THE NUTRITIONAL VALUE OF RYEGRASS - WHITE CLOVER LEAF PROTEIN CONCENTRATE

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University.

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1983

ERRATA

Incorrect Data

1. The energy intake values presented in Table VI p. 23 should read as 7218.0, 6079.8, 7550.9 and 6043.2 respectively and the energy values present in Table VIII p. 27 should read 9304.9, 9445.6, 9313.4 and 8384.2. The amount of cornstarch added in Diet L presented in Table XIII, Appendix A, p. 167 should read 4.20.

Typographical and Editing Errors

1. p. 65 Second paragraph - the phrase "soybean enzyme" should read "soybean enzyme inhibitor".

2. p. 163, Table IX Appendix A: The phrase "average phosphorus" should read "available phosphorus."

3. p. 196, Table XVI, Appendix B: The phrase "trypsinogen and chymotrypsinogen levels" should read "trypsin and chymotrypsin activities."

4. pp. 155,158,161 : The words lupin or lupins refer to lupin meal.

5. p. 154: The title "Ruakura Animal Research Centre" should read "Ruakura Agricultural Research Centre."

6. The reference Heywang, B.,C.R. Thompson and A.R. Kemmerer (1957) Poult. Sci., 38: 968 should read Heywang, B., C.R. Thompson and A.R. Kemmerer (1959) Poult. Sci., 38:968.

7. p. 199 The reference to Bondi et al. (1973) should read: Bondi, A., Y. Birk and B. Gestetner (1973) In: Chemistry and Biochemistry of Herbage (ed. Butler, G.W. and R.W. Bailey) Vol. 1, Academic Press, London, p. 511.

8. The following typographical/spelling errors should be noted:
   comparitive (comparative) p. 53
   titerperoids (triterpenoids) p. 67
   nutrtional (nutritional) p.70
   interum (interim) p. 134
   proteloytic (proteolytic) p. 143
   Tokoyo (Tokyo) p. 154
   glysine (glycine) p. 155, 158
   mamalian (mammalian) p. 204
   Olfield (Oldfield) p. 69
   Schleine (Scheline) p. 81

9. p. (ii) The phrase " ... estimates for the LPC diet was ..." should read ".... estimates for the LPC diet were ..."

   p. 21 The sentence "The results of Trial 1 were summarised in Table V." should read "The results of Trial 1 are summarised in Table V."

10. The following headings should read:-
    p. 52 Effect of Pelleting, Methionine on Increasing Inclusion Levels of LPC (15%, 20%) on Chick Growth.

    p. 54 Effect of Pelleting, Methionine Supplementation and of Different Levels of LPC (15%,20%) on Chick Growth.

    p. 113 Comparison of the "Corrected"(CAAA) and Apparent (ApAAA) Amino Acid Availability of LPC and SBM Ingredients and of Diets Containing LPC and SBM.

    p. 123 Comparison of Amino Acid Digestibility Measured at the Ileum with Amino Acid Availability Measured by Excreta Analysis.

11. The following words should read:-

    p-Nitroaniline (p-nitroaniline) p. 75

    Inhibitor (inhibitor) p. 144

    Cornstarch (cornstarch) p. 154

12. The symbol (G)* should be included on Table VII, p. 26 and Table IX, p. 32. Also the symbol (g) should be included on Table XXVI, p.131.

ABSTRACT

Leaf Protein Concentrate (LPC), which was manufactured from a mixture of Ryegrass and White Clover (Lolium perenne and Trifolium repens) at the Ruakura Agricultural Research Centre, was evaluated as a possible protein source for feeding to chickens. The nutritional value of LPC was compared to that of soybean meal (SBM).

LPC was shown to have a lower nutritional value than SBM in the growth trials. The addition of methionine or cystine to the diet containing LPC improved both food utilisation and weight gain of the chickens. These growth parameters showed the greatest improvement when 2 g methionine/kg diet was added to the LPC diet. The additional quantity of 2 g methionine/kg diet was similar to the amount of sulphur amino acid contributed by LPC to the diet; 1.8 g sulphur amino acid from LPC/kg diet. When an equivalent amount of cystine (1.6 g) to methionine, on a sulphur basis, was added to the LPC diet and fed to chickens, it was shown to support the same amount of growth and maintain a similar food utilisation level as additional methionine. LPC contributed only 0.6 g cystine/kg of diet. As this was much lower than the added cystine and/or methionine, it was concluded that the availability of cystine in the whole diet was reduced by the presence of LPC rather than the lack of availability of cystine in LPC alone.

The following information was also obtained:-

(i) Pancreatic hypertrophy and increased pancreatic enzyme activity (trypsin and chymotrypsin) occurred due to feeding the LPC diet.

(ii) The in vitro exhaustive enzyme digestibility study indicated that while the overall digestibility of LPC was approximately 6% lower than that of SBM, none of the individual amino acid digestibility estimates in LPC diverged markedly from the mean. All LPC amino acids were released equally by enzyme hydrolysis.

(iii) In contrast to the *in vitro* findings, the *in vivo* mean amino acid availability estimates for the ingredient LPC (as measured in the excreta) were lower than the corresponding SBM estimates by approximately 15%. The cystine availability estimate for the ingredient LPC was only 51.2% in terms of corrected amino acid availability (CAAA), and 11.9% in terms of apparent amino acid availability (ApAAA). By comparison the cystine availability estimates for the ingredient SBM were 80.8% CAAA and 75.7% ApAAA. When the diets containing LPC or SBM were assayed by the same technique, the differences in the amino acid availability estimates were markedly reduced. The availability estimates of cystine in the LPC diet were still lower than the other amino acid availability estimates for the LPC diet. These however were only 8-10% lower than the corresponding estimates for the SBM diet.

(iv) The mean amino acid digestibility estimates, derived by analysis of the ileal contents of chickens fed with the LPC diet were 26% lower than those for chickens fed the SBM diets. The cystine digestibility estimates for the LPC diet was approximately 45% lower than the corresponding cystine digestibility estimate for the SBM diet. These results indicated that digestion and/or absorption of the LPC diet was probably being retarded as compared with the SBM diet.

(v) Supplementation of the LPC diet with the antibiotic, Neomix, gave an improvement in growth and an increase in the mean amino acid availability (measured by excreta analysis) of approximately 7%. This indicated that the gut microflora were influencing the nutritional value of LPC.

Feeding the LPC diet in comparison to feeding the SBM diet also tended to increase the level of C_{19} cyclopropane fatty acid in the excreta. This indicated that feeding the LPC diet was influencing the nature and/or activity of the microfloral population.

The physiological and metabolic effects of feeding raw soybean meal and/or trypsin inhibitors, which have been reported in the literature, included pancreatic hypertrophy, increased pancreatic proteolytic enzyme activity, retardation of ileal protein digestibility and an influence by gut microflora. Each of these factors were characteristic of chickens fed the LPC diet. It was therefore concluded that the additional need for cystine or methionine by chickens fed the LPC diet, was due to the presence of trypsin inhibitors in the LPC.

It was demonstrated, by feeding L-(methyl ^{14}C) methionine that phenolic compounds were being methylated. However the need for detoxification of aromatic compounds, which required methionine (as a methyl donor) and/or arginine (ornithine), could not explain the growth depression experienced by chickens fed the unsupplemented LPC diet.

The feeding of L-(methyl ^{14}C) methionine in conjunction with the LPC diet also indicated that the digestibility of methionine was not being hindered during the digestive process by preferential binding with other compounds in the LPC diet.

It was concluded from the results of this study that LPC adequately supplemented with methionine, could be a useful addition to the range of ingredients available for use in poultry feeds.

ACKNOWLEDGEMENTS

I would like to thank my Supervisors Dr. M.N. Wilson and Mr. M.R. Patchell for their interest and co-operation during this study. In particular I wish to express my appreciation to Dr. S. Bornstein for his encouragement during the early stages of this study and to Dr. D.R. Husbands and Dr. J.C. Hawke for their helpful advice and assistance.

I am also indebted to Dr. P.E. Donnelly and his team at Ruakura Animal Research Centre for supplying the Leaf Protein Concentrate and to the Poultry Research Centre staff for their help in both the management of the trials and in the analysis of the amino acids.

Special thanks is also given to my wife for all her work typing this thesis and for the encouragement given.

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APPENDIX A: Summary of Tables continued:

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LIST OF ABBREVIATIONS

AME	Apparent Metabolisable Energy
ApAAA	Apparent Amino Acid Availability
ApAAD	Apparent Amino Acid Digestibility
Arginine	L-Arginine (used to supplement diets)
CAAA	Corrected Amino Acid Availability
Histidine	L-Histidine (used to supplement diets)
Lysine	L-Lysine. HCl (used to supplement diets)
LPC	Leaf Protein Concentrate
Methionine	DL-methionine (used to supplement diets)
N.S.	Not Significant
PVP	Polyvinyl pyrollidone
SBM	Soybean meal
SEM	Standard Error of the Mean
TLC	Thin-layer Chromatography
TME	True Metabolisable Energy

SECTION 1

CHAPTER 1: INTRODUCTION

Since the 1920's the nutrient requirements of chickens have been identified one by one so that today some 40 nutrients are recognised as being essential for optimum growth and development of the chicken (Scott et al., 1969). The development of this knowledge, together with major advances in the fields of genetics and veterinary medicine plus complimentary work by engineers working with physiologists and producers, has led to the growth of large intensive egg and meat chicken industries in many parts of the world.

Although much is known about the nutritional requirements of the chicken much still remains to be learnt, to enable the industry to capitalise on the continued genetic improvement. Not only is a detailed and precise knowledge of the chicken's nutrient requirements necessary but an adequate knowledge of the nutrient levels, interactions and inadequacies of various feed ingredients is required to increase the efficiency of intensive livestock farming.

In 1977 Ryan, speaking on behalf of the N.Z. Feed Compounding Industry, indicated that to feed the layer population of 4.8 million and the meat chicken population of 18 million, 183,000 tonnes of layer chicken feed and 80,000 tonnes of meat chicken feed would be required. Between 1977 and 1982 the number of layers declined to 3.5 million whereas meat chicken production increased to 33 million. In addition, the nation's pig industry has changed from one which relied on large volumes of liquid by-products from the dairy industry to one which uses meal diets similar to those employed in poultry production. The pig therefore now actively competes with the chicken for available feed ingredient supplies.

New Zealand has problems with feed ingredients peculiar to itself. Compared to the Northern Hemisphere, New Zealand has only a limited supply of soybean meal and fishmeal. Both these products are high in protein and the protein is nutritionally balanced. New Zealand's protein sources are largely animal by-products such as meat and bone

meals. These by-products are variable in protein quality, tend to be low in isoleucine compared to the level in soybean meal and fishmeal and some have substantial levels of calcium and phosphorus present. These minerals are important in the nutrition of the chicken, but balancing the amino acid, phosphorus and calcium requirements in feed formulations is more difficult when these components are "tied up in the same package."

In most countries of the world either synthetic methionine or synthetic lysine are used to supplement poultry rations. The former is usually the first limiting amino acid when soybean meal is available and the latter when either cotton seed, ground nut or sunflower are the major protein source in a feed (Bornstein, 1977). The large market available to synthetic forms of methionine and lysine enables them to be produced at economic prices. Bornstein (1977) concluded that the first two limiting amino acids in New Zealand poultry rations are methionine and isoleucine. Since synthetic methionine is available, the main economic problem is the inclusion of adequate levels of isoleucine which is not available in synthetic form at a reasonable price. The feed materials available in New Zealand are predominantly meatmeals. These tend to be low in isoleucine, as shown in Bornstein's (1977) data reproduced in Table I, compared to vegetable protein sources. It is therefore important that new vegetable feedstuffs be investigated. There is also a need for an increase in the total supply of feedstuffs. In 1978 Patchell forcasted that if the production of meat and bone meals in New Zealand did not increase by 10% per year, home production of meals would be insufficient to meet the feed requirements of the poultry and pig industries in New Zealand by 1985.

Leaf Protein Concentrate: Fresh green leafy plants are excellent sources of the protein, energy, vitamins and other nutrients needed by monogastrics including humans. The majority of the essential amino acids consumed by monogastrics as plant or animal food are

Table I: The Lysine and Isoleucine Content of Various Feed Ingredients (Bornstein, 1977)*

Ingredient	Protein Content (%)	g amino acid/100 g protein	
		Lysine	Isoleucine
Maize	9.0	3.2	3.2
Barley	11.0	3.7	3.0
Wheat	12.0	3.3	3.5
Wheat Bran	15.0	4.0	2.6
Pollard	15.0	4.3	3.1
Brewers' Grain	16.5	5.9	5.8
Lucerne Meal	17.0	6.0	4.2
Pea Meal	22.0	6.8	4.3
Lupin Meal	26.5	4.8	4.9
Soybean Meal	50.0	6.1	4.2
Meat & Bone Meal	50.0	5.0	2.4
Fish Meal	51.0	8.0	4.2
Liver Meal	71.0	5.9	3.0
Blood Meal	87.0	8.6	0.8
Leaf Protein Concentrate	48.0	6.3	4.2

* Based on Payne (1976) and Anderson and Warnick (1969)

derived from amino acids initially synthesised in the leaves of growing plants. The presence, however, of crude fibre in leaves prevent their direct utilisation as a major source of food protein by non-ruminants (Kinsella, 1970).

Osborne and Wakeman in 1920 stated:

> "If we can learn to separate the contents of the plant cell from the cell wall and water we shall obtain a food product of great value."

The pioneering work of Pirie (Morrison and Pirie, 1961; Pirie, 1963; 1966) demonstrated that the extraction of leaf protein concentrate (LPC) was practical. The methods used in extraction and partial purification of LPC have been reviewed by Morrison and Pirie (1961) Pirie (1963, 1966, 1971, 1978), Byers and Sturrocks (1965) and Chayen et al.,(1961).

In 1967, Allison, working at Lincoln College, proposed that research into techniques for the production of edible protein from green leafy materials should begin in New Zealand (Hove and Bailey, 1975). By 1977, a team at the Ruakura Agricultural Research Centre had developed a large pilot plant to extract protein from pasture which was surplus to other requirements at favourable growth periods of the year. It was hoped that the residue of herbage fibre from the juice extraction could be fed to ruminant animals. Reports have shown that lucerne residue was satisfactory for feeding to sheep (Vartha and Allison, 1973) and cattle (New Zealand Poultry World, 1980). The pilot plant was capable of processing 800-1000 kg of fresh grass or lucerne per hour and of yielding 18-20 kg LPC per tonne of raw material.

The nutritional value of LPC for use in animal diets has been extensively reviewed by Woodham (1971), Morris (1977) and Pirie (1978). Woodham (1971) concluded that the safety of feeding LPC to monogastric animals was proven and nutritional studies since 1971 have confirmed his work (Morris, 1977).

Table II: The Amino Acid Content [g (amino acid)/ 16 g N] of Leaf Protein Concentrate (LPC) and Soybean Meal(SBM)

Amino Acid	General LPC[1]	Ruakura LPC[2]				Soybean Meal[3]
		Ryegrass	White Clover	Ryegrass-White Clover	Lucerne	
Isoleucine	4.5 - 5.5	4.6	4.7	4.6	4.6	5.2
Leucine	8.8 - 10.2	8.0	8.6	7.8	8.4	6.4
Lysine	5.6 - 7.3	5.7	6.0	5.4	6.1	6.4
Methionine	1.6 - 2.6	2.4	2.1	2.3	2.1	1.4
Phenylalanine	5.5 - 6.8	5.8	5.7	5.3	5.8	5.4
Threonine	4.7 - 5.8	4.8	5.4	4.8	5.0	4.0
Tryptophan	1.2 - 2.3	2.5	2.5	2.5	2.5	1.3
Tyrosine	3.7 - 4.9	4.4	4.8	4.1	4.7	4.0
Valine	5.9 - 6.9	5.2	5.8	5.2	5.4	5.4
Arginine		6.5	6.5	5.7	6.1	7.6

[1] From Byers, (1971): The range of amino acid analyses on 56 unfractionated leaf proteins made from 31 species of plants

[2] From Donnelly and McDonald (1978)

[3] From Scott *et al.* (1969) Soybean Meal (50%) dehulled

Values of the amino acid content for several LPC preparations reported in the literature are summarised in Table II and are shown to be similar to the values for soybean meal (SBM). Morris (1977) stated that biologically, the first limiting amino acid in LPC's is "methionine or cystine or the total of these two," and if adequately prepared, LPC is of high biological value (Byers, 1971). However, the nutritional value of the product is variable, even when it is produced from the same plant species (Woodham, 1965). Improper processing has produced variable nutritional results, so that care in interpretation of such results needs to be taken. Pirie, a long-time worker in the field of LPC production has commented:-

> "... the results of feeding experiments are now of little interest unless precise information is given, not only about the species from which the LPC was made, but more significantly about the details of the separation procedure and method of preservation or drying. As the preceding sections show, it is easy to damage a protein by inept handling; poor nutritive value is probably more often the result of technical incompetence on the part of the processor than of synthetic inconsiderateness on the part of the plant. Nevertheless I have often been amazed at the good results claimed with some products which I knew, having seen the techniques used, to be heavily contaminated or damaged." (Pirie, 1978)

Pirie (1978), Morris (1977) and Donnelly (1980) have reviewed the influence of processing variables on the quality of LPC. These factors include the rate of processing, pH of extraction, heat of coagulation and heat of drying. The Ruakura process (described in the Materials and Methods Section)was developed to meet the operational and economic requirements which would allow it to fit into the farming scene as an adjunct to animal grazing enterprises (Donnelly, (1980). The purpose of this present study was to evaluate the nutritional value of the unique Ruakura product within the limits of restricted supply.

The LPC supplied by Ruakura was a dark green, powdery (spray-dried) product with a crude protein content of about 46% (air-dried) (N x 6.25), and was produced from Ryegrass-White Clover pasture (Lolium perenne and Trifolium repens). It was supplied to the Poultry Research Centre in several batches, the analysis of which is summarised on page 12. The LPC batches were prepared specially for the Poultry Research Centre. Processing conditions were maintained as standard as possible though some variation in quality was unavoidable e.g. seasonal variation in pasture growth.

This thesis describes work carried out to assess LPC in terms of its nutritional properties using chickens. The research work consisted in the first place of a number of independent experiments or growth trials. The results of these early experiments, plus the limitation of the LPC supply, determined the design and extent of further trials. For clarity of presentation each group of experiments is described and evaluated in sequence. Because the methods used vary greatly between experiments a Materials and Methods section has been included in the description of each experiment.

Initially three preliminary chick growth trials were carried out to define the general nature of possible problems associated with the use of LPC in practical formulated diets e.g. appetite depressing factors, toxicity and utilisation by the chicken for growth These growth trials are described in Chapter 3. The preliminary trials were followed by four more growth trials which studied more specifically the factors that affect the utilisation of LPC. This was done by supplementing LPC diets with lysine, methionine, cystine and arginine and also by pelleting LPC diets. These trials are described in Chapter 4.

