a very small proportion of the total tissue present.

In other words it is possible to envisage a system which has only a small degree of protein turnover perhaps located at specific areas within the muscle. In the growing animal the rate of synthesis should be very high in these areas. The results presented in figures 4.5.2, 4.7.1, 4.8.1 and 4.9.1 indicate a high rate of synthesis, since a substantial percentage of total label taken up is done so within the first few hours after injecting the tracer.

Millward (1980) proposed a theory which may explain such a system. It must be noted he was concerned only with myofibrillar turnover, although his ideas might nonetheless be applied to some sarcoplasmic proteins if it were assumed that they were at least in part associated with the myofibrils (see section 5.4).

5.3 Myofibrillar Growth

The main difficulty in explaining growth in the muscle is probably accounting for myofibrillar growth. How can the myofibrils grow? Do the fibrils split and are new sarcomers added?

One of the leading theories concerning myofibrillar growth was presented by Millward (1980).

Millward concentrated initially on the fact that the myofibril is made up of many different proteins, each with an individual

turnover rate (Millward 1980). He concluded that the synthesis and degradation of myofibrils must therefore involve separation of the individual proteins of the myofilament. For this to occur, Millward proposed that myofibrillar degradation involved removal of individual subunits from intact myofibrils at varying rates, or some degree of myofibrillar disassembly, producing a pool of individual contractile protein subunits, some of which would be broken down by the proteolytic system. Some of these subunits would be replaced by newly synthesised proteins, while others would be returned to another myofibril. Thus while protein subunits remain bound to the myofibrillar apparatus they may be protected for the degradative processes which attack the "soluble" or dissociated proteins.

Growth in this system could be explained if the synthetic rates were greater than the dissociation or degradation rates. However, organisation into new sarcomeres is more difficult to explain, unless the system occurred mainly at the ends of myofibrils. This has been suggested by many workers (Holtzer et al 1957; Kitlyakara and Angerine 1963; Ishikawa 1965; Goldspink 1968; Mackay et al 1969).

New sarcomeres could be formed by the same mechanism of association-dissociation of myofibrillar turnover described for the non-growing system. Therefore, Millward's theory may allow for a large fraction of myofibrillar protein which does not get broken down during growth, while there may be a small fraction of myofibrillar protein turning over at the ends of the myofibrils.

If this were the case it may explain the labelling patterns described in this thesis, which indicate that only a fraction of the

label gets incorporated into myofibrillar protein.

It must be asked, however, if these theories can also explain the similar labelling patterns found for sarcoplasmic proteins. The answer is yes, if it can be shown that at least some of each individual sarcoplasmic protein is associated with the myofibrils and synthesis is concentrated in the area at the ends of the myofibrils. If this were the case, at least some sarcoplasmic proteins may fall under the same association-dissociation control mechanism as that for myofibrillar proteins described by Millward.

Therefore, if it can be shown that a fraction of some sarcoplasmic proteins may not be truly soluble but simply less strongly associated to the myofibril than other "myofibrillar" subunits, then it may explain why a large fraction of sarcoplasmic protein as well as myofibrillar protein does not become labelled during the 72 hour experimental period.

5.4 Association of sarcoplasmic proteins with the myofibrillar apparatus

There is some evidence for sarcoplasmic protein association not only with the myofibrillar protein but also with other sarcoplasmic proteins, which may be in turn bound to the myofibrils by "piggy back" binding. These ionic interactions are affected by factors such as pH and ionic strength and are thought to be centered on specific areas (i.e. binding sites) on each protein. Transketolase, transaldolase and GAPDH were shown by Wood et al (1985b) to be associated with each other, as were aldolase, pyruvate kinase and phosphoglycerate mutase by Clark et al (1985). While GAPDH and LDH were shown by Brandau and Pette (1966) to bind to isotropic zones of

cross striated muscle, Pette and colleagues also indicated that most of the glycolytic enzymes were located in the I band through histochemical (Sigel and Pette 1969), immunofluorescent (Dilken et al 1975) and biochemical studies (Arnold and Pette 1968, 1970; Arnold et al 1971).

Trypomycin has distinct sites for binding of aldolase and GAPDH (Clark et al 1985), while enolase, creatine kinase aldolase and pyruvate kinase have been found associated with actin-containing structures in nerve axons (Brady and Lasek 1981). In addition, CPK has been found associated with the M-line protein of muscle (reviewed by Ottaway and Mowbray 1977).

The sarcoplasmic proteins studied in this experiment, namely, aldolase, LDH, CPK and PGM, have all been found associated in some way with the myofibrillar apparatus. They may therefore, in part at least, fall under control mechanisms similar to those proposed by Millward (see section 5.3). This may explain the plasma and protein ^{14}C labelling patterns, which indicated that a fraction of the studied "soluble" muscle proteins were not labelled over the 72 hour experimental period.

If this conclusion is correct, it may require a reappraisal of those isotope labelling techniques which assume all the particular protein under investigation is susceptible to labelling. From this assumption the rate of synthesis is calculated from the degree of isotope incorporation in a given time-interval, for a specific amount of protein. However if only a fraction of that protein is actually being labelled, then the rate of synthesis will be estimated to be less than it actually is.

5.5 Shapes of the ^{14}C specific activity labelling curves

There are two main observations that can be made about the shape of the labelling curves. These are; firstly - that, once synthesised, the label appears to be retained within the protein or group of proteins, at least over the experimental period.

Secondly, the individual enzymes labelling curves do not follow a simple precursor-product relationship with the tracer.

It has been noted that although each of the individual proteins studied had ^{14}C specific activities of around 500dpm/ μmol leucine, there were characteristic differences between each. If only a percentage of each enzyme population is available for synthesis, then these differences in ^{14}C specific activity may be due to the different percentage of each enzyme available for synthesis. Moreover differences in each enzyme's content of leucine should be taken into account.

5.5.1 Myofibrillar, sarcoplasmic and summed enzyme labelling curves

The myofibrillar protein ^{14}C specific activity values over the experimental period follow a bimodal pattern (see fig. 4.8.1) with peaks at around 15 hours and 48 hours after the initial injection of the isotope. A possible explanation for such a pattern is that because a group of proteins are being monitored, each peak is due to a different protein. The two peaks may simply indicate the maximum labelling point of two major proteins from the group which make up the myofibrils, or the resultant of several. Moreover, the fact that little label is lost over the 72 hours experimental period does not necessarily mean that none of the proteins are being degraded, it may simply be due to a very slowly synthesised protein which is

at or near its maximum labelling point at 72 hours (see fig. 5.5.1).