The information obtained in the growth trials was examined further by chemical and biochemical studies to explore the reasons for the observed nutritional effects. These studies included digestibility studies; the feeding of a radioactive marker compound and

subsequent analysis of the compounds excreted; pancreas and pancreatic enzyme studies; and studies into the possible influence of the gut microflora. These are described in Chapters 6 and 7 and the overall conclusions are discussed in Chapter 8.

CHAPTER 2: MATERIALS AND METHODS FOR GROWTH TRIALS

This chapter describes the Materials and Methods used in Growth Trials 1 to 7. The details of the management of chicks etc. applies also to the experiments described in later chapters which involve the use of chicks.

a) Leaf Protein Concentrate

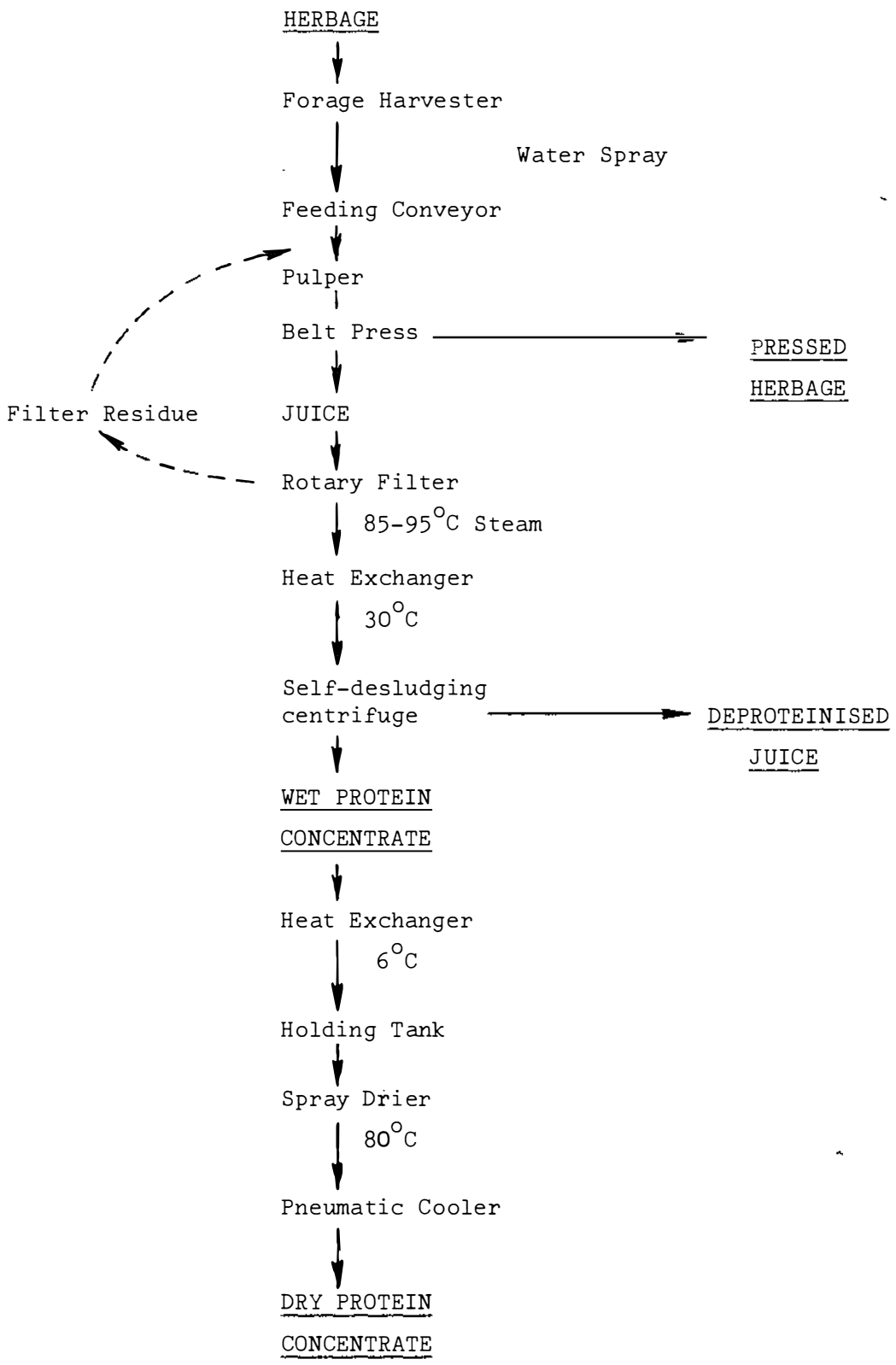
All ingredients except LPC were supplied by the Feed Processing Centre, Massey University or as listed in Appendix A, page 154. The LPC supplied by Ruakura Agricultural Research Centre was produced from Ryegrass-White Clover pasture (*Lolium perenne* and *Trifolium repens*). Reference to LPC in the present work refers to Ryegrass-White Clover LPC. The LPC was prepared by the method as described below and by Donnelly (1980).

The basic procedure for the production of LPC at the Ruakura Agricultural Research Centre (shown in Fig. 1) was described by Donnelly and McDonald (1978) as follows:-

"1. Harvesting the herbage with a forage harvester.
2. Wetting the herbage to 10% dry matter and pulping in a rotary pulper.
3. Expressing juice with a belt press.
4. Coagulating protein in the juice by in-line steam injection to raise the temperature to 80-90°C.
5. Partial dewatering of the juice to approximately 15% dry matter with a self-desludging centrifugal separator.
6. Drying of the protein coagulum by a spray process."

The analyses of the batches of LPC supplied to the Poultry Research Centre are summarised in Table III. The analysis of the SBM used in this work is summarised in Table IV.

Fig. 1: Extraction and Processing System Used At The Ruakura Agricultural Research Centre (Donnelly,1980)



b) Diets used to evaluate the nutritional value of LPC

Two types of diet design were used to compare the ingredients LPC and SBM in this study:

(i) Compounded diets

(ii) "Basal" + "Test" Ingredient (SBM or LPC)

In the preliminary trials (1-3) the experimental diets (type i) were compounded diets. A compounded feed is formulated using a list of nutrient requirements and ingredient gross nutrient values. The specified nutrients in the diets are present at the required concentration but no regard to the source of the nutrients is made. For this reason the use of compound diets has the limitation that an unspecified ingredient or a particular level of an unspecified ingredient may be present in one test diet but not in another. Therefore if several of these diets are used, it is not possible to compare directly the effect of LPC or SBM alone. The purpose of using compounded diets in trials 1-3 was to indicate any "practical problems" due to using LPC in a formulated diet, as LPC would ultimately be used in commercially compounded diets. These "practical problems" could then be studied more "directly" in later trials.

In the direct comparison of the effects of LPC or SBM in a diet (type ii) (Trials 4-7) a procedure similar to that of the Total Protein Efficiency (TPE) method of Woodham (1968) was used. This method was designed to test various protein sources under "practical-type" conditions. In Woodham's method, twelve percent of the test dietary protein was supplied by the test supplement and the remainder by a basal containing cereals and yeast. Such a procedure was preferred to the classical protein evaluation methods such as the Protein Efficiency Ratio method, as these exclude any potentially useful complementary effects which may be present when mixed diets are fed (Hackler, 1977).

Table III: Analysis of Leaf Protein Concentrates Supplied During the Period of this Study

	Batches				
	1	2	3	4	5
Energy kJ/g		12.49			12.10
Crude Protein %	46.52	42.78	48.75	38.94	40.30
Calcium %	0.94				
Phosphorus %	2.42				
Sodium %	0.05				
Chloride %	0.05				
Potassium %	0.89				
Fat %	11.60				
Ash %	12.92		9.50	13.48	
Crude Fibre %	1.40				
Cystine %	0.40	0.40	0.40	0.40	0.33
Threonine %	2.07	1.93	2.07	1.87	1.91
Serine %	1.84	1.70	1.90	1.74	1.79
Glycine %	2.46	2.24	2.49	2.27	2.27
Valine %	2.57	2.41	2.68	2.27	2.40
Methionine %	0.76	0.73	0.82	0.80	0.93
Isoleucine %	1.98	1.88	2.03	1.80	1.86
Leucine %	4.02	3.72	4.10	3.69	3.72
Tyrosine %	1.93	1.72	1.86	1.84	1.82
Phenylalanine %	2.58	2.37	2.69	2.32	2.41
Histidine %	1.02	0.90	0.97	0.88	0.90
Lysine %	2.72	2.93	3.01	2.43	2.45
Arginine %	2.74	2.32	2.93	2.44	2.68
Tryptophan % *		1.37			

* Analysed at the Ruakura Agricultural Research Centre

Table IV: Analysis of Soybean Meals Supplied During the Period Of This Study

	Batches			
	1	2	3	4
Energy kJ/g	11.72			11.63
Crude Protein %	50.0	49.0	53.0	45.2
Calcium %	0.30			
Phosphorus %	1.30			
Sodium %	0.01			
Chloride %	0.03			
Potassium %	2.00			
Fat %	0.80			
Ash %	5.00			
Crude Fibre %	3.00			
Cystine %	0.64	0.69	0.67	0.69
Threonine %	1.82	1.90	1.82	1.80
Serine %	2.48	2.49	2.48	2.44
Glycine %	2.10	2.01	2.10	2.29
Valine %	2.29	2.33	2.29	2.11
Methionine %	0.52	0.62	0.64	0.61
Isoleucine %	2.10	2.09	2.13	1.98
Leucine %	3.86	3.78	3.86	3.57
Tyrosine %	1.88	1.80	1.88	1.70
Phenylalanine %	2.46	2.49	2.40	2.33
Histidine %	1.22	1.16	1.22	1.29
Lysine %	2.91	2.93	2.91	2.88
Arginine %	3.51	3.57	3.51	3.63
Tryptophan %	0.60*			

* Theoretical Value (Scott et al., 1969)

In this present work a basal diet consisting of grain, soybean meal, synthetic lysine and cornstarch was formulated (Appendix A, Table XII) using the constraints given in Appendix A, Table XI. The constraints for amino acid content used in the formulation were taken as 50% of the recommended National Research Council (NRC, 1971) levels for meat chickens 0-6 weeks of age. The relationship between the amino acid values was slightly modified using the Hurwitz et al.,(1978) calculation for amino acid requirements for growing chickens. Experimental values from Trial 2 were substituted in the calculation in order to apply it to growing Sykes cockerel chickens.

The total protein level was maintained below 16% (N x 6.25), as Woodham (1968) found statistically, that the discrimination between different proteins was optimised at lower levels of supplementary protein. The "test proteins" contributed approximately 7% protein (N x 6.25) to the "test diets " when included on an equivalent isoleucine basis. The purpose of equalising the test proteins on this basis, was to not only reflect more closely the "practical conditions" experienced by New Zealand feed manufacturers, as discussed in Chapter 1, but this method was also considered to equate more closely the amino acid content of LPC and SBM. The test proteins, SBM and LPC were therefore incorporated into the basal diet at equivalent isoleucine levels in place of an equivalent amount of cornstarch to give the total test diet. The final formulation was such that in the final test diet, adequate levels of minerals, vitamins, linoleic acid and energy were present.

c) Chickens

Day-old Sykes cockerel strain chickens were used for all the growth trials. The chickens were either hatched and sexed at the Poultry Research Centre or supplied as day-old (already sexed) by Fairfield Hatcheries, Levin. Before being randomly assigned to battery brooders the chickens were individually wing-banded.

d) Management

The battery brooders were three tiers high, with each tier divided into four compartments. All compartments had their own feed trough, and a common water trough was shared between two adjacent compartments. Each tier of a battery had a common heating element across the four compartments. Each compartment held 14 chickens. Feed and water were supplied ad libitum with the feed weighed out at intervals to reduce spillage. A background wire grid was placed over food after the first week as a further measure to reduce spillage. Light was supplied for 23 hours a day except in Trial 1, where it was supplied for 16 hours a day. The light intensity was reduced after the first week to decrease chicken antagonism and toe pecking. A detailed daily diary of all activities was kept. The shed temperature was maintained at 21°C with supplementary heating. A thermostatically controlled fan was used when necessary. The temperature of the battery brooders was at 32°C for the day-old chickens and reduced progressively with age to 21°C.

Chickens were weighed individually on a Mettler P3 Balance, reading ± 1 g. A Mettler P3 Balance was used to weigh feed during the experimental periods. In the initial experiments (Trials 1-3) the experimental diets were fed from one to 28 days of age. This was modified in Trial 4 and subsequent trials to allow a preliminary period of one week on a commercial 20% crude protein (N x 6.25) "chick starter diet",before the three weeks on the experimental diets. The one week preliminary period was used to allow:-

(i) rejection of sickly or "runty" chickens;
(ii) reduction of the level of feed spillage (the inability of small chickens to easily reach the food meant that food had to be kept high up in the troughs for the first few days);
(iii) reduction of the carryover effect of the yolk sack;
(iv) reduction of the amount of LPC required for an experiment.

In a later experiment (Experiment 6) a modified battery brooder was used. The brooder was operated normally except that the size of each compartment which contained 4 chickens was reduced, so that there was a gap between compartments to avoid cross-contamination of the excreta during the collection period.

The individual cages used in Experiment 2 and 8 were modified stainless steel rat cages with individual water and feed troughs. The chickens used in these experiments were initially reared in the battery brooders. At 2 weeks of age the chickens were transferred to the modified rat cages. The chickens were then allowed a week to settle in their new environment before the experiment began.

e) Chemical Analysis

(i) Proximate Analysis:

Moisture, crude fat, ash and fibre were determined by methods of the Association of Official Agricultural Chemists (1960).

Protein (N x 6.25) was determined with a Coleman Nitrogen Analyser based on the Dumas method and minerals were determined with a Coleman Atomic Absorption Spectrophotometer, using standard techniques in each case.

(ii) Amino Acid Analysis:

Samples (125 mg) were hydrolysed in 150 mls 6 M HCl by refluxing for 24 hours at 130^{o}C in a nitrogen atmosphere. The solution was then filtered, reduced in volume on a rotary evaporator and made up to 25 mls with pH 2.2 sodium citrate buffer. The samples (0.1 - 0.5 mls depending on crude protein content) were analysed with a Beckman 120 C Amino Acid Analyser. All samples were hydrolysed and analysed in duplicate. If the duplicate results were outside the analytical limits of $\pm$ 3%, the samples were rehydrolysed and reanalysed. In the latter stages of this study two Beckman 120 C Amino Acid Analysers were available for use.

Both analysers gave comparable results except some difficulty was experienced analysing tyrosine in the excreta samples. Because tyrosine, by this stage was not considered to be influencing the experimental results, these excreta samples were not reanalysed to improve the estimates of tyrosine. Norleucine was used as an internal standard and was not added until the sample was applied to the column of the analyser.

(iii) Methionine and Cystine Analysis (Performic Acid Oxidation Method):

One millitre of 30% hydrogen peroxide was added to 9.0 mls of 88% formic acid and allowed to stand for 1 hour. The mixture (performic acid) was then cooled to $0^{o}C$ and used immediately. A sample (size dependent on expected cystine content) was weighed out in a flask, cooled in an ice-bath, and then 5 mls of performic acid was added for each 10 mg of sample. The flask was maintained at $0^{o}C$ for 16 hours after which time 0.75 mls of a solution of 5.8 M HBr in 5 mls of performic acid was added. The performic acid solution was then removed by rotary evaporation; 150 mls of 6 M HCl was then added to the sample and open flask hydrolysis carried out as described in (ii) above, except that the time for hydrolysis was 20 hours. The samples were then analysed with the Beckman 120 C Amino Acid Analyser.

f) Determination of Metabolisable Energy

The metabolisable energy of the foodstuffs was determined by using the True Metabolisable Energy (TME) method (Sibbald, 1976).

The metabolisable energy was calculated from the following formula:

$$\text{True Metabolisable Energy (TME) (kJ/g)} = \frac{100}{1} \times \frac{\text{Total gross energy fed (kJ/g)} - (\text{Energy excreted (kJ)} - \text{Endogenous energy excreted (kJ)})}{\text{Total feed (g) eaten}}$$

From the equation it can be seen that the following three energy values are needed for the calculation of the TME value of a feedstuff:

(i) The total gross energy of the feed which is fed to the cockerel
(ii) The total energy excreted by a fed cockerel
(iii) The total endogenous energy excreted.

The excreta of a starved cockerel was used to estimate the endogenous excretion level. The determination of the excreted energy values (ii) and (iii) both required the collection of excreta.

The TME method as described by Sibbald (1976) was modified slightly. Instead of using one group of adult cockerels as an endogenous control for the other groups of "test" cockerels, each cockerel was used as its own endogenous control.

Sixteen adult Sykes cockerels were housed in single bird cages in an environmentally controlled room. The cockerels were maintained on a 14% crude protein (N x 6.25) commercial chick grower diet during all non-experimental periods. Any effects, therefore, due to the maintenance diet were common to all metabolisable energy estimates.

Two separate experimental "feeding" periods of 24 hours each were carried out a week apart. Before each experimental "feeding" period, each cockerel was starved for 24 hours. In order to have an endogenous energy value for each individual cockerel a "cross-over" design was adopted. For each experimental "feeding" period two groups of 4 cockerels were starved for a further 24 hours for the estimation of endogenous energy. The excreta collection period was 24 hours after which the excreta were placed in individual pots and frozen.

The following week, during the experimental "feeding" period the order of the cockerels was reversed, i.e. the previously fed cockerels were now starved and the previously starved cockerels were force-fed with the relevant ingredient. The excreta collection period was again 24 hours. The frozen excreta was freeze-dried, ground, weighed and pooled for analysis by bomb calorimetry. The gross energy of the ingredients was also determined by bomb calorimetry. The bomb calorimeter used was a Gallenkamp Automatic Adiabatic Bomb Calorimeter.

g) Statistical Analysis

Standard statistical procedures were used which included analysis of variance to test the significant differences in values of weight gain, feed intake and food utilisation (i.e. feed intake/ weight gain) (Snedecor and Cochrane, 1967). In testing the significance of mean comparisons, Duncan's Multiple Range Test was used (Duncan, 1955). The statistical package Minitab (1980) available on the University's Prime Computer system was used for one-way analysis of variance, correlations and regressions.

CHAPTER 3: GROWTH TRIALS - PART I

Preliminary Growth Trials

Three preliminary chick growth trials were carried out to evaluate the general nature of LPC as a constituent of compound diets.

As the LPC supplied by the Ruakura Agricultural Research Centre was a unique product, incorporating specific plant species and made by special manufacturing procedures, its nutritional properties had to be evaluated using published findings for other LPCs as only a guide.

The first trial was a simple feeding trial to ascertain the chicken's acceptance of LPC diets. Trials 2 and 3 examined the effect of lysine and methionine supplementation of LPC diets as well as factors such as dustiness which may affect appetite.