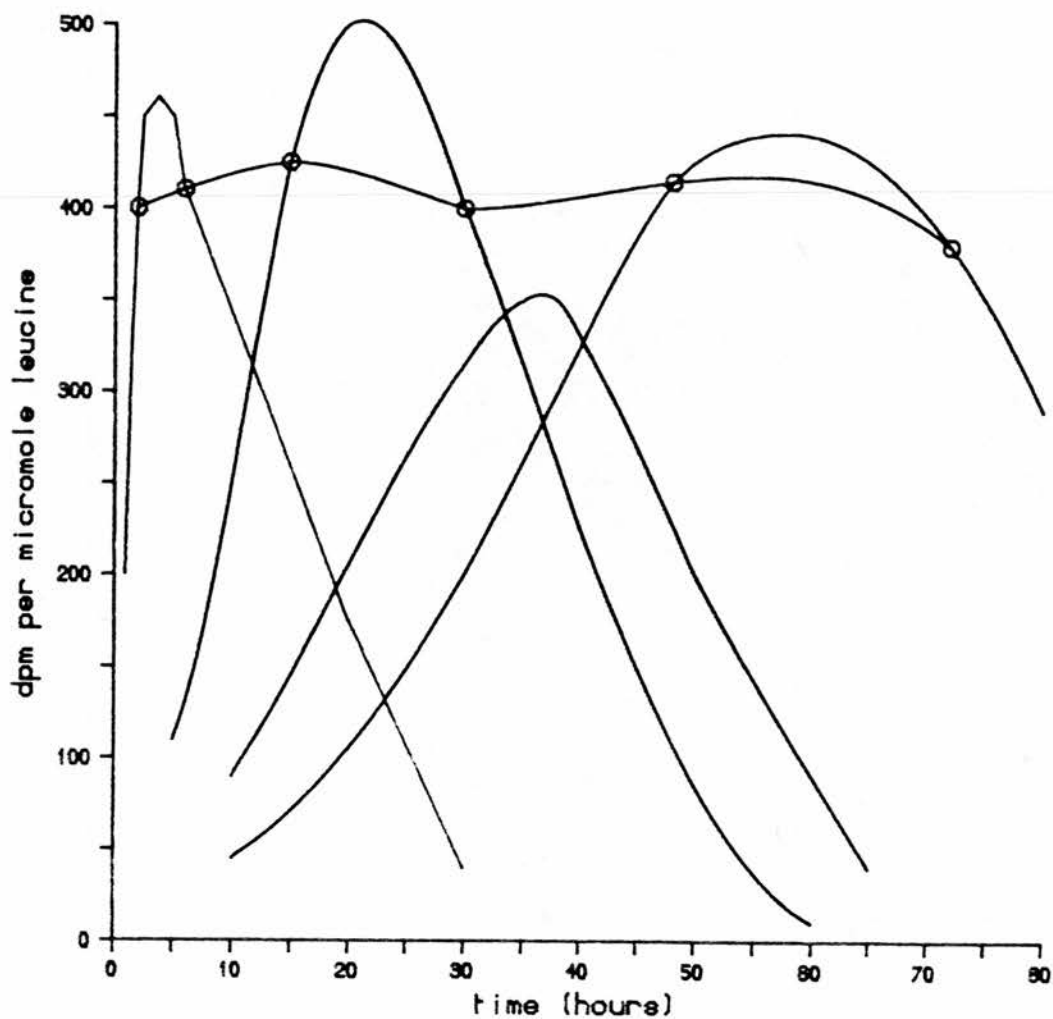
The whole muscle protein and sarcoplasmic protein ^{14}C specific activity labelling patterns can be explained in the same way, although as fig. 4.7.1 and 4.9.1 illustrates, the whole muscle protein and sarcoplasmic protein ^{14}C curves show the possibility of three peaks (at 2 hours, 15 hours and 48 hours for whole muscle protein and at 6 hours, 30 hours and 72 hours.)

Taking these factors into account, studies which deal only with whole muscle or fractions of muscle cannot be said to give a very clear picture of what is happening to individual proteins within the tissue. All that may be concluded from the myofibrillar and sarcoplasmic labelling patterns is that little label is lost from each fraction over the experimental period.

In an attempt to compare the data obtained from the individual enzyme ^{14}C labelling with that of the sarcoplasmic ^{14}C labelling pattern, the specific activity values, from all the enzymes were added together for each of the sampling times to produce the pattern shown in fig. 4.6.1.

However, as only 4 sarcoplasmic proteins (constituting some 30% of the total), were studied, it is not possible to make a direct comparison. Perhaps in a future, more comprehensive experiment it might be possible to isolate, if not all, at least 80 - 90% of the sarcoplasmic proteins. In this particular experiment, however, insufficient time was available for this.

FIGURE 5.5.1.



5.5.1. THEORETICAL LABELLING PATTERN OF A SAMPLE CONTAINING A GROUP OF PROTEINS

Each point represents the possible specific radioactivity derived from measuring dpm and calculating the amount of leucine in the total protein present. The diagram shows that in a sample containing a group of proteins little information can be gained concerning the labelling pattern of the individual proteins which make up that sample.

Nevertheless, the labelling pattern for the summed enzymes could be interpreted as indicating peaks at two time-points at least, these being 6 hours and 72 hours.

This may be said to be a somewhat subjective observation, however, since the degree of scatter of ^{14}C specific activity data points is very wide.

5.5.2 Individual sarcoplasmic protein ^{14}C labelling patterns

Looking at the ^{14}C specific activity labelling curves for the purified enzymes aldolase, LDH, PGM and CPK, the most striking observation is that none of them follows a single precursor-product relationship. The fact that the plasma specific activity curve does not appear to cross the enzyme specific activity curve in fig. 4.5.2 does not mean that the lines do not intersect in reality, since, as has been explained, the labelled protein may only be a fraction of the total. If the actual amount of protein which is labelled were known then a new specific activity curve could be drawn which should cross the plasma curve, provided that the plasma specific activity curve takes growth into account. If this were so, Zilversmit's criteria could be used.

The main problem, then, is explaining why the peaks are bimodal. Zilversmit would have predicted a single smooth peak (fig. 4.1.1).

The most immediate explanation is that there is more than one protein in each sample. That is to say that the isolated protein might contain some contaminant which has a different rate of synthesis. However, as most of the samples were shown to be at least 90% pure (see Chapter 3), this explanation is very unlikely.

One possible theory is that the rate of protein synthesis,

and/or the rate of protein degradation, may have been influenced by diurnal rhythms. The experiments reported in this thesis involved birds which were reared on a 23 hour light:1 hour dark cycle. It may be that even with only one hour of darkness, diurnal patterns were set up. If this were the case these rhythms may have continued to operate during the 72 hour experimental period, when the birds were kept in continuous lighting. Since it has been shown that if birds which have been previously kept on a light/dark cycle are then kept in continuous light they show residual free running diurnal rhythms (Duncan and Hughes, 1975; Fujita, 1973).

There are a number of hormones known to be involved in influencing protein metabolism which may be subject to diurnal rhythms set up by light/dark cycle or in some cases from the subsequent feeding behaviour derived from this. The level of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) may be particularly influenced by light/dark cycles. The concentration of T_4 and T_3 have been shown to be inversely related and show rhythmic changes during the daily light/dark cycles (Newcomer, 1974; Klandorf et al, 1978). Plasma T_3 levels increase during light periods and decrease during the dark while T_4 levels decrease during light periods and increase during the dark. The increase in T_4 during darkness may be due to a raised concentration of thyroid stimulating hormone which may be regulated by a circadian rhythm (Jordan et al, 1980; Chain, 1978). The thyroid gland of the chicken contains mainly T_4 (Astler, 1975) and much of the T_3 found in the circulation is probably formed from T_4 after monodeiodination in the peripheral tissues (Borges, 1980). Thus daily changes in the level of plasma

T_3 is a reflection of the rate of peripheral conversion of T_4 to T_3 . Therefore T_4 responds directly to light and dark whereas T_3 may be controlled by another mechanism.

It has been suggested that a number of physiological phenomena show diurnal rhythms, many of which are related to the cyclic pattern of food intake (Garlick et al, 1973). This pattern is set up because birds kept on light/dark cycles rarely if ever feed during the period of darkness (Savory, 1976). This may have led Sharp and Klandorf (1981) to suggest that the increase in the level of T_3 caused by a high rate of monodeiodination is unlikely to be directly affected by daylength but by the level of food intake.

If feeding behaviour is influenced by light/dark cycles then not only T_3 and T_4 levels will alter rhythmically throughout the day but also effectors such as insulin and corticosterone.

The mechanisms by which these hormones influence the rate of protein metabolism is complex. T_3 has been shown to be involved in the modulation of both protein synthesis and degradation. T_3 acts on the elongation phase of protein synthesis (Mathews et al, 1973) and may increase the number of ribosomes by increasing RNA synthesis (Brown et al, 1981). T_3 also regulates the capacity for proteolysis (De mantino and Goldberg, 1978). When the thyroid state is manipulated the rate of degradation varies directly with the T_3 levels (Brown et al, 1983). T_3 may act on lysosomal proteinases, in particular it may increase the rate of synthesis of cathepsin D, acting at the level of transcription (Millward, 1983).

The plasma insulin concentration may also affect protein metabolism. A raised insulin concentration has been shown to

increase protein synthesis by acting at the initiation stage (Garlick et al, 1973). In addition insulin may modulate the activity of other hormones. The effect of T_3 on translation is dependant on some insulin being present whereas insulin is able to stimulate transcription independently (Garlick et al, 1973).

The insulin antagonist corticosterone has been shown to be associated with starvation and refeeding and may be an important factor in the control of protein turnover (Reeds and Palmer, 1984). Corticosterone has also been shown to increase the catabolic effect on muscle (Munro, 1964) as well as inhibiting protein synthesis, probably through its inhibitory action on ribosomal RNA synthesis.

If the level of these hormones are influenced by diurnal rhythms then could these daily changes explain the bimodality of the peaks observed for the specific radioactivity of the purified enzymes shown in figs. 4.5.1-4.5.5.?

If the birds studied in this thesis were subject to diurnal rhythms set up by a 23 hour light 1 hour dark cycle, one might expect the specific radioactivity time curve for these enzymes purified (i.e. Aldolase, PGM, LDH and CPK) to have a bimodal pattern. One might even predict that either a peak or a trough would occur at or around every 24 hours, during the period of darkness.

In such a case one might expect a fairly high rate of protein synthesis during the light period, due to a high concentration of T_3 which would presumably increase the rate of protein synthesis to a greater extent and less specifically than it would the rate of protein degradation. In addition plasma insulin would be fairly

high also helping to maintain a high rate of protein synthesis. However during the expected dark period, feeding may stop and plasma T_3 and insulin concentration may fall, perhaps allowing corticosterone to inhibit protein synthesis and increase proteolysis. This would cause the uptake of labelled amino acids to decline and release some of the amino acids already taken up into protein. The specific radioactivity peak of a purified enzyme from such a labelling experiment would therefore be expected to decline. However when the darkness cycle has passed the insulin and T_3 levels may rise as refeeding begins causing the anabolic processes to take over and the specific radioactivity of the purified protein in each labelling experiment to rise again.

If one looks at the labelling patterns for the protein isolated in this thesis (fig. 4.5.1-4.5.5) it is apparent that for Aldolase and PGM the specific radioactivity does appear to peak and trough at the expected times (i.e. 24 and 48 hours). However the labelling patterns for CPK and PGM do not follow this cycle. If diurnal rhythms are important in influencing protein metabolism it would appear that not all proteins are affected in the same way. It may be that other hormonal factors are involved which are as yet unknown. In addition there are many other cues which affect feeding behaviour such as, air temperature, sounds and servicing times (Savory, 1976). Moreover the birds studied here were housed in a fume cupboard in a working laboratory which had large windows. Thus any number of cues might have been involved and although the laboratory lights were on the total light intensity must have varied from daytime to nighttime. These facts make it very difficult to

draw any conclusions on whether diurnal rhythms are involved in producing the results observed or whether some other explanation might be more plausible. One thing that can be said is that any future experiments should take into account the environment the birds are housed in and attempt to reduce as many external cues as possible. This should include rearing the birds in continuous light from hatching as well as carrying out the experiment in the same room in which the birds were reared.

An alternative theory which might explain the bimodal nature of the peaks shown in figs. 4.5.1-4.5.5 is that the protein is being synthesised at two different rates in different parts of the muscle cell. However, this could not be from the same amino acid precursor pool, since the second peaks on the LDH, CPK and PGM specific activity curve are higher than the first peaks. If both peaks represent protein synthesised from the same pool, the decreased precursor specific activity which is assumed to cause the first peak to fall would mean that the protein in the second peak would always have a lower specific activity and could never reach a higher specific activity than the first peak (see fig. 5.5.2).

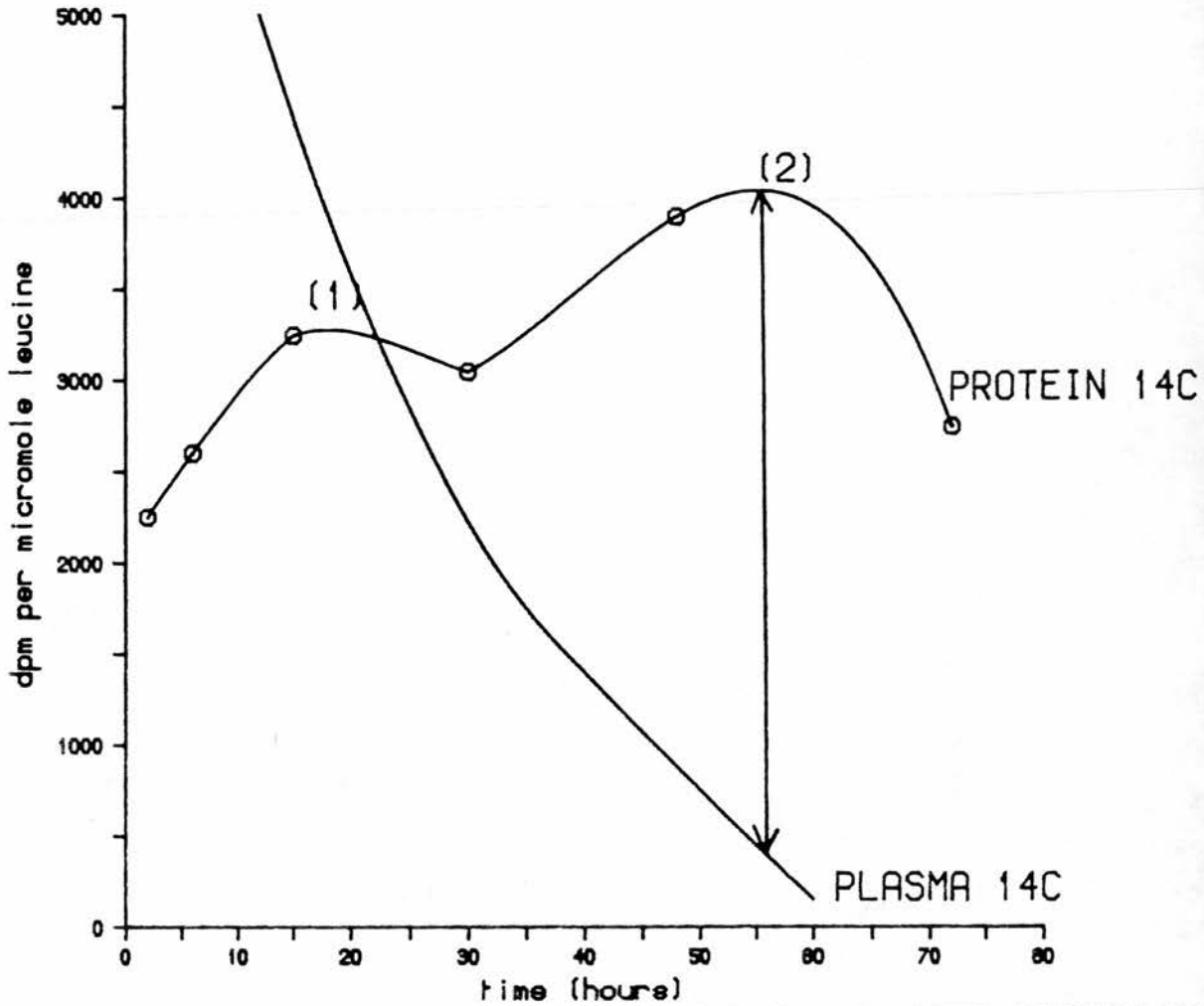
This argument forces the conclusion that not only are there two populations of each of the individual enzymes studied, but also that each each population is synthesised from a different amino acid precursor pool.

The model for such a system is outlined in fig. 5.5.3. The first peak might indicate synthesis at the membrane region of the cell where the muscle is at or near equilibrium with the plasma. The second peak could be from a slower movement of the label to

become compartmented into the intracellular amino acid pool before being taken up by protein synthesis, or perhaps be from a highly labelled protein from the membrane region moving towards the interior of the cell where the protein is rapidly degraded and the label released for uptake by another group of less rapidly synthesised proteins.