Preliminary Trial 1: Acceptability of LPC to Chickens

The experimental diets were designed to test the chickens acceptability of LPC. The four experimental diets used were computer formulated and were designated as listed below:-

A	SBM	"80% NRC"
B	LPC	"80% NRC"
C	SBM	"70% NRC" (+ 2% tallow)
D	LPC	"70% NRC" (+ 2% tallow)

The amino acid compositions of soybean meal (SBM) and LPC are given in Tables III and IV on pages 12 and 13 . Batch 1 of both ingredients was used in Trial 1. The computer formulation constraints are listed as 80% NRC and 70% NRC i.e. 80% and 70% respectively of the NRC (1971) amino acid requirement for broilers 0-6 weeks of age. The 80% NRC level was considered adequate for the crossbred egg-type cockerel chicks used in the growth trials. The 70% NRC constraint together with 2% tallow added to the diet giving a higher energy density (11.71kJ/g versus 12.13 kJ/g

for 80% and 70% NRC constraints respectively) was designed to widen the calorie-protein ratios (Appendix A, Table I). This was expected to emphasize any differences in amino acid availability. It should be noted that all metabolisable energy values used for the formulation of diets in Trials 1 and 2 were only estimated using published values (e.g. Scott et al., 1969).

An Apparent Metabolisable Energy (AME) value of 11.71 kJ/g was used for LPC which was equivalent to the published energy level of SBM. The mineral and amino acid composition of the LPC had been determined (Table III).

SBM was used as the standard protein in the control diets because the protein has a high consistent nutritional value and is extensively used commercially. Both SBM and LPC were held to the 25% level of inclusion in the experimental diets. All diets were formulated to be isocaloric and isonitrogenous.

The ingredient and calculated nutrient composition of the diets is summarised in Appendix A, Tables II and III. Each diet was fed to 4 replicates of 14 chicks each (total 224 chicks) from hatching to four weeks of age.

Results

The results of Trial 1 were summarised in Table V. The statistical analyses for Trial 1 are summarised in Appendix B, Tables I and II. There was a significant difference ($P < 0.05$) between the growth rate of chickens fed each of the diets. Chicks, whose diets included LPC, gained the least weight. The "70% NRC" nutrient constraint level with the higher energy level improved the growth of chicks fed diets containing soybean, (treatment A versus C) presumably because of an improved energy/protein ratio. With diets containing LPC (treatments B versus D) growth was depressed by 10% with chicks fed diet D compared to B. This was not due to any significant difference in overall food intake between the two treatments (519.2 g versus 498.2 g). Rather the chickens were unable to

Table V: Initial Evaluation of LPC in Comparison to SBM (days 0 to 28)

Treatment		Mean Weight Gain (g) (G)*	Mean Food Intake (g) (F)*	Mean Food Utilisation (F/G)
A	SBM "80% NRC"	295.56^{b}	616.39^{a}	2.09^{b}
B	LPC "80% NRC"	210.14^{c}	519.16^{b}	2.37^{c}
C	SBM "70% NRC" + 2% tallow	310.23^{a}	622.50^{a}	2.01^{a}
D	LPC "70% NRC" + 2% tallow	196.72^{d}	498.20^{b}	2.53^{d}
	SEM	4.99	9.65	0.14

Values with common superscript letters do not differ significantly ($P<0.05$)

* F = Mean food intake (g) per chicken in 28 days

G = Mean weight gain per chicken in 28 days

SEM = Standard Error of the Mean

Table VI: Summary of Amino Acid Intakes and Energy Intakes in Trial 1

Treatment	"80% NRC" (25% SBM)	"80% NRC" (25% LPC)	"70% NRC"+ 2% Tallow (25% SBM)	"70% NRC"+ 2% Tallow (25% LPC)	"70% NRC equivalent[1] level"
Food Intake/period (g)	616.4	519.2	622.5	498.2	622.5
Mean weight gain of birds (g)	259.6	219.1	310.2	196.7	
Arginine (g)	8.13	5.81	8.22	5.38[2]	6.10
Histidine (g)	3.08	2.49	3.11	2.24	1.99
Isoleucine (g)	4.93	4.05	4.85	3.78	3.73
Phenylalanine + Tyrosine (g)	9.74	9.24	9.65	8.52	6.54
Methionine + Cystine (g)	4.25	3.58	3.73	2.99[2]	3.74
Threonine (g)	4.50	4.20	4.85	3.83	3.49
Valine (g)	5.42	5.66	5.41	5.08	4.36
Tryptophan (g)	1.54	1.76	1.49	1.59	0.99
Lysine (g)	6.53	5.19	6.47	4.73[2]	5.48
Energy Intake kJ/period[3]	7224.6	6265.2	7256.9	6220.4	

[1] "70% NRC equivalent level" = 70% of NRC (1971) amino acid constraints for broilers 0-6 weeks of age multiplied by the intake level of Diet C. (Diet C gave the greatest growth gain. These birds were therefore consuming at the "ideal" intake level).

[2] Significantly lower intake level ($P < 0.05$) when compared with "70% equivalent level" and SBM.

[3] The "energy intakes" were calculated using True Metabolisable Energy (TME) data which was available after the completion of Trial 2.

utilise diet D as well as diet B (2.53 versus 2.37). This indicated that greater "stress" was being placed on the amino acid composition of the LPC diet with the increased energy:protein ratio in Diet D compared to the SBM diets.

The intake of amino acids and energy for chickens fed the experimental diets is given in Table VI. It should be noted that the energy intakes are calculated using the True Metabolisable Energy (TME) values for all ingredients including LPC and SBM.

While both the overall diet intake and the amino acid intake of chickens fed the LPC diet was lower than that of the chickens fed the SBM diet, only the intakes of arginine, the sulphur amino acids and lysine were lower than the "70% NRC equivalent level" when the chickens were fed diet D. The "70% NRC equivalent level" was calculated using the dietary formulation constraints for the amino acids (Appendix A, Table I) and converted to a "requirement" level using the food intake level of treatment C (25% SBM at "70% NRC" + 2% tallow). Diet C was used as a standard for calculation because the birds fed this diet achieved the greatest growth gain. The "70% NRC equivalent level" indicated that theoretically at least, the most likely amino acids in Trial 1 to be limiting in diets containing LPC were arginine, the sulphur amino acids and lysine.

Preliminary Trial 2: Lysine and Methionine Supplementation

The purpose of preliminary Trial 2 was to examine the effect of supplementing the LPC diet with additional lysine and methionine. In Trial 2 the proportion of SBM and LPC in the diets was reduced from 25% to 15%, but all other constraints were the same as for Diet C in Trial 1. The inclusion level of LPC and SBM was decreased to reduce any possible effect on the energy content of LPC which was still unknown at this stage of the work. The True Metabolisable Energy value (TME) became available at the completion of this trial.

The 15% SBM and 15% LPC diets acted as the two main comparative treatments. For a third treatment 0.03% methionine and 0.1% lysine were added to the 15% LPC diet. The level of amino acid supplementation was chosen to allow for an arbitrary 15% lower digestibility for LPC compared to SBM. In a fourth treatment an additional 5% LPC was allowed in the formulation to compare the effect of increasing the amino acid supply via additional LPC in the diet.

The four diets were coded as follows:-

A 15% SBM
B 15% LPC
C 15% LPC + 0.03% methionine + 0.1% lysine
D 15% LPC + 5% LPC

The four diets were each fed to three replicates of 14 chicks (total 168 chicks) from hatching to four weeks of age. The constraints, ingredients and calculated nutrient composition of the diets are shown in Appendix A, Tables V and VI. LPC from the second batch and SBM from the first batch (p13) were used for Trial 2.

Results

The results of Trial 2 are summarised in Table VII. The statistical

Table VII: Trial 2- Effect on Growth of Supplementation of LPC with 0.1% Lysine + 0.03% Methionine and with an Additional 5% LPC, in Comparison with SBM (1-28 days)

Treatment		Mean Final Weight(g) (G)	Mean Food Intake (g) (F)*	Mean Food Utilisation (F/G)
A	15% SBM	326.2^{a}	767.1^{a}	2.68^{a}
B	15% LPC	277.3^{b}	778.7^{a}	3.28^{b}
C	15% LPC + 0.03% methionine + 0.1% lysine	283.7^{b}	767.8^{a}	3.15^{b}
D	15% LPC + 5% LPC	251.0^{c}	691.4^{b}	3.38^{b}
	SEM	2.1	6.5	0.07

Values of common superscript letters do not differ significantly ($P < 0.05$)

*F = Mean food intake per chicken in 28 days

*G = Weight gain (g) (mean day-old chick weight = 40 g)

Table VIII: Amino Acid and Energy Intakes in Trial 2

Treatment	A 15% SBM	B 15% LPC	C 15% LPC + .03% methionine + 0.1% Lysine	D 20% LPC	"70% NRC[1] equivalent level"
Food Intake (g)	767.1	778.7	767.8	691.2	767.1
Weight of birds (g)	326.2	277.3	283.7	251.0	
Arginine (g)	7.82	7.63	7.52	6.08[2]	7.52
Histidine (g)	3.53	3.34[3]	3.30[3]	2.83[3]	2.45
Isoleucine (g)	4.76	5.06	4.99	4.70	4.60
Phenylalanine + Tyrosine (g)	10.51	11.91	11.74	10.85	8.05
Methionine + Cystine (g)	4.91	4.98	5.14	4.49[2]	4.91
Threonine (g)	4.83	5.45	5.37	4.97	4.29
Valine (g)	6.29	7.24	7.14	6.67	5.37
Tryptophan (g)	1.61	2.49	2.45	2.10	1.23
Lysine (g)	6.75	6.93	7.60	5.67[2]	6.75
Energy Intake (kJ/period)[4]	9307.7	9610.2	9475.9	8578.0	

[1] "70% NRC equivalent level" = 70% of NRC (1971) amino acid constraints for broilers 0-6 weeks of age multiplied by the intake level of Diet A.

[2] Significantly lower intake ($P < 0.05$) when compared with "70% NRC equivalent level"

[3] Significantly lower intake level ($P < 0.05$) when compared to SBM but higher than "70% NRC equivalent level"

[4] The "energy intakes" were calculated using True Metabolisable Energy (TME) data which was available after the completion of Trial 2

analyses for Trial 2 are summarised in Appendix B, Tables III and IV.

Chicks fed the 15% LPC diets had a significantly ($P<0.05$) lower weight gain and food utilisation than the chickens fed the 15% SBM diet even though there was no significant difference in overall food intake.

The addition of methionine and lysine to the 15% LPC diet (diet C) tended to improve growth rate and in particular the food utilisation. This improvement, however, was not statistically significant.

The addition of 5% LPC (diet D) resulted in significantly poorer growth rate and lower food consumption. The lower food intake with 20% LPC suggested that a factor was depressing appetite as food utilisation was unaffected. This indicated that the growth rate with LPC was a reflection of food intake as well as food utilisation.

Estimates of the energy value of SBM (batch 1) and LPC (batch 2) using the True Metabolisable Energy (TME) assay method (described p. 17) became available at this time. The TME value was established to be 11.72 $\pm$ .02 kJ/g for SBM and 12.84 $\pm$ .04 kJ/g for LPC. These differed little from the AME values used to formulate diets in Trials 1 and 2 (11.71 kJ/g for both LPC and SBM). When the TME of the 15% LPC or 15% SBM diets (12.34 and 12.13 kJ/g diet respectively) were considered, little difference was apparent.

This indicated that an energy difference between these ingredients was probably not affecting results. The calculation of the energy intake of chickens in Trials 1 and 2 (Tables VI and VIII) also indicated that the small differences in the energy values of the LPC and SBM diets could not explain the difference in growth results reported in the first two trials.

The amino acid intakes, summarised in Table VIII, demonstrated for the chickens fed the 15% LPC diet with or without methionine, that

the only amino acid which had a lower intake than the 15% SBM fed chickens was histidine. In comparison to the "70% NRC equivalent levels" the chickens fed these diets (15% LPC and 15% SBM) had an adequate supply of all essential amino acids. However when an additional 5% LPC was fed, arginine, methionine + cystine and lysine intakes were below both the 15% SBM and "70% NRC equivalent levels". This suggested that arginine, as well as lysine and the sulphur amino acids, may be limiting the growth of chickens fed diets containing LPC. Also as supplementation of the LPC diet with lysine and methionine improved food utilisation, although not significantly, it is possible that higher supplementary levels of these amino acids were required.

Preliminary Trial 3: Lysine and Methionine Supplementation continued

For Trial 3, the treatments were designed to further study the reasons for the low food utilisation of chicks fed LPC diets compared to SBM diets. The trial was composed of six diets, each one being fed to three replicates of 14 chicks (total 252 chicks) from hatching to four weeks of age. The ingredient and calculated nutrient composition of the diets are given in the Appendix A, Tables VIII and IX. LPC from Batch 2 and SBM from Batch 1 (p 12-13) were used in Trial 3.

The diets used in Trial 3 are as set out below:-

A 15% SBM
B 15% LPC
C 15% LPC + 5% SBM
D 15% LPC + 0.15% L-lysine + 0.05% DL-methionine
E 15% LPC pelleted
F 20% LPC pelleted

Diets A (15% SBM) and B (15% LPC) were the same as those used in Trial 2. Diet C was diet B (15% LPC) with 6% maize replaced by 5% SBM + 1% tallow. The replacement of maize with SBM was intended to improve the amino acid pattern and the tallow maintained the energy content. Diet D was diet B (15% LPC) to which 0.15% L-lysine and 0.05% DL-methionine was added to investigate again if either were limiting. The supplementation levels were equivalent to the additional lysine and sulphur amino acid contributed via the 5% SBM added in treatment C. If any of the three amino acids (lysine, methionine and cystine) were limiting then diets C and D should give similar results. Diet E was diet B dry pelleted and diet F was 20% LPC dry pelleted i.e. no steam was introduced during the pelleting process. The dustiness of the diets containing LPC may be adversely affecting the food intake of chickens fed these diets. If this is so, pelleting should improve food intake.

Results

The results are summarised in Table IX and the amino acid intakes are shown in Table X. The statistical analyses for Trial 3 are summarised in Appendix B, Tables V and VI.

As in Trial 2, the chickens fed the 15% SBM diet achieved significantly ($P<0.05$) larger body weights than the 15% LPC fed chickens. The addition of lysine and methionine to the 15% LPC diet did not improve chicken body weights although it slightly improved food utilisation in comparison to the unsupplemented diet. However, the addition of 5% SBM to the 15% LPC diet significantly improved food utilisation and also increased body weight gain, although the latter was not statistically significant.

The results with pelleting varied. Feeding the pelleted 15% diet (diet E) significantly improved the final body weights to a value comparable to that obtained with feeding the SBM diet. In contrast the chickens fed the pelleted 20% LPC diet had significantly lower final body weights than those fed the pelleted 15% LPC (diet E) and their body weights tended to be lower than those fed the unpelleted 15% LPC diet (diet B), though not significantly. Also the chickens fed pelleted 20% LPC diet had significantly lower food intakes than the chickens fed the unpelleted 15% LPC diet (diet B). The pelleting of the 15% LPC diet, however, improved food intake. This improvement indicated that LPC did not have a toxic effect on chickens. However, this preliminary study into the effect of pelleting was inconclusive due to the poor response with feeding the 20% LPC diet. A further study was carried out in Trial 7 to assess the effect of pelleting.

The intakes of lysine and methionine+cystine given in Table X did not vary significantly for any of the diets even though the growth gains varied. These results therefore indicate that an amino acid other than lysine and/or the sulphur amino acids may be limiting the growth of chickens fed the LPC diet. Comparison of the amino acid intakes of the 15% SBM and 15% LPC diets in Table X indicated that arginine and/or histidine could be implicated because the intakes of these were lower for chickens fed the 15% LPC diet.

Table IX: Trial 3 - Effect on Growth of Supplementation of LPC with 0.15% Lysine + 0.05% Methionine or with SBM, and the Effect of Pelleting in Comparison with SBM (days 0 to 28)

	Treatment	Mean Final Weight (g) (G)	Mean Food Intake (g) (F)*	Mean Food Utilisation (F/G)
A	15% SBM	347^{a}	617.2^{ab}	2.01^{a}
B	15% LPC	292^{bc}	610.1^{ab}	2.42^{c}
C	15% LPC + 5% SBM	315^{ab}	616.4^{ab}	2.24^{b}
D	15% LPC + 0.15% Lysine + 0.05% Methionine	294^{bc}	597.7^{bc}	2.35^{c}
E	15% LPC pelleted	327^{a}	641.0^{a}	2.23^{b}
F	20% LPC pelleted	275^{c}	560.3^{c}	2.38^{c}
	SEM	9	12.0	0.02

Values with common superscript letters do not differ significantly ($P < 0.05$)

*F = Mean food intake per chicken in 28 days

*G = Weight gain (g) (Mean day-old chick weight = 40 g)

Table X: Amino Acid Intakes in Trial 3

	15% SBM	15% LPC	15% LPC + 5% SBM	15% LPC + 0.15% Lysine + 0.05% Methionine	15% LPC Pelleted	20% LPC Pelleted	"70% NRC equivalent[+] level"
Food Intake (g)	617.2	610.1	616.4	597.7	641.0	560.3	617.3
Lysine (g)	5.31	5.43	6.28	6.22	5.70	5.20	5.41
Arginine (g)	6.66	5.97[1]	6.72	5.86[1]	6.28	5.70[1]	6.04
Isoleucine (g)	3.95	4.14	4.99	4.27	4.35	4.43	3.71
Methionine + Cystine (g)	3.95	3.90	4.25	4.12	4.10	3.75	3.95
Threonine (g)	4.38	4.70	5.12	4.42	4.93	4.71	3.45
Valine (g)	5.86	6.10	7.15	6.16	6.60	6.39	4.32
Histidine (g)	2.96	2.62[1]	3.02	2.70[1]	2.88	2.46[1]	1.98
Leucine (g)	10.73	10.67	11.77	10.46	11.22	11.26	6.91
Tyrosine (g)	4.19	4.15[1]	4.93	4.12[1]	4.49	4.37	3.02
Phenylalanine (g)	5.86	5.73[1]	6.47	5.44[1]	6.09	5.26[1]	3.46

[+] "70% NRC equivalent level" = 70%of NRC (1971) amino acid constraints for broilers 0-6 weeks of age multiplied by the intake level of Diet A.(Diet A gave the greatest growth gain so those birds were consuming at the "ideal" intake level).

[1] Significantly lower intake level ($P < 0.05$) when compared with 15% SBM.

An alternative possibility was that the addition of SBM to the LPC diet not only changed the level of amino acids but also altered the relative balance of amino acids and/or other compounds, where as additional LPC only maintained or worsened such a balance. This may mean that the growth response of LPC fed chickens to additional methionine and/or lysine may be dependant on another amino acid and/or on other compounds.

DISCUSSION (Trials 1-3)

The main conclusion from the preliminary trials was that the nutritional value of LPC was less than that of SBM. The fact that dietary amino acid patterns and intake levels overall for LPC and SBM were similar indicated that the availability of one or more LPC amino acids may be lower than for SBM. Supplementation of LPC with methionine + lysine gave no apparent improvement in growth and a slight, but insignificant, improvement in food utilisation. By contrast 15% LPC + 5% SBM tended to increase chicken growth and to improve food utilisation. With this diet the "overall" amino acid intake level tended to be improved. Since supplementation of the 15% LPC diet with lysine and methionine had no apparent affect on growth gain, it was suggested that:

(i) an amino acid other than the sulphur amino acids or lysine was first limiting,

or (ii) that the response to methionine or lysine was dependant on another amino acid which was closely second limiting,

or (iii) that the sulphur amino acids and/or lysine were required at higher supplementary levels than that used in Trials 1-3. In which case an anti-nutritional compound(s) may be reducing the availability of one or more amino acids for growth.