The model may explain the data, but does it have any physiological significance? It may if different areas of the growing muscle cell require different amounts of each protein. For example, the enzyme aldolase might be associated with the membrane

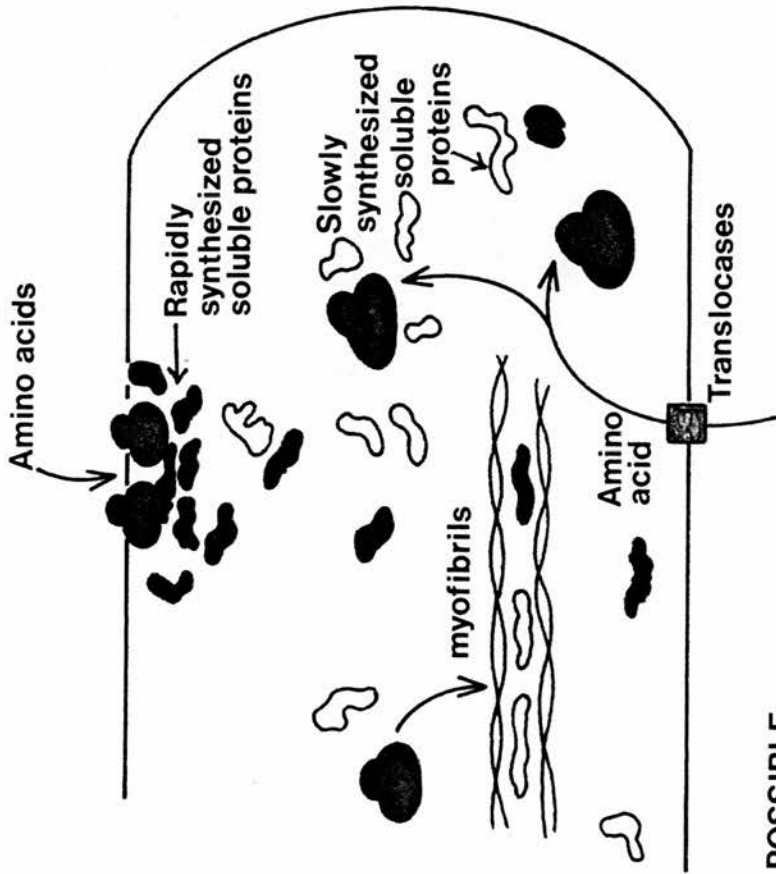
FIGURE 5.5.2.



5.5.2 THE FIGURE SHOWS THAT THE SECOND "PRODUCT PEAK" CANNOT BE DIRECTLY SYNTHESIZED FROM THE SAME "PRECURSOR POOL THAT THE FIRST "PRODUCT PEAK" WAS (i.e. THE PLASMA).

The peaks represent a possible specific radioactivity labelling pattern for a purified protein synthesized from a single precursor pool (i.e. the plasma amino acid pool). The decreasing plasma specific radioactivity would force the specific radioactivity of peak (1) to fall. This would also mean that the specific activity of peak (2) should always be lower than peak (1) since there is no source of highly labelled leucine left to take up.

FIGURE 5.5.3.



5.5.3. POSSIBLE
ORGANISATION
OF PROTEIN
SYNTHESIS IN MUSCLE

to help provide ATP for ribosomes or membrane-bound energy linked pumps, while the other interior population of enzyme might be useful in providing ATP for the myofibrils.

It may be the case that both groups are susceptible to association with the myofibrillar apparatus and become laid down during muscle growth. Perhaps once within the myofibrillar apparatus these proteins become protected from proteolytic attack. This could be an explanation as to why most ^{14}C label is retained within the muscle and why a large unlabelled pool could exist.

5.6 Carbon:nitrogen ratio analysis

5.6.1 Plasma $^{14}\text{C}:^{15}\text{N}$ ratio

From fig. 4.10.1 it can be seen that the plasma $^{14}\text{C}:^{15}\text{N}$ ratio remains fairly constant over the experimental period. This tends to suggest that, overall, ^{14}C and ^{15}N are removed from and returned to the plasma at the same rate, although this may not necessarily be carried out by the same tissues.

The plasma has a higher $^{14}\text{C}:^{15}\text{N}$ ratio than that of the injected solution. This may indicate that the liver removes ^{15}N by transamination with, for example, glutamate, at a very early stage, and that ^{15}N is rapidly incorporated into uric acid and NH_3 , then excreted.

This is consistent with the initial rapid loss of ^{15}N to the excreta discussed in section 4.3. .

5.6.2 Whole muscle, sarcoplasmic muscle and summed enzyme protein $^{14}\text{C}:^{15}\text{N}$ ratio analysis

The $^{14}\text{C}:^{15}\text{N}$ ratios of the whole muscle, sarcoplasmic protein, and the summed enzymes, shown in fig. 4.10.1, have higher values than

those of the plasma, as do those of all the other individual muscle proteins measured. This suggests that the amino acid protein precursor pool of muscle has a different $^{14}\text{C}:^{15}\text{N}$ ratio than that of the plasma. This may be due to transamination reactions occurring within the muscle, causing the ^{15}N to be rapidly lost from the leucine carbon skeleton. However, the carbon skeleton may also pick up ^{14}N from other transamination reactions, and become incorporated into protein. The ^{15}N may be incorporated into another amino acid available for protein synthesis or as seems more likely from the data, be incorporated into some molecule which may then be removed from the muscle (e.g. alanine) or incorporated into some molecule which is not involved in protein synthesis (e.g. creatine).

The values for the $^{14}\text{C}:^{15}\text{N}$ ratio are fairly constant for both whole and sarcoplasmic muscle protein apart from an exceptionally high value at 2 hours, for both protein groups. This is due to a fairly low ^{15}N specific activity at this time interval (see table 4.10.6). This may well be due to the initial high rate of loss of ^{15}N via the excreta (outlined in section 4.3).

This may have been highlighted in these protein groups by one or more particularly rapidly synthesised proteins which would have taken up a large proportion of the label at this early time when the $^{14}\text{C}:^{15}\text{N}$ ratio was at its highest.

5.6.3 Creatine kinase, LDH and PGM $^{14}\text{C}:^{15}\text{N}$ ratio analysis

The shape of the curve of $^{14}\text{C}:^{15}\text{N}$ ratio values for CPK is very erratic. However, if one refers back to the ^{14}C specific activity pattern over the experimental period (fig. 4.5.5) it can be seen that CPK apparently has at least three peaks indicating the

possibility of 3 sub populations of CPK. Since each population may be synthesised from its own specific amino acid precursor pool, the ratio pattern may reflect the differences in each pool which would be most obvious at each groups' highest degree of labelling - that is, the specific activity peaks. These occur at around 6 hours, 30 hours and 72 hours.

The shape of the curve for $^{14}\text{C}:^{15}\text{N}$ ratio values for LDH and PGM are similar in that they closely follow their own bimodal ^{14}C specific activity labelling patterns. This could be due to differences in the composition of their precursor pools as outlined for CPK. Alternatively it may be due to hormonal influences on the rate of uptake of labelled amino acids and the rate of transamination. These hormones may be controlled by diurnal rhythms (see section 5.5.2). It may be that each enzyme is affected differently producing the patterns shown in figure 4.10.1.

5.7 Summary of conclusions

In summary, the results obtained from this study suggest a system in the growing breast muscle of broiler chickens which retains the tracer ^{14}C over the experimental period. In addition, some of the ^{15}N appears to be specifically removed from the muscle during this time. It has been suggested that the lighting regime in this study may have affected the birds feeding behaviour and in turn the levels of some hormones known to influence protein metabolism, namely, triiodothyronine, insulin and corticosterone. It may be that diurnal rhythms explain the bimodal labelling patterns observed for purified aldolase and P.G.M.

A more tentative theory has also been proposed: it is suggested

that some individual proteins of chicken breast muscle might exist as distinct populations within the muscle and that they are synthesised from individual amino acid precursor pools. It is also proposed that only a fraction of each protein takes up the label, thereby implying that over the experimental period a large proportion of muscle protein is not being turned over.

6. Suggestions for further work

This thesis set out to answer too many questions. If anyone wished to repeat this experiment they would be wise to concentrate on the main area, namely the isolation and determination of ^{14}C specific radioactivity of individual sarcoplasmic proteins. If this was done one could increase the number of proteins isolated giving a more representative sample of sarcoplasmic protein (~80-90%). One could also increase the number of animals used at each time to, for example, 10 birds per interval and increase the number of time intervals within the 70-80 hour experimental period. This should give a much clearer picture of the ^{14}C specific radioactivity labelling pattern.

To cut down on the number of cues which might possibly initiate diurnal rhythms, it would be advisable to rear the birds in the fume-cupboard in which the experiment is to be carried out and to have constant lighting throughout. It might also be useful to measure plasma triiodothyronine, and perhaps plasma insulin, over the experimental period to see if changes in their concentration reflect the ^{14}C labelling patterns of isolated proteins.

Additional work could also be done, perhaps as part of a post-doctoral programme, which should aim to look at the system in more

detail. Amino acid analysis followed by scintillation counting could be carried out on the plasma and intracellular amino acid pools. This may determine whether the ^{14}C remains within leucine or is present in other amino acid. It would also give a more accurate estimate of the plasma and intracellular free amino acid ^{14}C radioactivity. In addition isolated proteins could be hydrolysed and their amino acids separated and counted for ^{14}C giving a more precise ^{14}C specific radioactivity value.