A summary of the comparative amino acid intakes with diets containing LPC used in Trials 1-3 is given in Table XI. This demonstrates that arginine, the sulphur amino acids, lysine and histidine all had lower intake levels than the comparable SBM diet on at least three occasions. However, only the intakes of arginine, the sulphur amino acids and lysine were lower than the "NRC equivalent level" in either the 20% or the 25% LPC diets.

While the intake of certain amino acids with diets containing LPC may be below the intake with SBM diets, their intake may be above the requirement for that particular amino acid. The

amino acid level of the LPC diets from Trials 1-3 were ranked by comparing the amino acid intake with these diets and the "70% NRC equivalent level"; these are summarised in Table XII. This showed that the sulphur amino acids and arginine consistently have the lowest intakes compared to the "70% NRC requirement" with lysine only dropping to fourth on one occasion. Therefore it appears that lysine, arginine or the sulphur amino acids were probably first limiting in the LPC diets.

Table XI: Summary of Trials 1-3: Amino Acids in the LPC Diet which have intakes below SBM Diet intakes and "NRC Equivalent Level."

Trial	Diet	Amino Acids with lower intake levels with LPC than with SBM diets	Amino Acids with lower intake levels with LPC than the "NRC Equivalent Level"
1	25% LPC (70% "NRC")	Arginine	Arginine
		Methionine + Cystine	Methionine + Cystine
		Lysine	Lysine
2	15% LPC	Histidine	-
2	20% LPC	Arginine	Arginine
		Methionine + Cystine	Methionine + Cystine
		Histidine	-
3	15% LPC	Arginine	-
		Histidine	-

Table XII: Ranking of Amino Acid Intake with LPC diets compared to "70% NRC equivalent Level" in Trials 1-3 (Comparison at different levels of LPC)

Trial 1 (25% LPC)		Trial 2 (15% LPC)		Trial 2 (20% LPC)		Trial 3 (15% LPC)	
Ranking	Comparative* Intake Level	Ranking	Comparative Intake Level	Ranking	Comparative Intake Level	Ranking	Comparative Intake Level
Methionine + Cystine	- 19.8	Methionine + Cystine	1.5	Arginine	- 19.1	Arginine	- 1.2
Lysine	- 13.6	Arginine	1.5	Lysine	- 19.1	Methionine + Cystine	5.0
Arginine	- 11.8	Lysine	2.7	Methionine + Cystine	- 8.6	Isoleucine	11.6
Isoleucine	1.3	Isoleucine	10.0	Isoleucine	2.2	Lysine	22.6
Threonine	9.7	Threonine	27.0	Histidine	15.5	Histidine	32.0
Histidine	12.6	Valine	34.8	Threonine	15.8	Threonine	36.2
Valine	16.5	Histidine	36.3	Valine	24.2	Valine	41.2
Phenylalanine + Tyrosine	30.3	Phenylalanine	47.9	Phenylalanine + Tyrosine	34.8	Phenylalanine	52.5

* Comparative Intake Level = $\frac{\text{LPC Intake - "70\% NRC equivalent level"}}{\text{"70\% NRC equivalent level"}} \times \frac{100}{1}$

CHAPTER 4: GROWTH TRIALS

Part II

Amino Acid Supplementation of LPC Diets

In the preliminary Trials 1-3 computer formulated diets were used. However as pointed out in the methods section, p 11 , such diets are of limited value. Following the preliminary trials a different procedure was used employing a basal diet as described in Chapter 2, p. 11 . The "test" proteins were added at equivalent isoleucine levels in place of cornstarch.

The four growth trials described below were carried out to study the effect of supplementing diets containing LPC with lysine, methionine, cystine and arginine.

(i) Trials 4 and 5 tested the effect of supplementing with lysine, methionine and arginine.

(ii) Trial 6 examined whether the need for additional methionine found in Trials 4 and 5 could be replaced by additional cystine since methionine can be used by the bird to synthesise cystine.

(iii) Trial 7 tested again the effect of a higher inclusion level of LPC in diets (20% inclusion level). This trial also tested whether additional methionine (or cystine) was required at the higher level of LPC inclusion. In addition the effect of dry pelleting on the utilisation and intake of LPC diets was examined.

DL-methionine was used and assumed to be equivalent to L-methionine. According to Boorman and Lewis (1971) the utilisation of D-methionine by growing chickens is "equivalent or almost equivalent" to the corresponding L-isomer. All other amino acids supplemented were the L-isomers.

Trial 4: Effect of Additional Lysine, Arginine or Methionine

The purpose of Trial 4 was to test whether LPC diets required additional lysine, arginine and/or methionine. The trial consisted of 12 treatments made up of the basal diet plus:-

A	15% SBM
B	15% LPC
C	15% LPC + 0.1% arginine
D	15% LPC + 0.2% arginine
E	15% LPC + 0.2% lysine
F	15% LPC + 0.6% lysine
G	15% LPC + 0.2% lysine + 0.2% arginine
H	15% LPC + 0.6% lysine + 0.2% arginine
I	15% LPC + 0.1% methionine
J	15% LPC + 0.1% arginine + 0.1% methionine
K	15% LPC + 0.2% arginine + 0.1% methionine
L	15% LPC + 0.2% arginine + 0.2% lysine + 0.2% methionine + 0.2% histidine

The control diet of 15% SBM was supplemented with methionine to give the same level of sulphur amino acids as in the 15% LPC diet.

The reasons for the choice of the experimental diets were as follows:-

a) D'Mello and Lewis (1970) showed that an adverse lysine:arginine ratio (2:1) depresses growth due to the imbalance of the amino acids.

b) In treatment E and F the level of lysine was increased to test not only the requirement for higher levels of lysine but also to increase the lysine:arginine ratio to a maximum of 1.5:1 (diet F) in order to increase "stress" on the availability of arginine. Such a "stress", if it occurred, should be relieved by the arginine supplementation in diets G and H.

c) Methionine was added at levels higher than in previous trials (Trials 1-3) in order to test if LPC diets required methionine and/or cystine at levels higher than would be expected as a result of a minor reduction in the amino acid digestibility of LPC.

d) Histidine was included in the last treatment because a direct comparison of SBM and LPC amino acid levels showed that the histidine level in LPC (Table III) was low compared to that in SBM. If this amino acid was closely second limiting it might affect the growth results and so require further study.

Each diet was fed to 3 replicates of 14 chicks (total of 504 chicks). The ingredient and nutrient composition of the diets is given in Tables XIII and XIV of Appendix A. Batches 2 of the LPC and SBM ingredients were used in Trial 4.

Results

The results are summarised in Table XIII. The statistical analyses are summarised in Appendix B, Table VII.

Treatments B to H indicated that supplementation of the LPC diet with arginine or lysine, or both, resulted in no significant differences in growth rate, compared to the LPC control diet. Therefore neither of these amino acids appeared to be first limiting, nor was the availability of arginine for growth apparently "stressed" by increasing the level of lysine (diets G and H).

When methionine was added to the LPC diets (diets I to L) the growth rate and food utilisation was significantly ($P < 0.05$) improved. At the highest level of methionine supplementation, (diet L), food utilisation was improved to a value similar to

TABLE XIII: Trial 4 - Effect of Supplementing LPC Diets With Arginine, Lysine and Methionine

Treatment	Diets	Mean Weight Gain (g) (G)	Mean Food Intake (g) (F)	Mean Food Utilisation (F/G)
A	15% SBM*	268.8^{a}	592.7^{abc}	2.20^{a}
B	15% LPC	203.0^{c}	565.6^{bcd}	2.78^{c}
C	15% LPC + 0.1% arginine	204.7^{c}	574.8^{abcd}	2.83^{cd}
D	15% LPC + 0.2% arginine	196.3^{c}	554.8^{cd}	2.83^{cd}
E	15% LPC + 0.2% lysine	201.0^{c}	555.9^{cd}	2.77^{c}
F	15% LPC + 0.6% lysine	189.5^{c}	552.6^{cd}	2.92^{d}
G	15% LPC + 0.2% lysine + 0.2% arginine	191.9^{c}	548.3^{d}	2.86^{cd}
H	15% LPC + 0.6% lysine + 0.6% arginine	194.3^{c}	552.6^{cd}	2.84^{cd}
I	15% LPC + 0.1% methionine	234.9^{b}	575.6^{abcd}	2.45^{b}
J	15% LPC + 0.1% arginine + 0.1% methionine	251.9^{ab}	609.7^{a}	2.42^{b}
K	15% LPC + 0.2% arginine + 0.1% methionine	244.7^{b}	600.9^{ab}	2.45^{b}
L	15% LPC + 0.2% arginine + 0.2% lysine + 0.2% methionine + 0.2% histidine	251.7^{ab}	572.3^{abcd}	2.27^{a}
SEM		5.8	12.5	0.03

Values with common superscript letters do not differ significantly ($P < 0.05$)

* This diet was supplemented with methionine to correct for the lower methionine level in SBM.

that of the chickens fed with SBM. The improvement in growth rate due to methionine addition was enhanced with additional arginine (diets J and K) although this was not significant. However, food utilisation was not improved by the addition of arginine to the 15% LPC + methionine diet.

Trial 5: Methionine and Arginine Supplementation

The purpose of Trial 5 was to further test the effect of feeding methionine and/or arginine supplemented LPC diets to chickens.

The seventeen diets consisted of basal plus:-

A 13% SBM
B 15% LPC
C 15% LPC + 0.1% arginine
D 15% LPC + 0.2% arginine
E 15% LPC + 0.3% arginine
F 15% LPC + 0.1% methionine
G 15% LPC + 0.1% arginine + 0.1% methionine
H 15% LPC + 0.2% arginine + 0.1% methionine
I 15% LPC + 0.3% arginine + 0.1% methionine
J 15% LPC + 0.2% methionine
K 15% LPC + 0.1% arginine + 0.2% methionine
L 15% LPC + 0.2% arginine + 0.2% methionine
M 15% LPC + 0.3% arginine + 0.2% methionine
N 15% LPC + 0.3% methionine
O 15% LPC + 0.1% arginine + 0.3% methionine
P 15% LPC + 0.2% arginine + 0.3% methionine
Q 15% LPC + 0.3% arginine + 0.3% methionine

A 4 x 4 factorial design was used with 4 levels of arginine and methionine supplementation of 15 % LPC diets plus a control diet of 13% SBM. Because of the factorial design, the 13% SBM diet was run separately, so that it was only a guide to growth. Each treatment had 3 replicates of 14 chicks (total chicks = 714). The experimental growth period was from the 7th day to the 28th day. The LPC used was a different batch (batch 3) from that used in the previous trials. The level of SBM was reduced to the 13% inclusion level so that both LPC and SBM contributed equal amounts of isoleucine to the diet (i.e. 130 g SBM/kg ration was equal to 150 g LPC/kg ration on an isoleucine basis). The ingredient and

Table XIV: Trial 5 - Effect of Supplementing LPC Diets With Methionine and Arginine

Treatment	Diets	Mean Weight Gain (g) (G)	Mean Food Intake (g) (F)	Mean Food Utilisation (F/G)
A	13% SBM*	227.7	597.2	2.61
B	15% LPC	195.5^{a}	558.8^{a}	2.86^{a}
C	15% LPC + 0.1% arginine	210.0^{ab}	598.1^{bcd}	2.85^{a}
D	15% LPC + 0.2% arginine	212.2^{b}	597.2^{bcd}	2.80^{ab}
E	15% LPC + 0.3% arginine	221.4^{bc}	602.1^{bcd}	2.72^{b}
F	15% LPC + 0.1% methionine	233.9^{cde}	593.9^{bcd}	2.54^{c}
G	15% LPC + 0.1% arginine + 0.1% methionine	230.8^{cd}	574.1^{ab}	2.49^{cd}
H	15% LPC + 0.2% arginine + 0.1% methionine	239.5^{def}	582.8^{abc}	2.43^{cde}
I	15% LPC + 0.3% arginine + 0.1% methionine	239.8^{defg}	609.8^{cd}	2.54^{c}
J	15% LPC + 0.2% methionine	249.4^{efgh}	593.9^{bcd}	2.37^{de}
K	15% LPC + 0.1% arginine + 0.2% methionine	250.9^{fgh}	606.1^{bcd}	2.41^{de}
L	15% LPC + 0.2% arginine + 0.2% methionine	255.5^{gh}	591.9^{abcd}	2.31^{e}
M	15% LPC + 0.3% arginine + 0.2% methionine	252.7^{fgh}	611.8^{cd}	2.42^{cde}
N	15% LPC + 0.3% methionine	248.7^{efgh}	587.9^{abcd}	2.36^{de}
O	15% LPC + 0.1% arginine + 0.3% methionine	253.3^{fgh}	599.8^{bcd}	2.36^{de}
P	15% LPC + 0.2% arginine + 0.3% methionine	258.9^{h}	618.2^{d}	2.39^{de}
Q	15% LPC + 0.3% arginine + 0.3% methionine	241.5^{defg}	573.6^{ab}	2.37^{de}
SEM	LPC diets	4.8	9.8	.03
	SBM diet	5.0		

Values with common superscript letter do not differ significantly ($P < 0.05$)

*This diet was supplemented with 0.03% methionine

nutrient composition of the diets are shown in Tables XV and XVI, Appendix A.

Results

The results and diets are summarised in Table XIV. The statistical analyses are summarised in Appendix B, table VIII. Figures 2 and 3 demonstrate more clearly some of the features of the responses obtained. It is evident from Figs. 2 and 3 that supplementation of up to 2 g methionine/kg of diet (0.2%) significantly improved both the weight gain and food utilisation of chickens fed diets F - Q when compared to the LPC control (diet B). The 2 g additional methionine/kg of diet was more than the total methionine contribution by LPC to the diet. The LPC contributed 1.2 g methionine/kg of diet compared to SBM which contributed 0.81 g methionine/kg diet. This indicated that a low availability for growth of the methionine in the LPC alone could not explain these results. Rather it appears that the availability for growth of the total methionine contributed by all dietary ingredients in the LPC containing diets must have been low.

To specify the additional methionine requirement of chickens fed the 15% LPC diet would have needed greater amounts of LPC than were available. This additional level, however is greater than 1 g methionine/kg diet, less than 3 g methionine/kg diet, and approximately 2 g methionine/kg diet.

The addition of arginine (diets C - E), to the LPC diet, in contrast to the previous trial, improved both the weight gain and food utilisation of the chickens fed the LPC diet. This improvement was statistically significant ($P < 0.05$) for both growth parameters when the 15% LPC + 0.3% arginine diet was fed.

Statistical analysis of the weight gain data (given in Table VIII, Appendix B) demonstrated that there was, not only a significant growth improvement due to arginine ($P < 0.01$), but also a significant ($P < 0.05$)

Fig. 2: The effect of Methionine and Arginine supplementation on weight gain of chicks fed LPC diets (Trial 5)

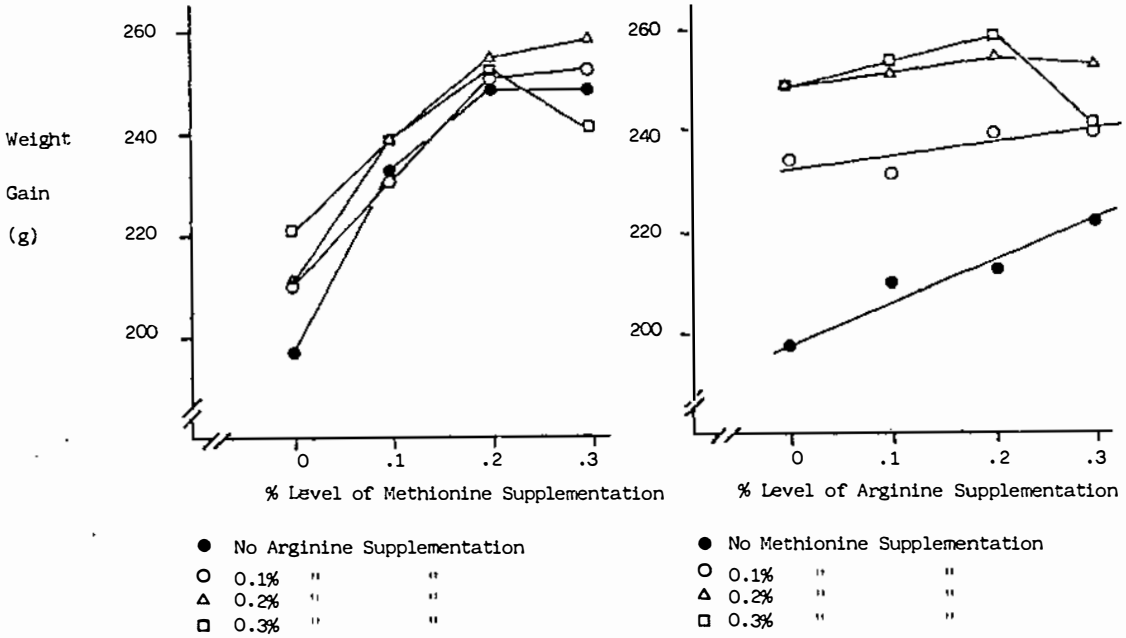
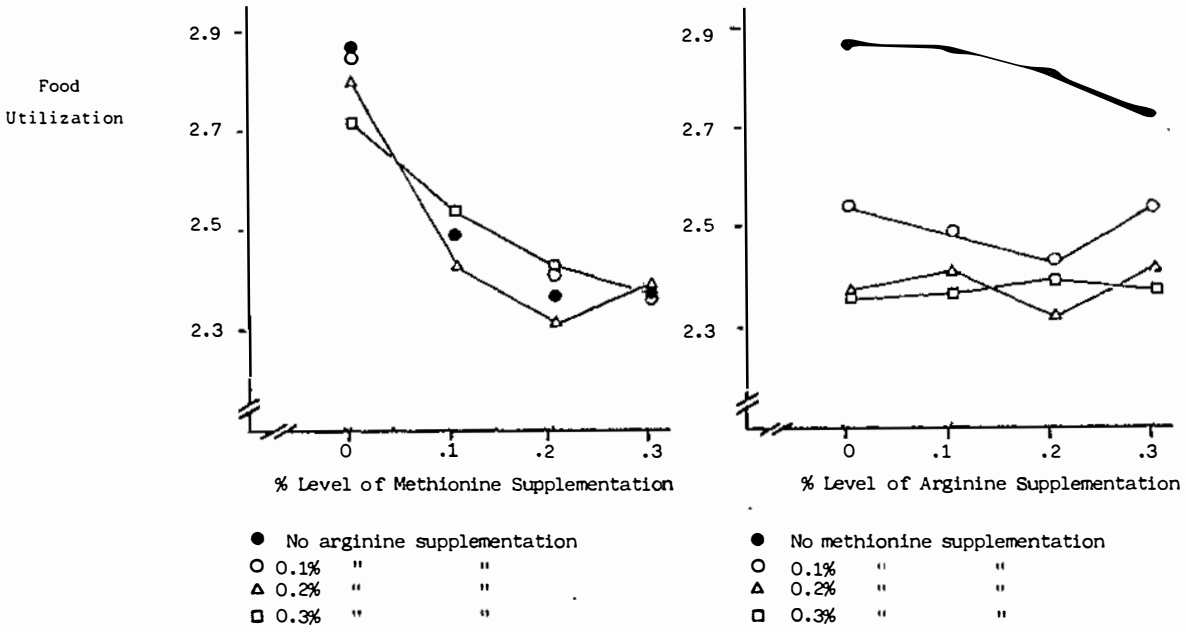


Fig. 3: The Effect of Methionine and Arginine Supplementation on Food Utilisation of 15% LPC diets (Trial 5).



methionine x arginine interaction.