If this line of investigation proved interesting one could then go back and look at the metabolism of the ^{15}N labelled leucine and compare the two.

Appendix

7. Appendix

The use of 1-[¹⁴C]-glycine as a tracer for chicken breast muscle protein metabolism

Introduction

Commercially bred chickens grow extremely rapidly. There has been speculation that for the first few weeks at least there is little turnover of proteins but only new synthesis (cf the ¹⁴C specific activity curve for myofibrillar proteins, shown in fig. 6). In mammals, cytoplasmic proteins are known to turn over more rapidly than myofibrillar proteins (Bates and Millward 1983), but little work has been done on this in birds, although short term ¹⁴C incorporation studies have been made (Leberz et al 1982). The present study has looked at specific activity changes in selected cytoplasmic proteins of breast muscle from 3 week old chickens of a commercial broiler strain; following a single injection over a 78 hour period of 1-[¹⁴C]-glycine.

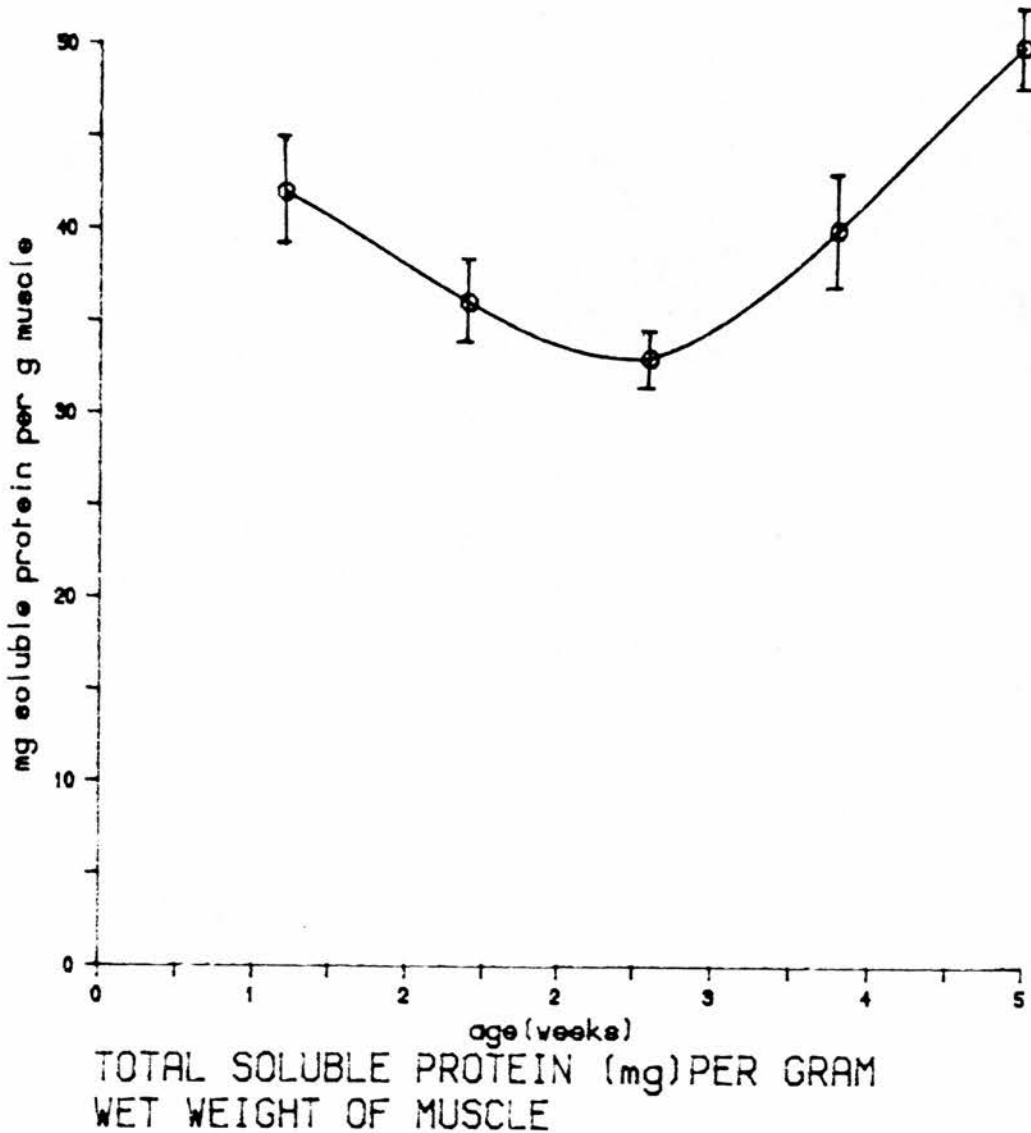
In this experiment there was a pause in weight gain by the birds (see fig. 1). This may have been due to the trauma of the experiment, which involved housing the birds in a fume cupboard and keeping 3 birds in each cage.

The fact that these birds were not "growing" proved to be advantageous, since mathematical analysis of the results is simplified if the system(s) are in the steady state (see Zilversmit et al 1943).

Methods

The birds were housed in perspex cages, 3 per cage, which were placed in a fume cupboard, and were injected intravenously with

FIGURE 1



Breast muscle homogenate in 50mm phosphate buffer pH7.5 was centrifuged at 12,000g for 10 min. The supernatant was assayed for protein by the method of Lowry et al (1951). Each point represents the mean, with error bars corresponding to \pm the standard deviation. 3 birds were used for each time interval.

50 μ Ci 1-[14 C]-glycine, dissolved in 1ml of saline. Three birds were killed at each time interval (i.e. 2, 6, 12, 24, 48 and 78 hours). Blood was removed by cardiac puncture into EDTA-coated tubes, and centrifuged. Breast muscle samples were rapidly excised and frozen in liquid nitrogen. 10g samples of muscle were later homogenised and cytoplasmic proteins separated and purified by methods based on those of Lebherz et al (1982).

Results

It has been assumed that the plasma pools of glycine and serine equilibrate rapidly. The specific activities quoted are therefore dpm/ μ mol (glycine + serine) for both plasma and protein.

It was not possible to purify all of the proteins to homogeneity within the time allocated to this experiment. It is therefore impossible to present complete evidence that each labelling pattern is as indicated by the curves shown. This experiment was intended as a pilot study to give some indication of the 14 C specific activity patterns over the 78 hour experimental period. In addition, optimal time intervals were then predicted which could be used for the more definitive experiment.

Taking into account the limited aims of this project, some unexpected and interesting data have nevertheless been produced. It appears that enolase and PGM (figs. 2 and 3) show single peak labelling patterns, while CPK and aldolase appear to have double peak 14 C specific activity curves (figs. 4 and 5).