The significance of the interaction was doubtful as it appeared to be due mainly to the 15% LPC + 0.3% arginine + 0.3% methionine treatment. Such an effect may have been a "real" effect and caused by feeding the relatively high combined level of supplementary amino acids or it may have been due to an "environmental effect" e.g. contamination of the diet Q.

Comparison of the effect of arginine and methionine addition on growth by calculating the correlation coefficients supported the conclusion that methionine was the major contributor to the recorded improved growth parameters in all methionine supplemented diets. The partial correlation coefficient between arginine level and weight gain with methionine held constant was $r_1 = 0.164$ (N.S.), whereas that between methionine level and weight gain was quite large $r_2 = 0.868$ ($P < 0.05$). The multiple correlation coefficient was $R = 0.883$ ($P < 0.05$).

Trial 6: Methionine and Cystine Supplementation

As the chicken is able to synthesise cystine from methionine this trial was designed, in the light of the results of Trial 5, to study whether cystine supplementation of LPC diets was as effective as methionine supplementation.

The following coded diets were fed to 3 replicates of 14 chicks (total chicks 336) from 1 to 4 weeks of age. The diets consisted of basal plus:-

A	15% LPC
B	13% SBM
C	15% LPC + 0.2% methionine*
D	15% LPC + 0.16% cystine*
E	15% LPC + 0.12% cystine + 0.05% methionine*
F	15% LPC + 0.08% cystine + 0.1% methionine*
G	15% LPC + 0.04% cystine + 0.15% methionine*

* Cystine and methionine were added on an equivalent sulphur basis i.e. 1 mole cystine = 2 moles methionine.

The ingredient and nutrient composition of the diets are shown in Tables XVII and XVIII, Appendix A. LPC batch 4 and SBM batch 3 were used in Trial 6.

Results

The results of the three week test period are shown in Table XV. The statistical analyses are summarised in Appendix B, Table IX.

The weight gains and food utilisation of the chickens fed either cystine and/or methionine supplemented diets were not significantly different ($P' < 0.05$) from those fed the soybean control diet (diet B) but were significantly higher than those fed the unsupplemented 15% LPC diet (diet A).

Table XV: Trial 6 - Results of Cystine Supplementation (21 days)

Treatment		Mean Weight Gain (g) G	Mean Food consumption (g) F	Mean Food Utilisation (F/G)
A	15% LPC	186.7^{a}	478.3	2.56^{b}
B	13% SBM	217.1^{b}	508.4	2.34^{a}
C	15% LPC + 0.2% methionine*	205.9^{b}	486.5	2.41^{a}
D	15% LPC + 0.16% cystine*	206.9^{b}	495.3	2.39^{a}
E	15% LPC + 0.12% cystine + 0.05% methionine*	206.9^{b}	477.6	2.31^{a}
F	15% LPC + 0.08% cystine + 0.1% methionine *	212.1^{b}	494.9	2.33^{a}
G	15% LPC + 0.04% cystine + 0.15% methionine*	212.4^{b}	497.5	2.32^{a}
SEM		3.65	8.09	.03

Values with common superscript letters do not differ significantly ($P < 0.05$)

* Added on an equivalent sulphur basis

These results therefore suggest that cystine and not methionine is the first limiting amino acid in the 15% LPC diets. Presumably supplementation of the LPC diet with methionine is beneficial only because it is converted to cystine. As LPC only contributed 0.6 g cystine/kg to the 15% LPC diets, the total dietary cystine available for growth was apparently decreased with the inclusion of LPC into the diet. This conclusion presumes that two moles of methionine are converted by the bird to 1 mole of cystine. It is possible that this conversion is less than 100% and that added L-cystine is used more efficiently than is the additional methionine.

However because of the large amount of methionine required to be added to improve growth, i.e. 2 g methionine/kg LPC diet in Trial 5, or 3 times the amount of cystine contributed by LPC to the LPC diet it is more than likely that the amount of cystine available in the whole diet was being decreased. The optimum level of cystine addition was not investigated.

Trial 7: Effect of pelleting, methionine supplementation on Increasing Inclusion Levels of LPC (15%,20%) on Chick Growth.

In the preliminary trials when LPC was included in diets at levels greater than 15%, dietary intake was reduced. This suggested the possible presence of a factor depressing appetite. In Trials 4 and 5 methionine supplementation of the 15% LPC diet markedly improved growth. As a lack of methionine may have been responsible for the low food intake with diets containing more than 15% LPC, this was examined in this trial, Trial 7. In addition,the effect of dry pelleting the diets was again investigated,as the results in Trial 3 were inconclusive.

Twelve diets as listed below were fed. These consisted of basal plus:-

A 13% SBM
B 13% SBM + pelleting
C 15% LPC
D 15% LPC + 0.2% methionine
E 15% LPC + pelleting
F 15% LPC + 0.2% methionine + pelleting

G 17.3% SBM
H 17.3% SBM + pelleting
I 20% LPC
J 20% LPC + 0.27% methionine
K 20% LPC + pelleting
L 20% LPC + 0.27% methionine + pelleting

Methionine was added at a ratio of 2 g methionine/150 g LPC and 2.7 g methionine/200 g LPC, at the 15%, and 20% inclusion levels of LPC respectively. The twelve treatments were each fed to 3 replicates of 14 chicks (total chicks 504).

Each treatment group with a given inclusion level of LPC or SBM was run as one battery unit and the results were analysed

separately. The ingredient and calculated nutrient composition of the diets are given in Table XIX and XX, Appendix A. Batch 3 of both SBM and LPC was used for Trial 7.

Results

The results are summarised in Table XVI and Figs. 4 and 5. The statistical analyses are summarised in Appendix B, Table X. Supplementation of the diets with methionine significantly ($P' < 0.05$) improved the body weight gain and food utilisation of birds fed with all levels of LPC.

Feeding the diets in pellet form increased the weight gain of chickens fed the 15% unsupplemented LPC diet (diet E). At the 20% LPC level pelleting the unsupplemented diet had no effect. Addition of methionine to the pelleted 15% LPC and 20% LPC diets significantly improved weight gain (diets F and L) in comparison to chickens fed the pelleted unsupplemented LPC diets (diets E and K).

Pelleting the methionine supplemented 20% LPC diet (diet L) also significantly improved weight gain of chickens in comparison to those fed the unpelleted methionine supplemented 20% LPC diet (diet J). A similar trend was also obtained with the comparitive 15% LPC diets (diets F and D) although the improvement with pelleting was not statistically significant. With regard to food utilisation, methionine supplementation gave significant improvement but on no occasion did pelleting alone do this (Fig. 5). This indicated that pelleting was improving growth by increasing food intake rather than by improving food utilisation. It seems probable that the powdery nature of the product was affecting the ability of the chicken to consume the LPC diets. It is also possible that the dry pelleting process may modify the nutritional properties of the diets and improve the availability of the dietary nutrients. However, the lack of improvement

Table XVI: Effect of Pelleting, methionine supplementation and of Different Levels of LPC (15 or 20%) on Chick Growth (21 days)

Treatment		Weight Gain (g) (G)	Food Intake (g) (F)	Food Utilisat. (F/G)
A	13% SBM	227.8^{cd}	585.3^{cd}	2.57^{b}
B	13% SBM + pelleting	241.2^{d}	598.6^{d}	2.48^{bc}
C	15% LPC	164.3^{a}	476.6^{a}	2.90^{a}
D	15% LPC + 0.2% methionine	223.5^{c}	529.9^{b}	2.37^{c}
E	15% LPC + pelleting	187.4^{b}	548.5^{bc}	2.93^{a}
F	15% LPC + 0.2% methionine + pelleting	234.2^{cd}	575.4^{cd}	2.46^{bc}
SEM		4.6	11.2	.04
G	17.3% SBM	254.6^{bc}	589.6^{bc}	2.31^{b}
H	17.3% SBM + pelleting	267.4^{cd}	606.6^{c}	2.26^{ab}
I	20% LPC	200.2^{a}	534.6^{a}	2.67^{c}
J	20% LPC + 0.27% methionine	253.8^{b}	541.5^{a}	2.13^{a}
K	20% LPC + pelleting	204.8^{a}	567.7^{ab}	2.77^{c}
L	20% LPC + 0.27% methionine + pelleting	273.4^{d}	583.0^{bc}	2.13^{a}
SEM		4.5	11.1	.03

Values with common superscript letters do not differ significantly ($P < 0.05$).

Fig. 4: Effect of Pelleting and Methionine Supplementation on Weight Gain with Two Levels of LPC Compared to Equivalent SBM Levels (Trial 6).

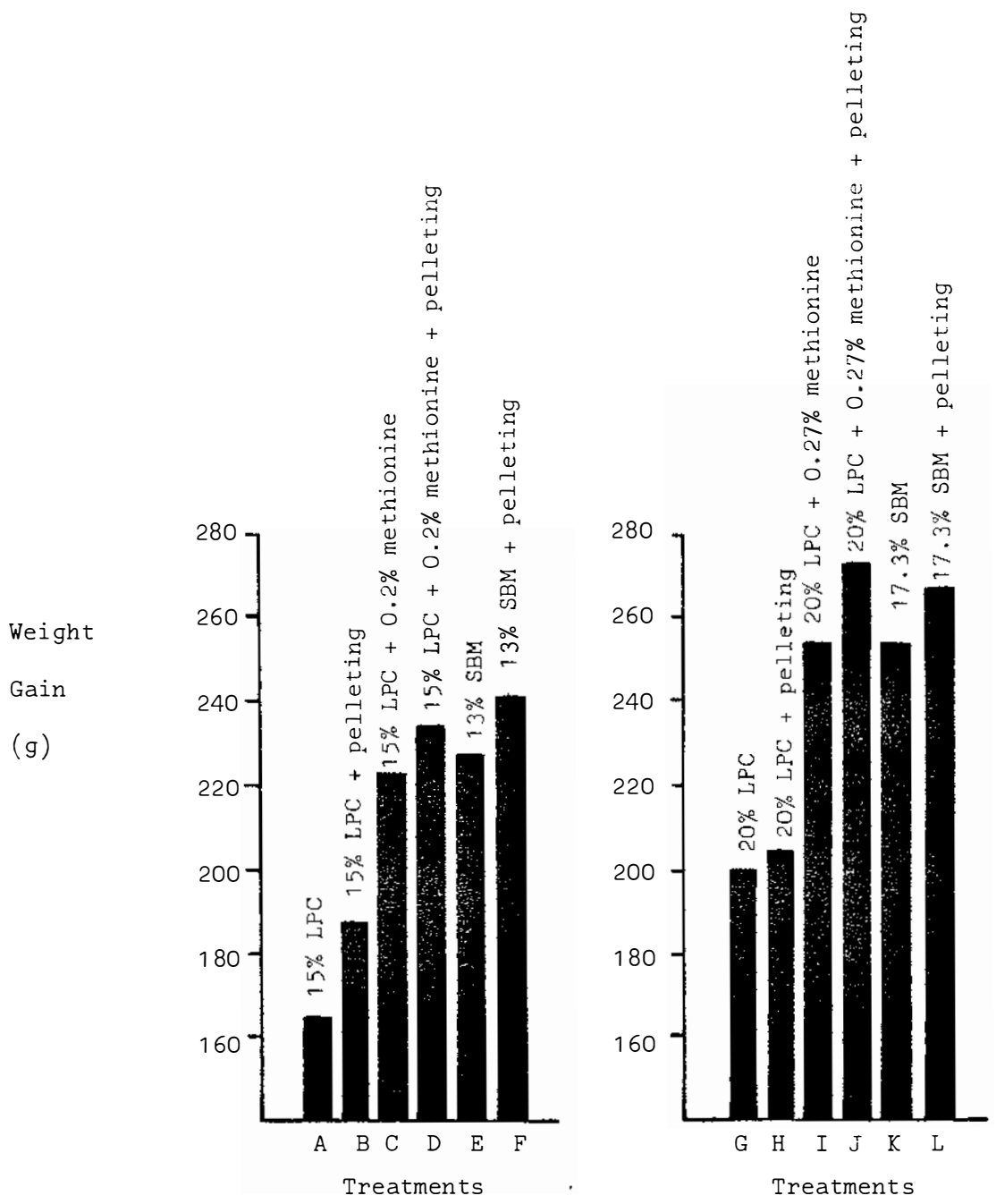
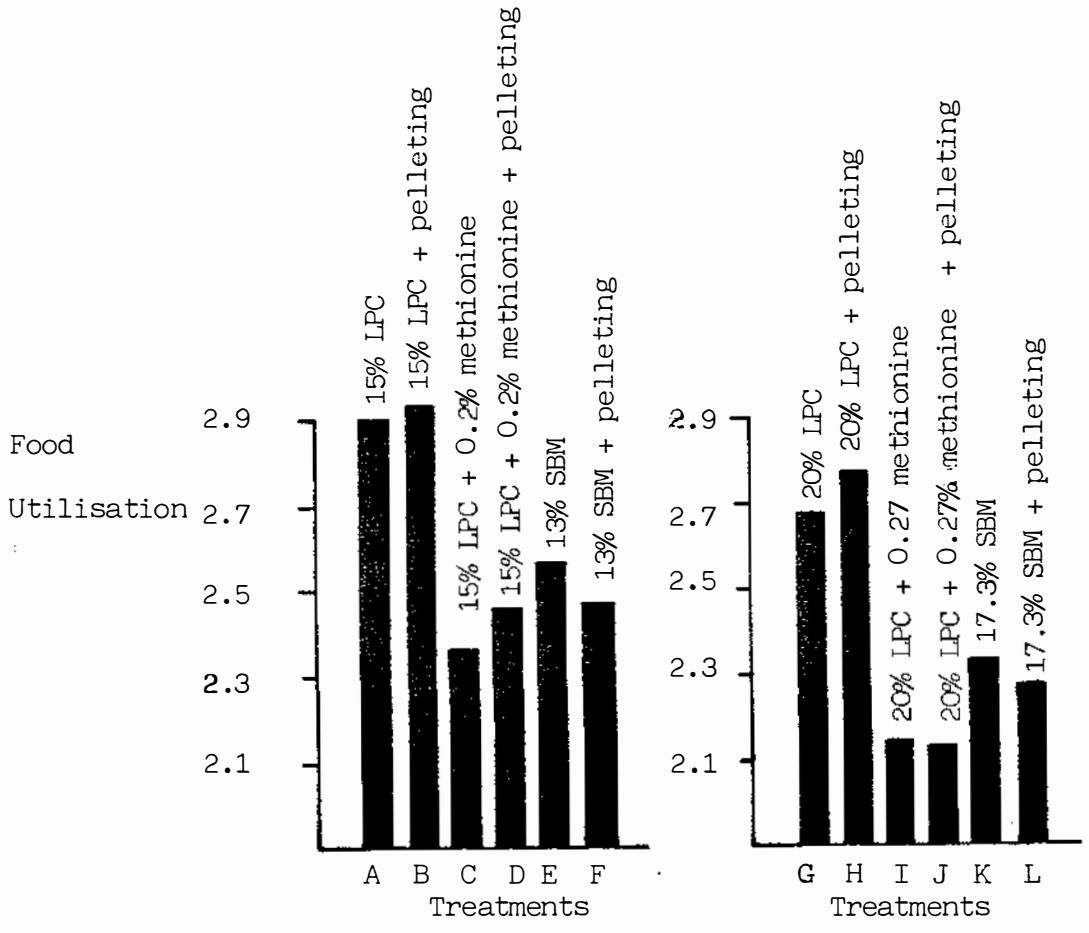


Fig. 5: Effect of Pelleting and Methionine Supplementation on Food Utilisation With Two Levels of LPC Compared to Equivalent SBM Levels (Trial 6).



in food utilisation suggests that there had been no significant change in the nutritional value of the diets with pelleting.

Since pelleting the 15% LPC diet and both the methionine supplemented 15% and 20% LPC diets improved both the growth and food intake of chickens fed these diets, it further confirmed the earlier observation (Trial 3) that LPC did not have a toxic effect on chickens.

Discussion: Trials 4 - 7

From the preliminary trials it was tentatively concluded that arginine or the sulphur amino acids or lysine was first limiting for chicks fed LPC diets. The results obtained in Trial 4 indicated that neither arginine nor lysine were the first limiting amino acid but that methionine and/or cystine was first limiting in the 15% LPC diet. The addition of methionine to the 15% LPC diet improved both weight gain and food utilisation of chicks fed diets containing LPC.

A similar result was obtained in Trial 5. Both the food utilisation and weight gain of chickens fed the LPC diet showed the greatest improvement when methionine (2g/kg diet) was added to the LPC diet. Supplementation with 2 g methionine/kg diet was similar to the total sulphur amino acid contribution by the LPC to the diet i.e. 1.8 g total sulphur amino acids from LPC/kg of diet. This supported the premise that the inclusion of LPC in a diet may be adversely affecting the ability of the chicken to adequately utilise all the sulphur amino acid sources in the diet rather than just the LPC sulphur amino acids alone. This conclusion was further supported in Trial 6. An equivalent amount of cystine, on a sulphur basis to methionine, when added to the LPC diet and fed to chickens was shown to be able to support the same amount of growth and maintain a similar food utilisation level as the additional methionine.

Also chickens fed either the SBM diet or the LPC diet containing an additional 1.6 g cystine or 2 g methionine/kg diet achieved similar growth results (Trials 6 and 7). LPC (150 g LPC/kg diet) contributed only 0.6 g cystine/kg to the LPC diet so that the addition of 1.6 g cystine or 2 g methionine/kg diet was approximately 3 times the amount of cystine that the LPC contributed to the 15% LPC diet. It is also of interest to note that the amount of sulphur amino acid in the SBM diet was similar to that in the LPC

diet (e.g. 6.6 g sulphur amino acid/kg SBM diet versus 6.5 g sulphur amino acid/kg LPC diet from Trial 6). Therefore not only was the amount of cystine available for growth from the LPC in the diet reduced, but the amount of cystine available from the other dietary constituents was also lowered.

The addition of methionine to LPC diets was also shown to be beneficial in Trial 7. Both growth and food utilisation improved when methionine was added to the 15% and 20% LPC diets. Dry pelleting these diets (LPC + methionine) also improved growth although this was only statistically significant for the 20% LPC diet. It was concluded that the dustiness of the diet possibly reduced the chicken's ability to consume the LPC diets at the higher inclusion levels.

In summary, the growth trials have demonstrated that the major cause for the growth depression of chickens fed LPC was a reduction in the amount of total dietary cystine available for growth and that cystine was the first limiting amino acid in the 15% LPC diet. Supplementary cystine and/or methionine relieved the adverse effects of feeding LPC. The ability of cystine or methionine to improve growth was also reported for lucerne LPC by Donnelly (1980) and James and Hove (1980) and for Ryegrass-White Clover LPC by Donnelly (1980).

In Trial 5, arginine supplementation of the LPC diet, in contrast to the Trial 4 results, tended to improve the weight gain of the LPC fed chickens. The statistical analysis of this data indicated that the evidence for a metabolic relationship between arginine and methionine, which may be contributing to the lower growth gains of chickens fed LPC, was slight. However the chicken growth studies reported by Lesley et al. (1976), on the effects of supplementing rapeseed meal diets with both methionine and arginine, showed that such a relationship is hard to detect.

A possible relationship between methionine and arginine was therefore further studied along with factors affecting the utilisation of cystine. These experiments are reported in Section II.

SECTION II

CHAPTER 5: INTRODUCTION - SOME COMPOUNDS INFLUENCING THE NUTRITIONAL VALUE OF LPC

The results of the growth trials previously reported in this thesis, suggested that the inclusion of LPC in a diet reduced the availability of the sulphur amino acids for growth in most if not all the protein constituents of the LPC diet. This may be due to any one or a combination of a variety of compounds and interactions which have been implicated by previous workers as possibly lowering the nutrtional value of LPC. Some of these are listed below:

1. Phenolic compounds
2. Carbohydrate - protein interactions
3. Oxides of methionine
4. Unsaturated fatty acids
5. (i) Saponins and
   (ii) Oestrogenic substances

The presence of some of these compounds will depend on the mix of species of pasture plants grown for LPC production e.g. saponins and oestrogenic compounds are present in legumes but not in grasses. However as N.Z. pasture used for LPC extraction contains a mixture of both legumes and grasses all compounds and reactions need to be considered in the present studies.

1. Phenolic Compounds

Phenolic compounds have been implicated in affecting the nutritional quality of all foodstuffs. They are widespread in plants and frequently occur in high concentrations (Harborne and Simmonds, 1964; Harborne, 1964; Bate-Smith, 1963). The classification of phenolics is reviewed by Ribereau-Gayon (1968). p-Coumaric and caffeic acid are amongst the commonest of all plant phenolics, and ferulic and sinapic acids are also widely distributed. Some of the major aspects of the possible nutritional

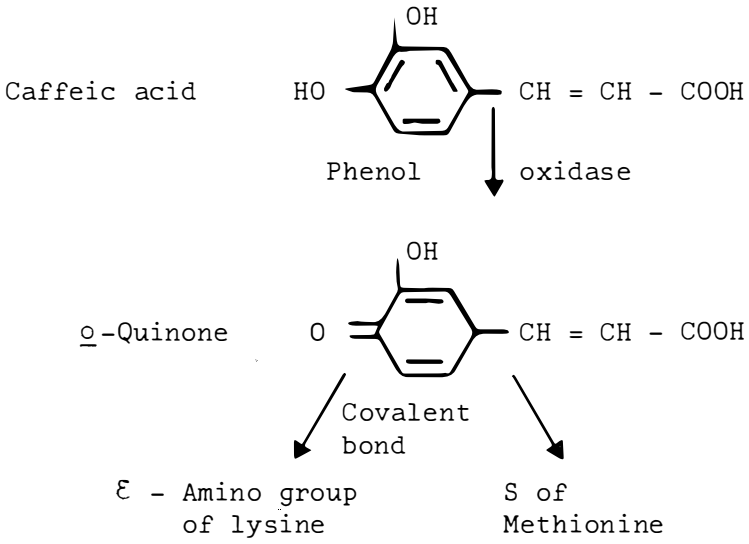
effects of phenolics discussed in the literature are described in the following sections (a) to (c).

(a) Detoxification: Plant phenolics are toxic to some organisms but their presence may protect the plant against pests and pathogens. Mechanisms which are able to detoxify the phenolic compounds, may be required to protect animals which ingest them as forage (see review by Singleton and Kratzer, 1969). The detoxification process may reduce the level of essential nutrients available to the animal for growth. For example, in the chicken, the protective mechanism of detoxification via methylation, dehydroxylation and conjugation may include an added requirement for a methyl donor (e.g. methionine) and arginine (ornithine) (Chang and Fuller, 1964; Conner et al., 1969; Lesley et al., 1976; Rayudu et al.,1970; Williams, 1959).

(b) Formation of Complexes: Phenolics take part in hydrogen bonding (Thomson, 1964; Flett, 1952) and form insoluble complexes with proteins (Sosulski, 1979). They may also be readily oxidised, non-enzymatically or catalysed by phenol oxidases or peroxidases, to form o-semiquinone radicals and o-quinones (Pierpoint, 1969 a,b). The quinones may in turn act as an oxidising agent on proteins or may covalently link with proteins (Loomis and Battaille, 1966) which will adversely affect the nutritional quality of a feedstuff (Pierpoint, 1974; Synge, 1975, 1976; Van Sumere et al., 1975; Hurrell and Finot, 1982).

An example of possible reactions as summarised by Sosulski (1979) is shown in Fig. 6 below.

Fig. 6 : Enzyme-Catalyzed Oxidation of Caffeic Acid to Caffeoquinone followed by Autolytic Bonding to Amino and Thiol Groups in Proteins (Sosulski, 1979)



Chlorogenic acid, in the presence of phenol oxidase, will couple with the thiol group of cystine residues and ε-NH_2 groups of lysine (Pierpoint 1969 a, 1974). Methionine has also been shown to react covalently with o-quinone (Vithayathil and Murphy, 1972). Horigome and Komadtsu (1968) have demonstrated in studies with rats that the treatment of casein with phenolic extracts from red clover lowered its digestibility and/or its biological value and its lysine availability. According to Lahiry et al. (1977) removal of chlorogenic acid from lucerne LPC improved the in vitro digestibility.

Both hydrolysable tannins, e.g. tannic acid (Van Buren and Robinson, 1969), and condensed tannins (Strumeyer and Malin, 1975) have been shown to be capable of complexing with protein. Eggum and Christensen (1975) have shown that tannic acid added to the diet significantly reduced the nitrogen digestibility of soybean protein. Such protein binding by tannins is believed to be responsible for the growth depression observed when rats and chickens are fed carobs (Ceratonia siliqua) (Tamir and Alumot, 1969). This was demonstrated by an increasing level of insoluble faecal nitrogen which correlated with the level of growth depression.

Seed coats of various horse beans (Vicia faba L) contain various condensed tannins and these have been attributed with causing a lower dietary nitrogen digestibility (Martin-Tanguy et al., 1977) when fed to chickens. When oak-leaf tannins were complexed with casein, the protein was almost completely protected against hydrolysis (Feeny, 1969), in vitro. High tannin sorghum has also been shown to reduce dietary nitrogen digestibility (Elkin et al., 1978) when fed to chickens. When these diets were supplemented with methionine and fed to chickens, a significant improvement in growth was reported (Elkin et al., 1978).

c) Enzyme Inhibition: Phenolic compounds have been shown to cause enzyme inhibition. Treatment of various plant extracts with polyvinyl pyrollidone (PVP) has resulted in improved activity of the enzymes trypsin, l-amylase, cellulase (Wynne-Griffiths, 1981), β-glucosidase (Goldstein and Swain, 1965) and pepsin (Jung and Fahey, 1981). PVP acts as a soluble absorbant for clearing protein extracts of phenolic compounds (Loomis and Battaile, 1966). Removal of phenolics with PVP, during the preparation of LPC, not only improves in vitro digestibility but also removes the enzyme (trypsin) inhibitor activity in Fescue and Italian ryegrass LPC (Humphries, 1980).

The effects of enzyme inhibitors, especially in soybean, have been widely studied in chicks (e.g. Liener and Kakade, 1969; Rakis, 1974). Soybean trypsin inhibitors are heat labile and protein in nature. The presence of soybean enzyme in a diet, increases the chicken's need for cystine and/or methionine (Liener, 1979). This increased cystine-methionine requirement is mainly due to an increased synthesis and secretion rate of the pancreatic cystine-rich proteolytic enzymes (Liener, 1979) and a retardation of the digestion rate (Bielorai et al.,1977). These enzyme inhibitors are referred to as "trypsin" or "protease" inhibitors.

Phenolic compounds have also been suggested as the cause for inconsistent results when comparing data from growth trials with various animals and in vitro digestibility methods (Akeson and Stahman, 1965; Saunders et al., 1973). Proteolytic enzymes can be affected by both free and bonded phenolic compounds in vitro (Milic et al., 1972; Griffiths and Jones, 1977; Feeny, 1969; Boudet and Gadal, 1965; Pierpoint, 1971).

The in vitro activity of papain under certain circumstances may be stimulated rather than inhibited by quinones (Pierpoint, 1971), whereas in vivo the opposite may occur. Boudet and Gadal (1965)

have suggested that differences in *in vitro* results may be due to differences in the stability of phenolic-protein complexes under different pH conditions.

2. Protein-Carbohydrate Interaction

Protein-carbohydrate complexes formed during processing have been suggested as an explanation of the lower nutrtional value of LPC (Osner and Johnson, 1968; Subba Rau *et al*., 1969; Subba Rau and Singh, 1970). The nutritional effects of some of these interactions have been reviewed in Nutrition Reviews (1978). Pirie (1978) suggested LPC was probably more susceptible to conjugation with carbohydrates than with phenolics during preparation, though such a reaction has not been demonstrated.

3. Formation of Oxides of Methionine

Partial oxidation of methionine to its sulphoxide and sulphone can occur during heat treatment of proteins. Oxidation depresses the availability of methionine to animals without affecting the total amount determined by chemical methods (Miller *et al*., 1965; Ellinger and Boyne, 1965; Woodham and Dawson, 1968). Methionine sulphone has been reported to have no methionine activity in the rat (Njaa, 1962; Miller and Samuel, 1968; Anderson *et al*.,1976), whereas the availability of the methionine sulphoxides (on a methionine equimolar basis) have been reported as 75-100% for DL-methionine sulphoxide, 50% for D-methionine sulphoxide and 60-100% for L-methionine sulphoxide in rats (Bennett, 1939; Njaa, 1962; Ellinger and Palmer, 1969; Miller *et al*., 1970; Anderson *et al*.,1976). In the chick, Kuzmicky *et al*.(1977) found that in relation to DL-methionine, L-methionine sulphoxide was 77% available and D-methionine sulphoxide was 52% available. Gjoen and Njaa (1977) found that when cystine was adequate, methionine sulphoxide was equivalent to methionine. The extent of oxidation may be affected by processing time. Byers

(1970) recorded that during processing 18% methionine appeared as methionine sulphoxide in wheat LPC but when the extract was allowed to stand for two hours, 30% methionine appeared as methionine sulphoxide.

The addition of sodium metabisulphite during processing improves the nutritional value of LPC (Bickoff et al., 1975; Donnelly, 1980). Snow et al. (1976) suggested that bisulphite may reduce the oxidation of methionine and that this was responsible for the improved nutritional value of LPC.

4. Oxidation of Unsaturated Fatty Acids

Oxidation of unsaturated fatty acids may take place readily in the presence of trace minerals and atmospheric oxygen and in the absence of antioxidants. Lea and Parr (1961) found that in crude leaf protein, the enzyme system for lipid oxidation was still active in the crude extract at room temperature but was inactive after freeze drying. Shah (1968) recorded that enzyme systems which cause changes in LPC lipids were inactivated by heating the protein to 100°C but that non-enzymatic oxidation of lipids still continued. More recent work with LPC produced from ryegrass (Hudson and Warwick, 1977) showed that the unsaturated fatty acids in this LPC have a high degree of resistance to auto-oxidation. This is probably due to the presence of potent antioxidants present in plants.

5. (i) Saponins

Saponins are plant glycosides which yield sugars and aglycones on hydrolysis and are derived from polycyclic ring compounds called sapongenins. The saponins are divided into two main groups according to the chemical nature of the sapongenins: steroids (C_{27}) or titerperoids (C_{30}) (Bondi et al., 1973). The biological effects of lucerne saponins on chickens and other animals have been reviewed by Cheeke (1971) and Bondi et al. (1973). Heywang (1950) showed that lucerne meal included in poultry mash

above the 5% level decreased the growth of chickens. This was attributed to the saponin fraction of lucerne (Peterson, 1950). Later Heywang et al. (1957) showed that lucerne saponins have a depressing effect on egg production.

Cheeke (1971), in his review on the "nutritional and physiological properties of saponins", states the the mechanism by which lucerne saponins cause growth depression has yet to be determined. He lists several possibilities which include depressed food intake, complexing with nutrients to render them unavailable, inhibition of digestive enzymes and inhibition of cellular metabolism. From the above list, Cheeke (1971) suggested that "the growth depressing activity is probably largely a result of effects on voluntary intake. Evidence to support the other possibilities mentioned is scanty."

Cholesterol complexes with saponin and alleviates the depressing effect on the growth rate of animals (Kuzmicky et al., 1972). No detectable effects were found with the addition of cholesterol to diets containing lucerne LPC (Subba Rau and Singh, 1970; Kuzmicky et al., 1972). On the other hand Colishaw (1956) found that a growth depression caused by feeding lucerne LPC could be counteracted by adding cholesterol. This effect was not found in LPC produced from Italian ryegrass or white clover. Reshef et al. (1976) concluded that the saponin toxicity is due to the medicagenic acid-containing saponins which are precipitated by cholesterol i.e. not all saponins are biologically active so that the type of saponins present needs to be defined.

Livingston et al. (1979) reported that saponins were concentrated at higher levels in lucerne leaf protein fractions compared to the original lucerne. This may mean, therefore, that the effects of saponins may be increased due to the processing of leaf protein.

5.(ii) Oestrogenic Substances

Most research into phyto-oestrogens has centered on the subterranean clovers, though oestrogenic compounds have been identified in wheat, rye, oats and soybean meal (Pieterse and Andrews, 1956), barley grain (Bradbury and White, 1954) and lucerne (Stob et al., 1957).

Environmental and plant factors have been shown to affect the oestrogenic potency of lucerne e.g. strain differences, foliar pathogens, environmental stress factors, stage of plant growth and seasons. The drying of lucerne for hay reduces the activity of the material (Bickoff et al., 1960); commercial dehydration, such as in the production of lucerne meal, has a similar but smaller effect (Francis and Millington, 1965). Phenolic compounds with oestrogenic activity such as isoflavones (e.g. Biochanin A, genistein) and coumestrol cause uterine enlargement in immature or ovariectomised mice (Wong and Flux, 1962; Munford and Flux, 1961; Olfield et al., 1966).

The effects of oestrogenic compounds on egg production was demonstrated by the implantation of 1, 2, 4 or 8 diethylstilbesterol pellets (15 mg) into New Hampshire females at nine weeks of age. The age at first egg was retarded, but not in proportion to the dosage. Maximum production was attained later in the treated females but their total production was depressed (Fraps et al., 1956). Knuckles et al. (1976) have reported that lucerne LPC may contain higher coumestrol contents than fresh lucerne so that the oestrogenic activity appears to be unaffected by processing.

Discussion

From this brief review it can be seen that there is a wide range of compounds that may influence the nutritional value of LPC. General discussions of LPC's do not take into account the varied herbage species that have been used. Firm conclusions

can only apply to a product of known plant origin, including the particular strains, as these can vary in chemical composition.

The growth trials (Chapter 4) indicated that the availability of cystine and methionine and possibly of arginine in the complete diet was adversely affected by the inclusion of LPC. Reactive compounds which influence the digestibility process may have been formed as a result of processing. Apart from this possibility the growth trial results remove as the major cause any suggestion of LPC protein damage due to processing. This is because interaction of protein material during processing with either carbohydrates or phenolic compounds (e.g. chlorogenic acid or quinones) would lower only the availability of amino acids in the LPC for the growth of chickens and not the availability of amino acids contributed by other protein sources in the diet. Likewise conversion of methionine to its oxides would only adversely affect the quantity of methionine in the LPC that is available to the chicken for growth.

The "saponin fraction" possibly reduces food intake and/or has a toxic effect. The growth results, however, indicate that low food intake is not the major factor reducing the growth of chickens fed the LPC diet compared to the growth of chickens fed the SBM diet. However as mentioned earlier saponins may adversely affect digestive enzyme activity. Digestive enzyme inhibition may increase the chickens need for methionine and/or cystine (Liener, 1979). Though Birk (1969) considered such saponin-mediated activity as being slight, this may be influencing the amount of sulphur amino acid available to the LPC fed chickens for growth. Similarly no definite conclusions about the presence or absence of oestrogenic substances can be made from the growth studies.

If the above substances are removed from the list of compounds suspected of influencing the nutrtional value of LPC, few possibilities

are left that are capable of explaining the results of the growth trials obtained in this study. Factors influencing the digestion process, however, may explain the growth results. The presence of trypsin inhibitors (Humphries, 1980) would explain the need for additional methionine and cystine (Liener, 1979) and the lower amount of sulphur amino acid that was available to the chicken for growth. Also complex formations in the intestine of the chicken between protein material and, for example, phenolic compounds may lower the overall protein digestibility of the LPC diet. Such a growth depressing effect may, as in the case of high tannin sorghum (Elkin et al., 1978), be influenced by the addition of methionine.

Detoxification of harmful compounds by the chicken may explain the possible additional requirement for arginine. Detoxification could also influence the quantity of dietary methionine that was available to the chicken for growth.

Two areas were therefore studied to see if either:

a) detoxification of aromatic compounds (Chapter 6) and/or
b) factors influencing digestion (Chapter 7)

could explain the lower growth pattern due to feeding unsupplemented LPC diets to chickens.

CHAPTER 6: METHIONINE AND ARGININE - DETOXIFICATION OF AROMATIC COMPOUNDS PRESENT IN LPC

In the chicken, the detoxification of foreign aromatic compounds has been linked with an increased need for methionine and/or arginine (e.g. Lesley et al., 1976). Methionine can act as a methyl donor for methylation of aromatic compounds and arginine acts as a source of ornithine; the chicken can only synthesise ornithine from arginine (Tamir and Ratner, 1963). Ornithine, in turn, can be used by the chicken for the excretion of some aromatic compounds in a similar fashion to the use of glycine for hippuric acid synthesis in mammals. The study outlined in this Chapter therefore had the following aims:-

1. a) To test if methylation of phenolics does take place, and if so

   b) to discover whether the excretion level of the methyl carbon from L-(methyl ^{14}C) methionine was in fact significantly higher due to feeding LPC diets compared to SBM diets.