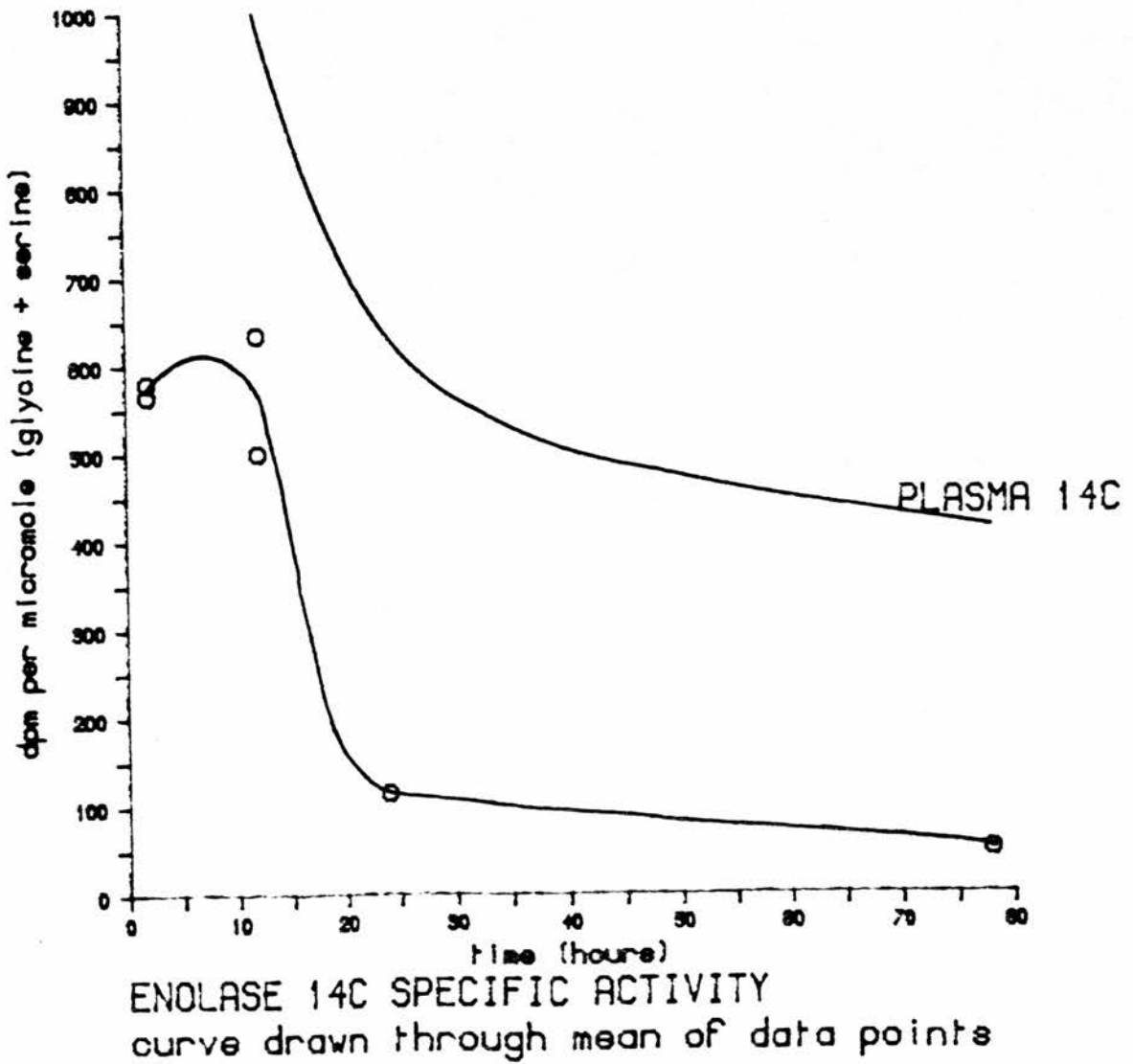
The myofibrillar fraction takes up very little label and does not appear to lose any label throughout the experimental period (fig. 6). The most striking feature from all the protein 14 C

specific activity curves, (except perhaps that of PGM) is that none are intersected by the plasma precursor ^{14}C specific activity curve (figs. 2 - 6 and fig. 7). An intersection would be expected if the plasma amino acid pool and the muscle protein pool follow a simple precursor-product relationship like that predicted by Zilversmit et al (1943).

Conclusions

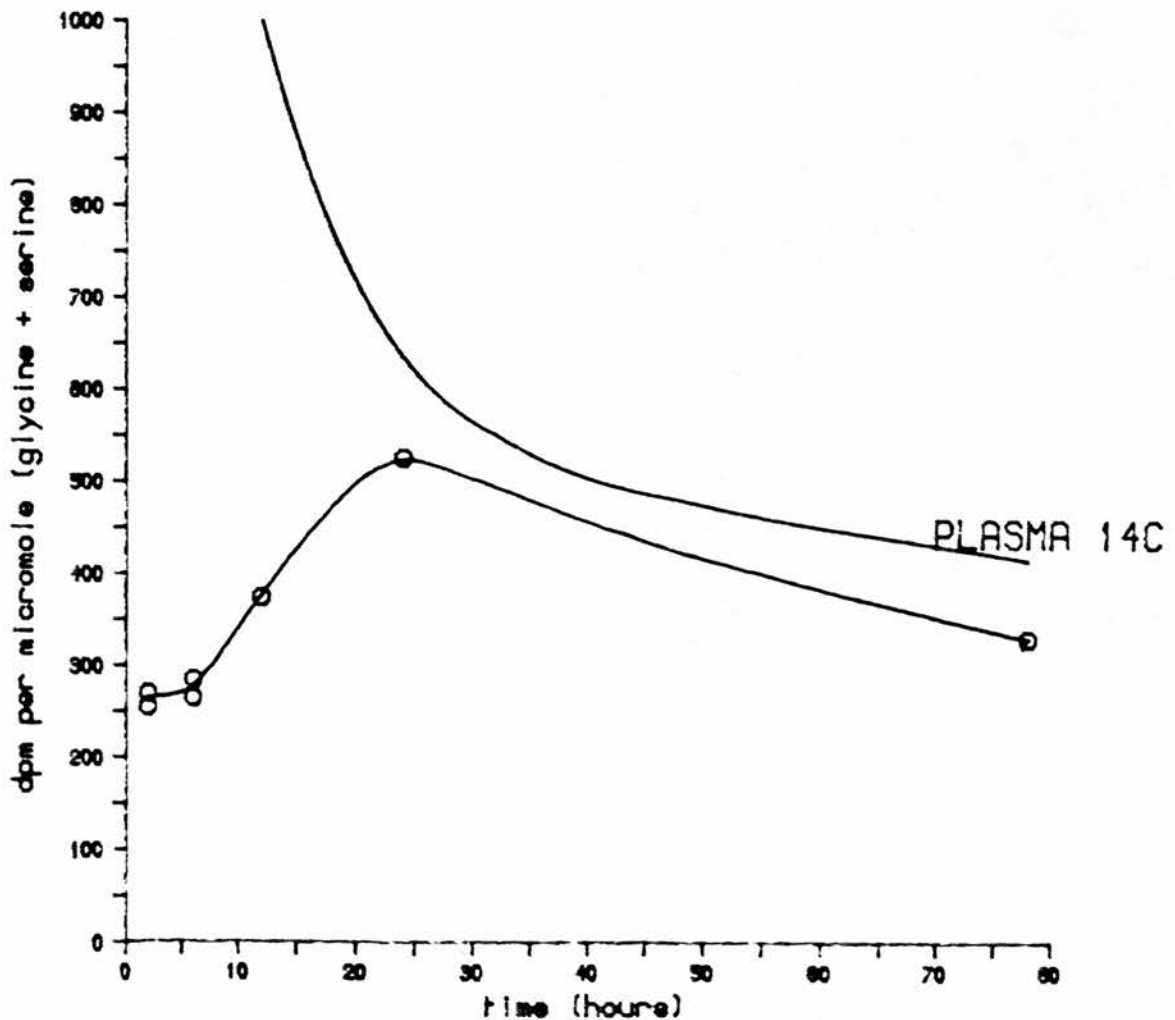
Although the data from this experiment are incomplete it has been shown that even in a "steady state" system the ^{14}C specific activity curves for amino acid precursors and protein products do not follow that predicted by Zilversmit et al (1943). This may be due to some form of compartmentation. In addition it appears that ^{14}C -labelled amino acids taken up by the myofibrillar proteins are not released, at least within the duration of this experiment.

FIGURE 2



The ^{14}C specific radioactivity of enolase which was purified from chicken breast muscle after pulse labelling with $[^{15}\text{N}]$ and $[^{14}\text{C}]$ labelled glycine. Each point represents the specific radioactivity derived from a single bird calculated from the amount of glycine + serine in enolase and the amount of enolase in 1g of muscle. The plasma ^{14}C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 7.

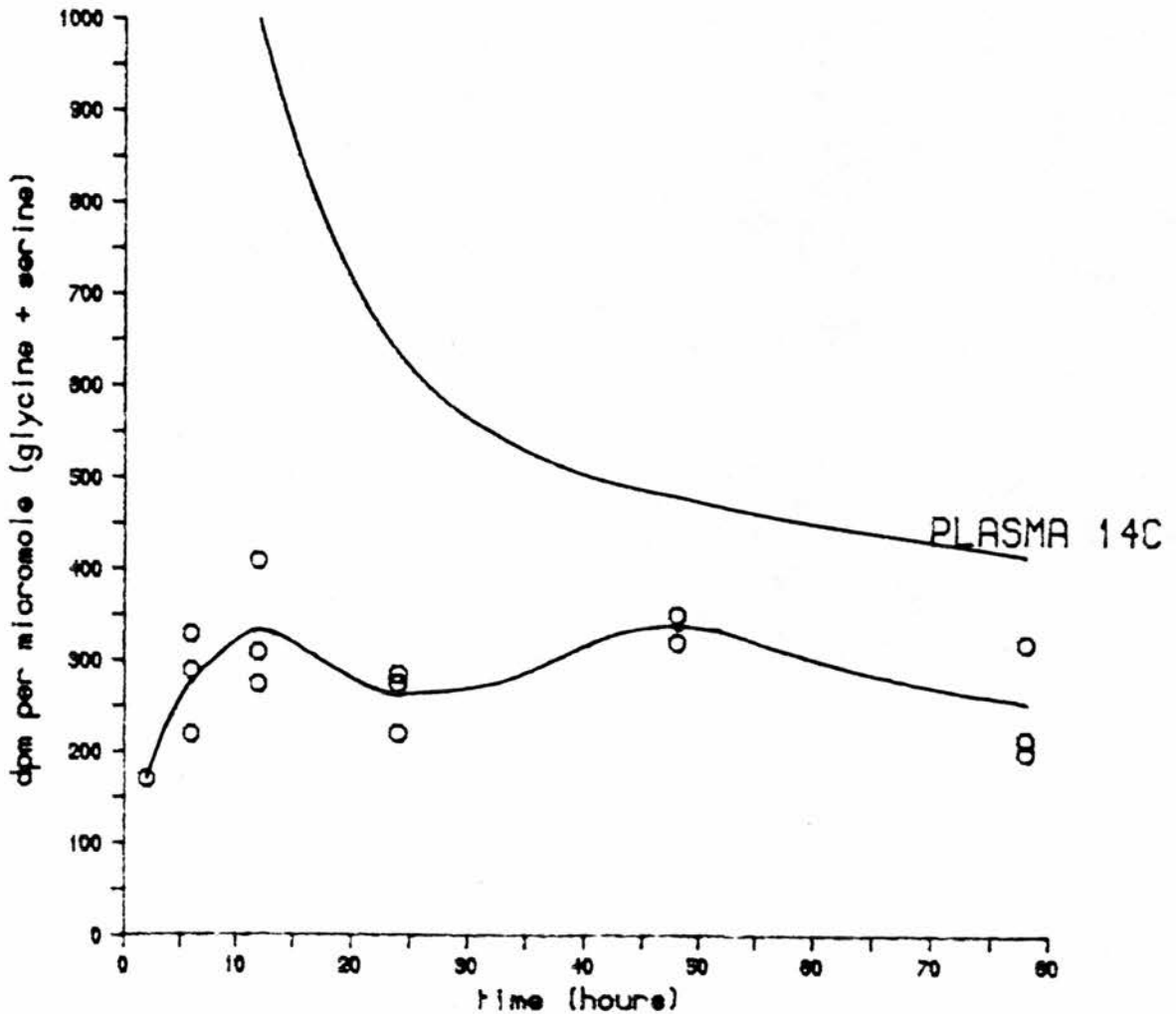
FIGURE 3



P.G.M. ¹⁴C SPECIFIC ACTIVITY
 curve drawn through mean of data points

The ¹⁴C specific radioactivity of PGM which was purified from chicken breast muscle after pulse labelling with [¹⁵N] and [¹⁴C] labelled glycine. Each point represents the specific radioactivity derived from a single bird calculated from the amount of glycine + serine in PGM and the amount of PGM in 1g of muscle. The plasma ¹⁴C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 7.

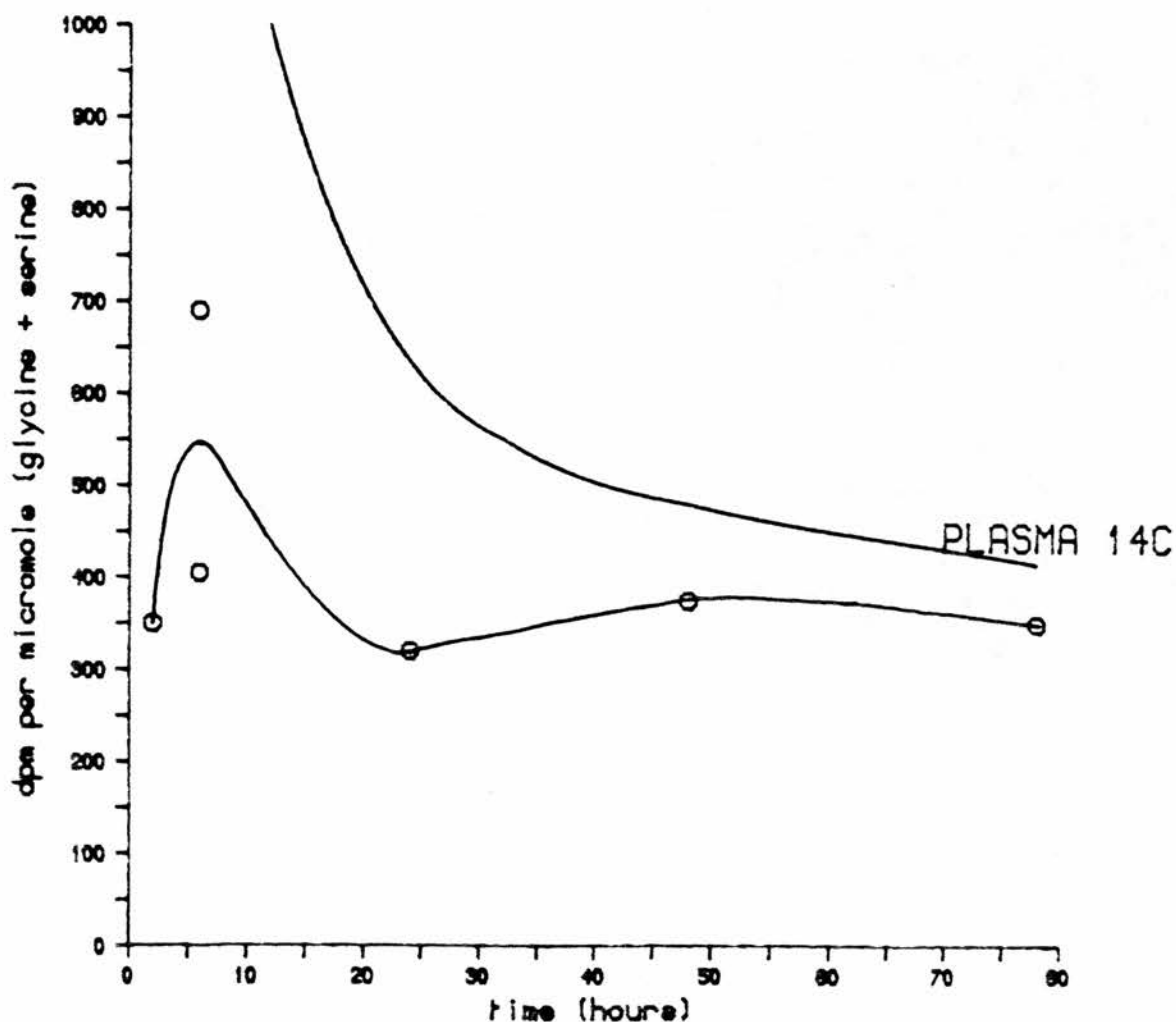
FIGURE 4



C.P.K. ^{14}C SPECIFIC ACTIVITY
 curve drawn through mean of data points

The ^{14}C specific radioactivity of CPK which was purified from chicken breast muscle after pulse labelling with [^{15}N] and [^{14}C] labelled glycine. Each point represents the specific radioactivity derived from a single bird calculated from the amount of glycine + serine in CPK and the amount of CPK in 1g of muscle. The plasma ^{14}C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 7.