2. To test whether the level of ornithine excretion (derived from arginine) was significantly higher in chicks fed LPC diets.

The laboratory techniques and experiments used to determine whether arginine and methionine are required for detoxification described under the following headings:-

II Experiments

Experiment 1 (p 79): "Does methylation of phenolic compounds occur?"

(a) Separate collection of faeces and urine
(b) Initial urine extraction and chromatography
(c) Excreta Extraction
(d) Initial feeding of L-(methyl ^{14}C) methionine
(e) Initial identification of ^{14}C labelled compounds
(f) Secondary identification of ^{14}C labelled compounds

(A) Lower Phase ("Lipid Fraction") Analysis
(B) Upper Phase ("Water Fraction") Analysis

Experiment 2 (p103): "Is the excretion level of the methyl group from methionine higher due to feeding LPC?"

Experiment 3 (p107): Requirement for conjugation - measurement of the ornithine excretion level due to feeding LPC and SBM.

Discussion (p 110)

I- Laboratory Techniques

(a) Paper and Thin Layer Chromatography

Paper chromatography was carried out in both ascending and descending direction using Whatman No. 1 and 3 MM paper. Small scale chromatography used glass jars and Whatman No. 1 paper. Larger scale chromatography used Whatman No. 3 MM paper and large glass chromatography tanks.

Lipids were separated by thin layer chromatography (TLC) on 0.25 mm silica gel G. (Merck, Germany) plates. The plates were prepared as specified by the manufacturer.

Silver nitrate plates were prepared by slurrying the absorbent silica gel G with 5% (w/v) $AgNO_3$ solution. Two mls of 5% (w/v) $AgNO_3$ (BDH Ltd., England) solution was used for every g of absorbent giving 10% (w/w) $AgNO_3$ in silica gel (Nichols and Moorehouse, 1969). The layers were then allowed to set for 30 minutes before activation at 110°C for 1 hour. Plates were freshly prepared each time they were required.

(b) High Voltage Electrophoresis

The samples were applied to Whatman 3 MM paper. The paper was moistened in pH 2.1 buffer (80 mls glacial acetic acid, 20 mls formic acid in 1 litre) and placed in a water cooled Michl solvent tank.

An electrical potential of 3.1 kV in pH 2.1 buffer was applied for 1 hour.

(c) Determination of Radioactivity

^{14}C radioactivity was determined with a Beckman Model LS 8000 Scintillation Counter. Aqueous samples were counted in

"Triton X-100 toluene scintillation solvent":-

9 g PPO (2,5 Diphenyloxazole, Sigma Chemicals, U.S.A.)
0.3 g of POPOP (1,4 Di 2-(5 phenyloxazolyl) benzene, Koch-Light Lab., England)
1 litre Triton X-100
2 litres toluene

and non-aqueous samples were counted in a Toluene scintillation solvent:-

6 g PPO
0.24 g POPOP
1 litre toluene

Radioactivity on paper chromatograms and TLC plates was detected by scanning on a Packard Model 7200 Radiochromatogram Scanner. Autoradiographs of the paper chromatographs were obtained by exposing the plates to Kodak R.P. Royal X - Omat X Ray film for 2 weeks, 1 month or 6 months in a light proof container. Films were developed and fixed with Kodak liquid X Ray developer and fixer. The movement of radioactivity on paper was also measured by cutting each chromatogram into 2 x 2 cm squares and each square was then counted in the appropriate scintillant.

(d) Stains

Due to the preliminary nature of the study in which the stains were used, only general stains were used. Their specificity is discussed below.

Phenolic Identification: Sulphanilic Acid and p-Nitroaniline Stains

The paper was sprayed first with:

(a) 0.3% (w/v) sulphanilic acid or p-Nitroaniline (BDH Ltd., England) in 8% (w/v) HCl and $NaNO_2$ (5% w/v) and then with

(b) 20% (w/v) sodium carbonate.

In reference to the specificity of sulphanilic acid and p-nitroaniline, Swain (1969) stated that "All compounds containing phenolic hydroxyl groups which have a free para or ortho position and which do not contain strongly deactivating substituents or stearically hindering groups react with diazotized amines at a suitable pH to give coloured azo dyestuffs."

Prussian Blue (Ferric chloride-potassium ferricyanide)

Aqueous 0.5% (w/v) solutions of each salt, potassium ferricyanide and ferric chloride (BDH Ltd., England), were mixed in equal proportions before spraying. If a permanent record was required after development the paper was washed in 0.1 M HCl.

The ferric chloride-potassium ferricyanide stain acts as an oxidising agent. Most phenols, except those which are highly substituted, are readily oxidised. Swain (1969) stated that "The most useful (oxidising agent) is the ferric chloride-potassium ferricyanide reagent which is fairly specific for phenolic compounds."

Protein/Amino Acid Identification: Ninhydrin

Solutions of cadmium acetate (15 g cadmium acetate, 300 mls glacial acetic acid and 600 mls water) and 1% (w/v) ninhydrin solution (Sigma Chemicals, U.S.A.) in acetone were mixed in the proportions of 3 to 17 respectively immediately before the strips were dipped in the stain.

"Ninhydrin is specific for the aliphatic or alicyclic primary amino groups. Secondary, tertiary, and quaternary amines, amides and amino-substituted aromatic compounds do not react. Exception: proline gives a yellow spot." Weil-Malherbe (1969)

Lipid Identification: 2',7'-Dichlorofluorescein

Lipids were sprayed with 0.05% (w/v) 2',7'-dichlorofluorescein (BDH Ltd., England) in methanol and viewed under U.V. light. This reagent is a general lipid spray (Olley, 1969) and reacts

with non-polar lipids, saturated and unsaturated (Mangold and Malins, 1960). The lipids become visible in ultraviolet light after spraying.

(e) Gas-Liquid Chromatography

The methyl esters of fatty acids were analysed using a Varian-Aerograph Model 1520 gas chromatograph fitted with a flame ionisation detector. The glass column (183 cm x 0.3 cm id) was packed with 9% (w/w) diethylene glycol succinate polyester (DEGS; Ann, Labs. Connecticut, U.S.A.) on Chromosorb Q (60-70 mesh) (Applied Sciences Laboratories, California, U.S.A.). The column was fitted with an effluent stream splitter diverting 3/4 of the sample to the collector jet and the remainder passed to the flame ionisation detector. Samples were injected on to the column using a 10 microlitre syringe (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia).

The column was held at 159°C with the injector and detector temperature at 200°C and 230°C respectively. The carrier gas was oxygen-free nitrogen flowing at 45 mls/min. A standard methyl ester mixture of 14:0, 16:0, 17:0, 18:0, 18:1, 18:2, 18:3 was used to standardise each determination.

Methylation of Fatty Acids

The lipid fraction was refluxed with methanol-benzene-concentrated sulphuric acid (20:10:1 v/v/v) for 90 minutes. The methyl esters of the component fatty acids were extracted with light petroleum spirits (b.p. 60-80°C) (Nichols et al., 1967).

Hydrogenation - (Brian and Gardner, 1968)

The methyl esters were hydrogenated for 3 hours in hexane under hydrogen, with 5% (w/w) Pt in charcoal (Koch-Light Lab., England) acting as a catalyst.

Bromination (Brian and Gardner, 1968)

After hydrogenation the hexane was removed with nitrogen. The methyl esters were dissolved in 2 mls anhydrous diethyl ether and cooled to zero ^{o}C. One millilitre of reagent grade bromine (BDH Ltd., England) in ether (1:5 v/v) was added. The ether and excess bromine were later evaporated with nitrogen.

Collection of Radioactive Effluent

Samples of the radioactive effluent were collected in a pyrex tube loosely packed with approximately 0.2 g of glass wool, which was moistened with toluene scintillant. The methyl esters were eluted from the collection tubes by 10 mls of toluene scintillation solvent into counting vials. A small hand-operated air pump was used to flush the solvent through the glasswool. Each sampling period (as monitored on the chart recorder) was collected in separate tubes.

II Experiments

Experiment 1: "Does Methylation of Phenolic Compounds Occur?"

The purpose of this experiment was to identify whether or not the methylation of phenolic compounds was occuring and to develop a basic method for subsequently testing the excretion level statistically. To aid the identification process an attempt was made to collect the urine and faeces separately by surgical modification of the chickens. This attempt was subsequently abandoned and L-(methyl ^{14}C) methionine was fed to an unmodified chicken. The procedures used for the identification of the radioactively labelled compounds and also the surgical procedures are described below.

(a) Separate Collection of Faeces and Urine

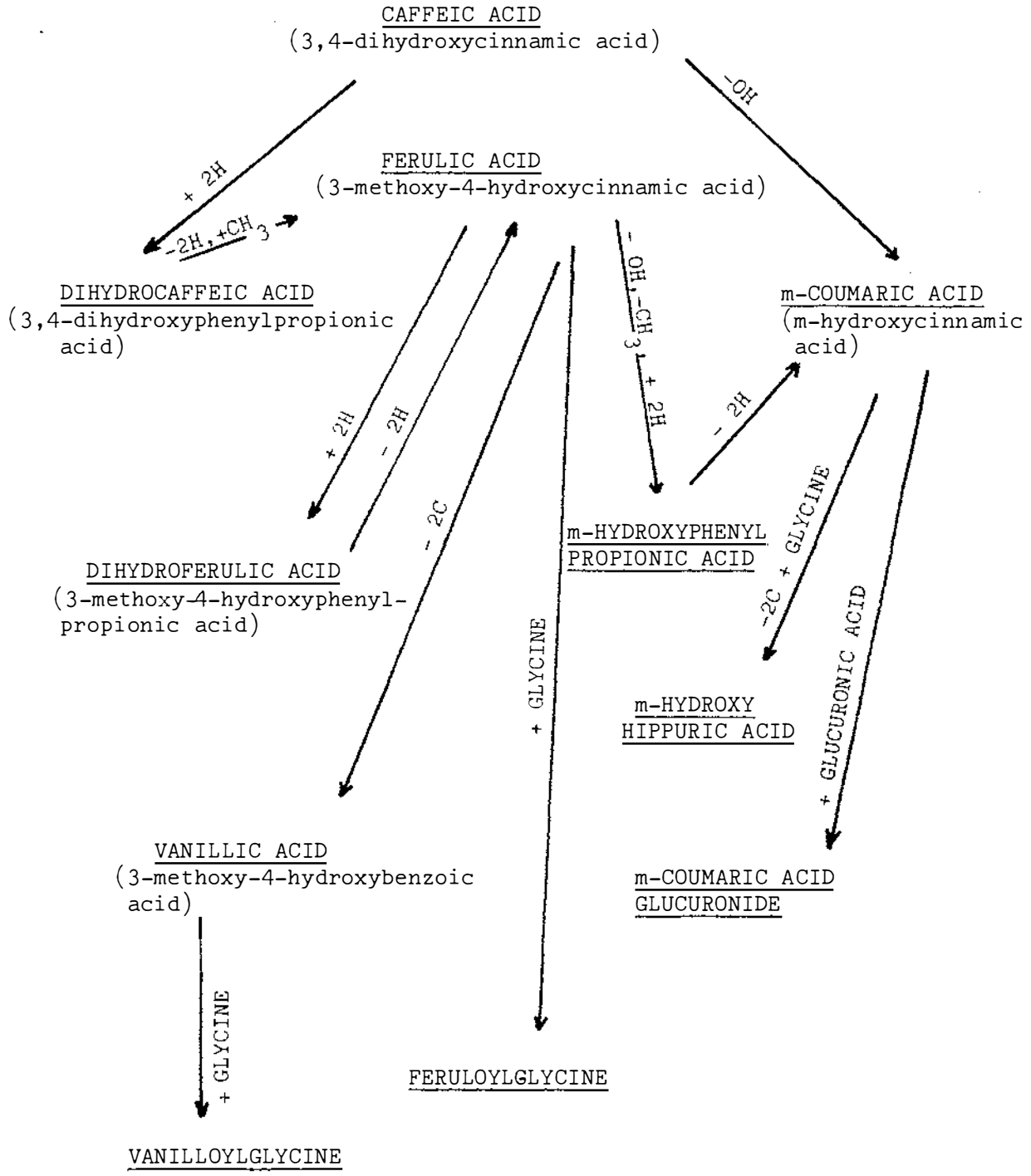
Two methods were investigated for the separate collection of faeces and urine.

(i) Mr A.M. Alexander, Department of Veterinary Pathology and Public Health, Massey University, carried out the surgical exteriorization of the rectum as described by Fussell (1969) and Okumura (1976) on 23 Sykes strain cockerels, aged five months plus. The operation presented little problem though the subsequent survival time was variable. The majority only survived a matter of weeks whereas 5 survived 1½ to 2½ months.

As suggested by Fussell (1969) a tube was inserted into the artificial anus during the operation, to aid post-operative healing. The tube was later removed. In some cockerels a tube was not inserted and this appeared to have no adverse effect on the subsequent healing.

The cockerels were either introduced to a normal diet directly after the operation (Okumura, 1979) or to a soluble milk protein diet (Fussell, 1969) which was fed for ten days before normal feeding. Neither diet appeared to affect the survivability of the cockerels. The cockerels excreted a solid faecal stool for 2-6 weeks

Fig. 7: Proposed Scheme for the metabolic transformations of caffeic acid (from Booth et al., 1957)



and then, for no apparent reason, the droppings became wet. The bowels then required daily flushing out. Over the subsequent weeks the cockerels slowly lost condition even though their food intake was still 80-120 g/day. Autopsy revealed that the cockerels were normal and had no apparent infection. Simkiss (1970) commented that adult cockerels are more difficult to surgically modify than the hen because the intestine is shorter and thinner. Pullets were unfortunately not available for use in the experiment.

Urine samples were collected from the four surviving cockerels using the method discussed by Fussell (1969). The cockerels were fitted with a harness to which was attached a collection bag.

(ii) Another method for separating urine and faeces from intact unanaesthetised chickens was also used. Four cannulae as described by Bokori (1961) were made by S.E. Compton, Agricultural Enginerring, Massey University. Short term urine collections of 1-3 hours were successfully made. Long term (greater than 4-6 hours), the faeces compacted in the faecal vent causing the cannula to shift from its original position.

Urine was successfully collected by both methods, i.e. surgical and cannula, from chickens fed with a 15% LPC diet. The ingredient and nutrient composition of the diet is described in Appendix A, Tables XV and XVI (Diet B was used).

Six urine samples were collected by both methods outlined above four samples from the surgically modified cockerels and two samples from the cannulated cockerels. These 6 samples were examined as described below.

b) Initial Urine Extraction and Chromatography

Of the numerous phenolic compounds in plants chlorogenic acid (3 - caffeoylquinic acid) is a commonly occuring plant ester (Schleine, 1978). The fate of the caffeic acid moiety was studied by Booth et al. (1957) and the resulting metabolic pathways and compounds are summarised in Fig. 7.

This indicates that the methylated metabolic products were vanillic acid, ferulic acid and dihydroferulic acid.

Examples of products which arise from the metabolism of plant flavonoids and tannins are protochatechuic acid and homoprotochatechuic acid (Parke, 1968). The possible metabolism of these compounds include methylation, dehydroxylation and conjugation reactions illustrated in Fig. 8 (Parke, 1968), with vanillic and homovanillic acid resulting from the methylation processes. Consequently these two acids were considered to be suitable standards to test for the presence of methylated compounds in the urine.

The urine collected by either the surgical or cannula techniques was separately prepared as described below by following the method of De Eds et al. (1957):-

a) Addition of 20% (w/v) NaCl solution to equal 25% of the urine volume

b) Acidified with H_2SO_4 (Bray et al., 1950)

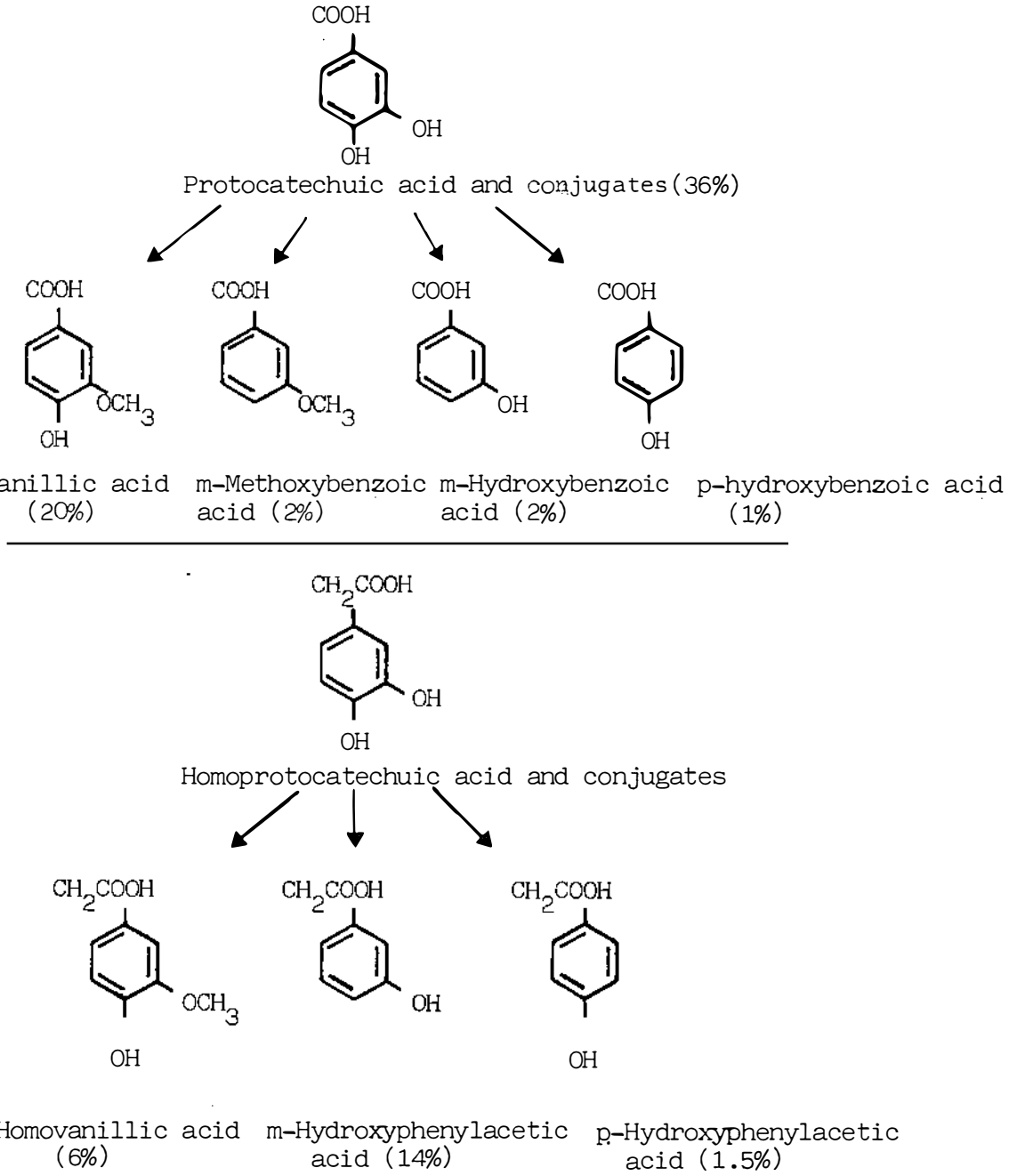
c) Partitioned 3 x with ether

d) Volume of ether extract reduced under nitrogen

e) The resulting residue was dissolved in acetone.