FIGURE 5

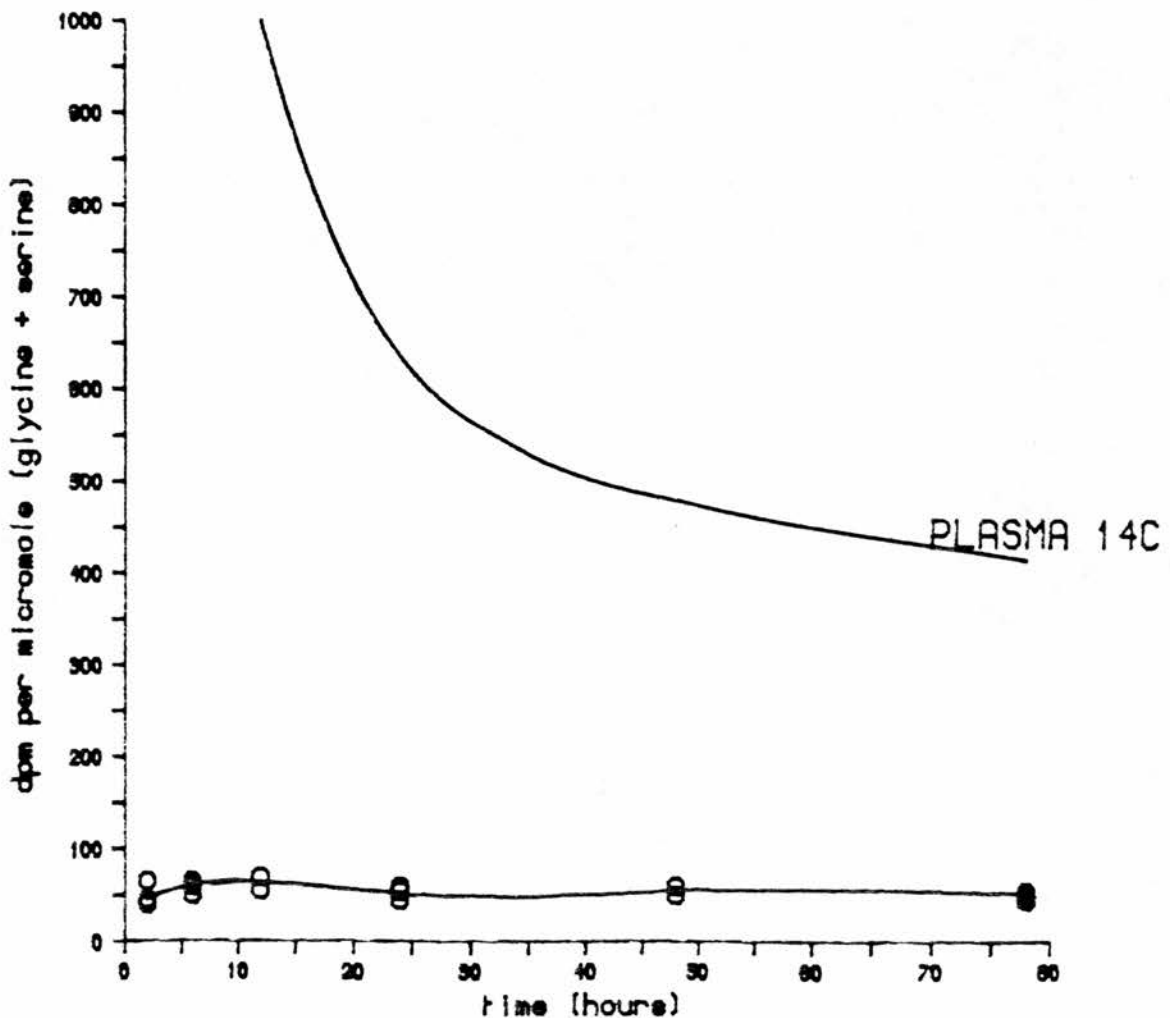


ALDOLASE 14C SPECIFIC ACTIVITY

curve drawn through mean of data points

The ^{14}C specific radioactivity of Aldolase which was purified from chicken breast muscle after pulse labelling with $[^{15}\text{N}]$ and $[^{14}\text{C}]$ labelled glycine. Each point represents the specific radioactivity derived from a single bird calculated from the amount of glycine + serine in Aldolase and the amount of Aldolase in 1g of muscle. The plasma ^{14}C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 7.

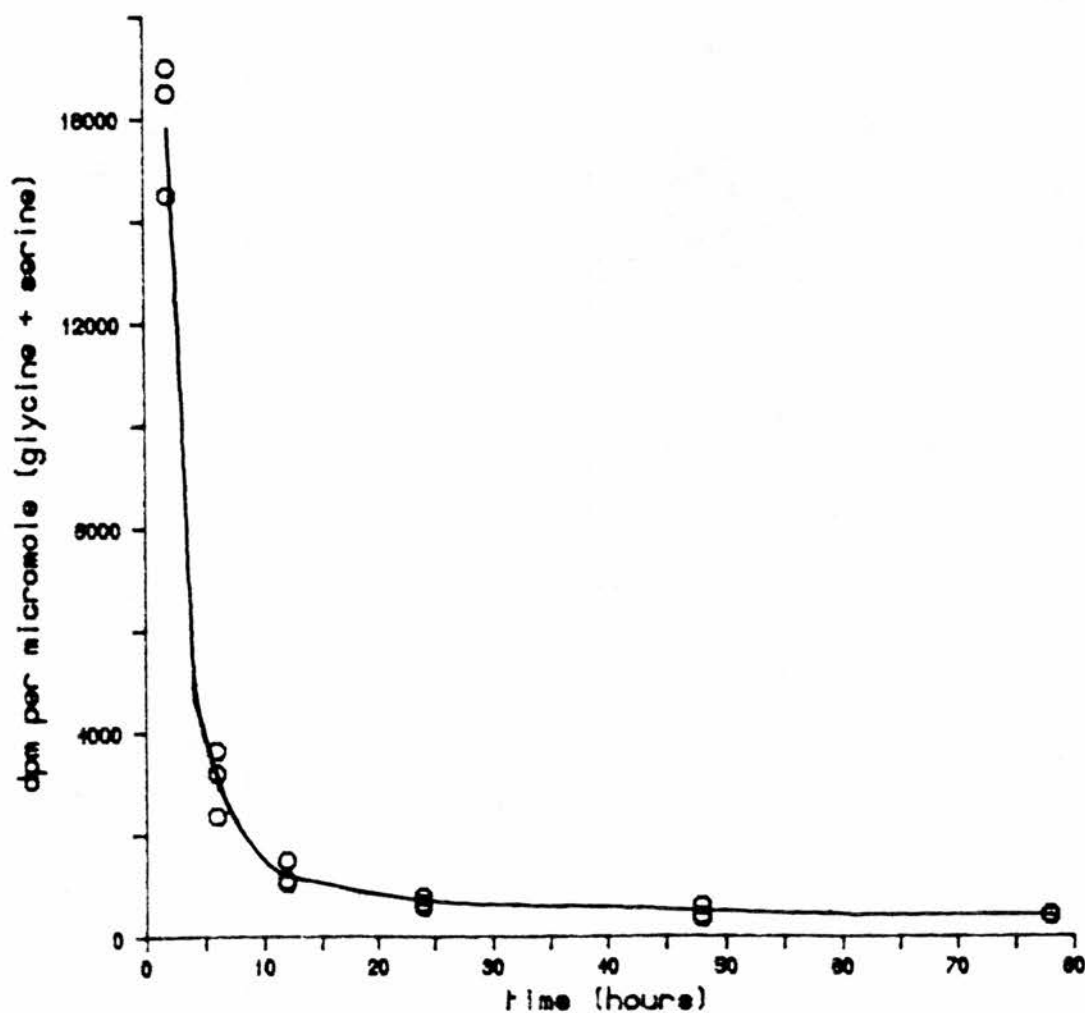
FIGURE 6



MYOFIBRILLAR 14C SPECIFIC ACTIVITY
 curve drawn through mean of data points

Chicken breast muscle homogenate in 50mM phosphate buffer pH7.5 was centrifuged at 12,000g for 10 min. The washed precipitate was counted and assumed to contain myofibrillar protein. Each point represents the specific radioactivity derived from a single bird calculated from the amount of glycine and serine in myofibrillar protein and the amount of myofibrillar protein in 1g of muscle. The plasma ^{14}C specific radioactivity is represented as an idealised curve derived from the data points presented in figure 7.

FIGURE 7



PLASMA ¹⁴C SPECIFIC ACTIVITY

curve drawn through mean of data points

The loss of label from plasma of chickens injected with a pulse of [¹⁴C] glycine. The dpm per micromole glycine and serine was determined in the 5000g supernatant from blood precipitated with 10% PCA. Each point represents the specific radioactivity calculated from a single bird.

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