The "urine ether fraction" was subjected to two dimensional descending paper chromatography using chloroform:acetic acid: water (2:1:1 v/v) followed by 20% (w/v) KCl on Whatman No. 1 paper (De Eds et al., 1957). The air dried chromatograms were then sprayed with sulphanilic acid or p-nitroaniline (Bray et al., 1950). Standards of vanillic acid (3-methoxy-4 hydroxybenzoic acid), homovanillic acid (3 methoxy-4 hydroxyphenylacetic acid) and hydroxybenzoic acid (B.D.H. Ltd., England) were also chromatographed under the same conditons.

Fig. 8: Metabolism of Protochatechuic Acid and Homoprotochatechuic Acid (-from Parke, 1968)



A typical chromatogram is shown in Fig. 9 and the colours developed with spraying are listed. Fourteen spots were developed with either sulphanilic acid or p-nitroaniline spray. Three of the areas had the same Rf values as the three standards vanillic acid, homovanillic acid and hydroxybenzoic acid. These metabolic products of plant phenolic compounds were therefore possibly being produced and excreted by the chickens fed diets containing LPC.

c) Excreta Extraction

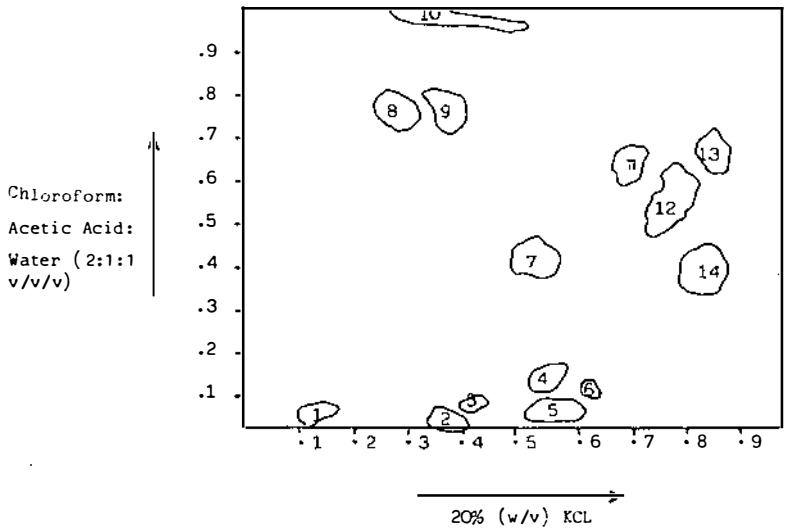
Even though urine was successfully collected from cockerels modified by either the surgical or the cannulae techniques, the health and life span of these cockerels was not considered to be reliable. Therefore the total excreta was collected from unmodified cockerels and examined to see if a similar result could be achieved to that obtained with the "urine ether fraction."

Three normal Sykes strain adult cockerels were maintained for a week on a 15% LPC diet (see diet B, Appendix A, Tables XV and XVI) after which 3 x 24 hours excreta collections were made. The three excreta samples were then extracted as described by Wong (1962) and Glencross et al. (1972).

The samples were refluxed in alcohol for 15 minutes, filtered and the residue re-extracted in boiling ethanol. The filtrate was concentrated and the ethanol was reduced to 70% (v/v) ethanol by the addition of water. The alcoholic suspension was extracted four times with light petroleum spirits (boiling range 60-65^{o}C). The alcoholic fraction was reduced under pressure. The residue was suspended in water and extracted four times with diethyl ether. The "excreta ether" samples were pooled and retained as the "excreta ether fraction."

The "excreta ether fraction" was chromatographed using the same developing solvents as described for the "urine ether fraction".

Fig. 9: Two-dimensional Schematic Chromatogram of the "Urine Ether Fraction." The Coloured Reactions of the Spots With Suphanilic Acid and p-nitroanaline are Shown Below. The Position of the Standards Used is also Shown.



Colour Reactions of the Corresponding Spots from the Above Chromatogram

	Sulphanilic acid	p-nitroanaline	Standard (corresponding position to numbered spots)
1	yellow	orange-brown	
2	yellow-orange	brown	
3	pink-purple	grey	
4	yellow	brown	
5	yellow	brown	
6	yellow	brown	
7	yellow	purple	hydroxybenzoic acid
8	pink	purple	vanillic acid
9	yellow	brown	
10	pink	brown	homovanillic acid
11	yellow	purple	
12	yellow	purple	
13	pink-red	purple	
14	orange	purple	

The resulting chromatographs were similar to Fig. 9 which indicated that the method used was suitable for the extraction of phenolics from excreta. Consequently, unmodified cockerels were used in the subsequent experiments, and the total excreta was analysed.

d) Initial Feeding of L-(methyl ^{14}C) Methionine

Dietary methionine L-(methyl ^{14}C) methionine was fed to an adult cockerel in order to determine whether phenolics were methylated and excreted.

An individually housed Sykes strain adult cockerel was fed the experimental 15% LPC diet for 5 days prior to feeding L-(methyl ^{14}C) methionine (Radiochemical Amsterdam, Australia Pty. Ltd). A 15% LPC pellet containing 200 µCi L-(methyl ^{14}C) methionine was force fed using the method described by Sibbald (1976). Six 12 hour excreta collections in alcohol were made. The excreta samples were pooled and extracted using the method described previously on page 84 .

The LPC diet used is described in Appendix A, Tables XV and XVI.

e) Initial Identification of ^{14}C Labelled Compounds

The "excreta ether fraction" was prepared and chromatographed as described above (Wong, 1962; Glencross et al., 1972). Three chromatographs were autoradiographed. Chromatographs were also cut into 2 x 2 cm squares and counted in Toluene Scintillation Solvent.

None of the phenolic compounds which were indicated as being present in Fig. 9 were labelled with radioactive ^{14}C. Following the above result, the movement of radioactive compounds in the "ether excreta fraction" was studied using one dimensional paper chromatography.

Each chromatograph was developed using a single developing solvent by ascending paper chromatography. The chromatograph was then scanned to indicate the position of the radioactive compound(s). This process was repeated 8 times using the different developing solvents listed in Figs. 10 and 11. The results of the radioactive movement are summarised in Figs. 10 and 11 and show that the radioactive material was associated with the non-polar fraction (Fig. 10), as there was virtually no movement with more polar solvents (Fig. 11). It was therefore concluded that the radioactive material was non-polar ("lipid") material.

f) Secondary Identification of ^{14}C Labelled Compounds

Because the ^{14}C labelled compound(s) appeared to be non-polar, a different partitioning of the initial ethanol extract was used. Partitioning of the 90% LPC excreta ethanol extract was carried out with chloroform:methanol:water (8:4:3 v/v/v) (Folch et al., 1957). The radioactive partitioning was 1:2.3 (upper:lower respectively).

The two phases were separated and examined. The examination of the upper phase is described on page 98 and that of the lower phase is described below.

(A) Lower Phase ("Lipid Fraction") Analysis:

The following tests were carried out:-

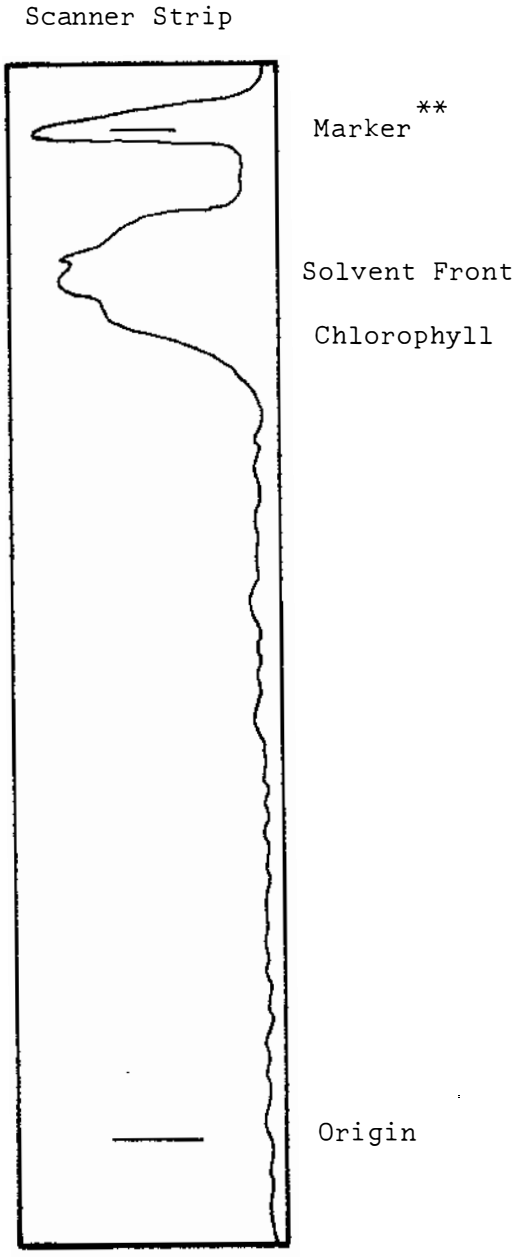
(a) Test for the presence of ^{14}C in phospholipids
(b) Test for the presence of ^{14}C in free fatty acids, monoglycerides, diglycerides and triglycerides
(c) Test for the ability of the unknown compounds to be methylated
(d) Test for the degree of saturation
(e) Gas liquid chromatography (G.L.C.) of methyl esters

The results of these tests were as follows:-

Fig. 10 : One Dimensional Development of the "Ether Excreta" Fraction Using Various Developing Solvents *

Developing Solvents Used:-

1. Chloroform:Acetic Acid: Water (2:1:1 v/v/v)
2. Toluene:Acetic Acid:Water (125:72:3 v/v/v)
3. Butanol:Acetic Acid:Water(5:4:1 v/v/v)
4. Chloroform:Methanol:Water(65:25:4 v/v/v)
5. Petroleum ether (60:80 v/v): acetone in propanol (90:10:0.45 v/v/v)



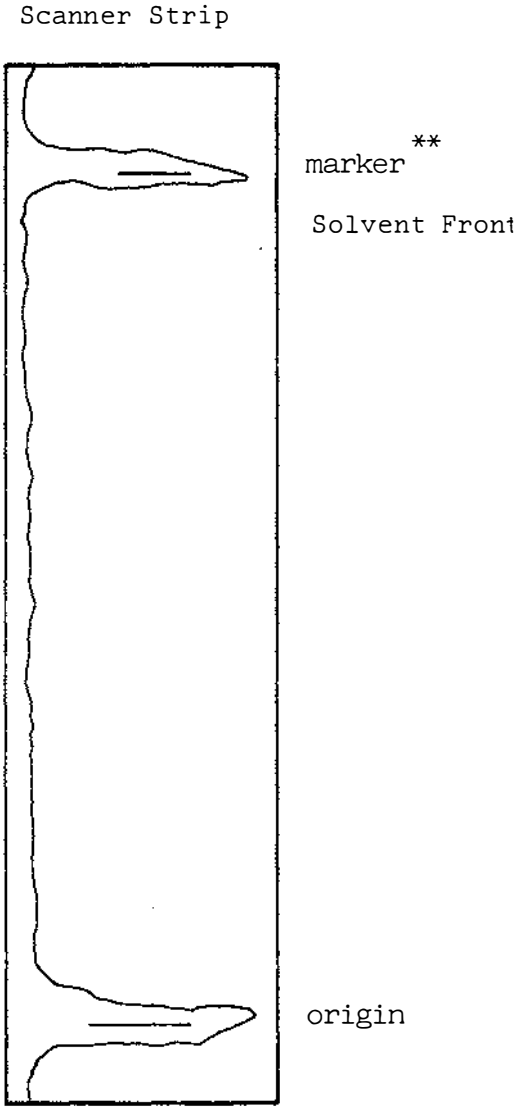
* Each chromatograph was developed using a single developing solvent by ascending chromatography. The chromatograph was then scanned to indicate the position of the radioactive compound(s). This process was carried out using 5 different developing solvents.

** A radioactive marker (^{14}C hexadecane) was applied to the chromatograph after the chromatography was complete and prior to scanning. This allowed correct comparison of the chromatograph and the scanner strip.

Fig. 11 : One Dimensional Development of the "Ether Excreta" Fraction Using Various Developing Solvents

Developing Solvents used:-

1. 15% (v/v) Acetic Acid
2. 20% (w/v) Potassium Chloride
3. 2% (v/v) Acetic Acid



* Each chromatograph was developed using a single developing solvent by ascending chromatography. The chromatograph was then scanned to indicate the position of the radioactive compound(s). This process was carried out using 3 different developing solvents.

** A radioactive marker (^{14}C hexadecane) was applied to the chromatograph after the chromatography was complete and prior to scanning. This allowed correct comparison of the chromatograph and the scanner strip.

a) Analysis of the presence of ^{14}C in phospholipids

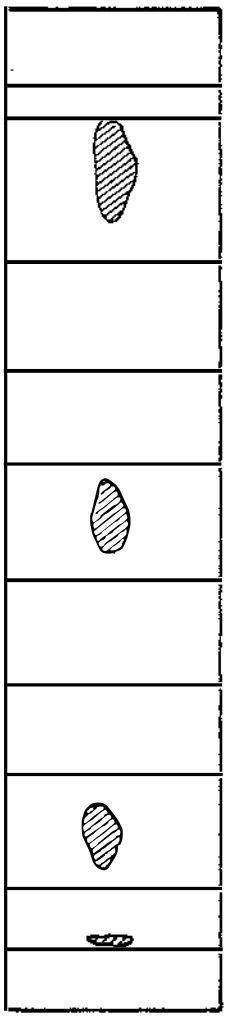
TLC plates were used. The developing solvent was chloroform: methanol:water (65:35:3 v/v/v). The standards phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin were identified by spraying with 2',7'-dichlorofluorescein. The 9 areas, as indicated in Fig. 12 below, were then scraped off, extracted in methanol:chloroform (1:2 v/v) and counted in Toluene Scintillant on solvent.

The results demonstrated that the radioactivity was not in the area of the neutral lipids or phosphatidyl choline. Phosphatidyl ethanolamine does not contain a methyl group and so the radioactivity detected in this region must be due to some other compound.

b) Test for the presence of ^{14}C in the free fatty acids, monoglycerides, diglycerides and triglycerides:

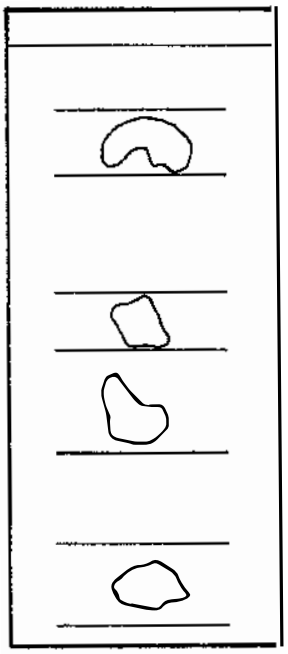
Thin layer plates of silica gel G were used. The developing solvent was diethyl ether: hexane: acetic acid (40:60:1 v/v/v). The standards used were palmitic acid (free fatty acid), monoglyceride, distearoylglycerol (diglyceride) and peanut oil (triglyceride) and these were detected by staining with 2',7'-dichlorofluorescein. Seven areas, indicated in Fig. 13 were scraped off the plate and extracted in chloroform:methanol (2:1 v/v). The major area of radioactivity was in the free fatty acid section of the plate.

Fig. 12: Test For ^{14}C in the Phospholipids



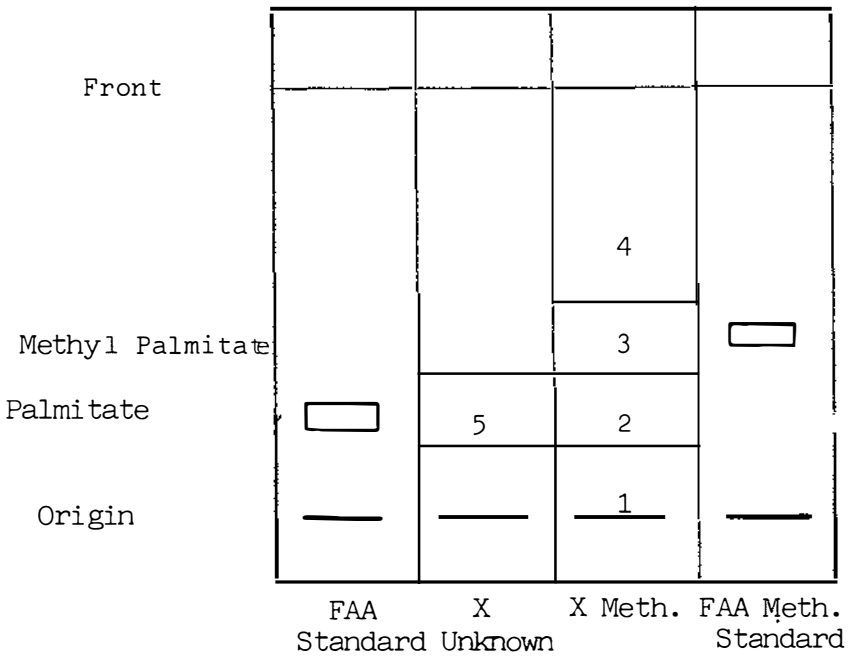
Standard	Area	% Radioactivity
Front	9	41.0
Phosphatidyl ethanolamine	8	57.1
	7	0 .4
	6	0 .5
Phosphatidyl choline	5	0 .5
	4	0 .5
	3	0
Sphingomyelin	2	0
Origin	1	0

Fig. 13: Test for the Presence of ^{14}C in the Free Fatty Acids

Standard	TLC plate	Area	Radioactivity %
Front			
		7	0.7
Triglyceride		6	12.7
		5	2.8
Free Fatty Acid		4	67.6
Diglyceride		3	3.0
		2	2.8
Monoglyceride origin		1	10.4

c) Methylation: The extract was then treated by the methylation procedure given in Methods, page 77 . Palmitate was also methylated using the same method to provide a check on the method, and to act as a standard. Methylated and unmethylated compounds (standard and extract) were chromatographed on a TLC plate. The developing solvent was diethyl ether:hexane:acetic acid (40:60:1 v/v/v). The standards were sprayed with 2',7'-dichlorofluorescein and from the position of radioactive area 3 compared to area 5 (Fig. 14) it was shown that an unknown compound(s) in the extract had been methylated as it now moved with methyl palmitate.

Fig. 14: The Effect of Methylation on the Movement of Radioactive Fraction



Area	% Radioactivity
5	0.5
4	1.2
3	87.2
2	4.5
1	7.1

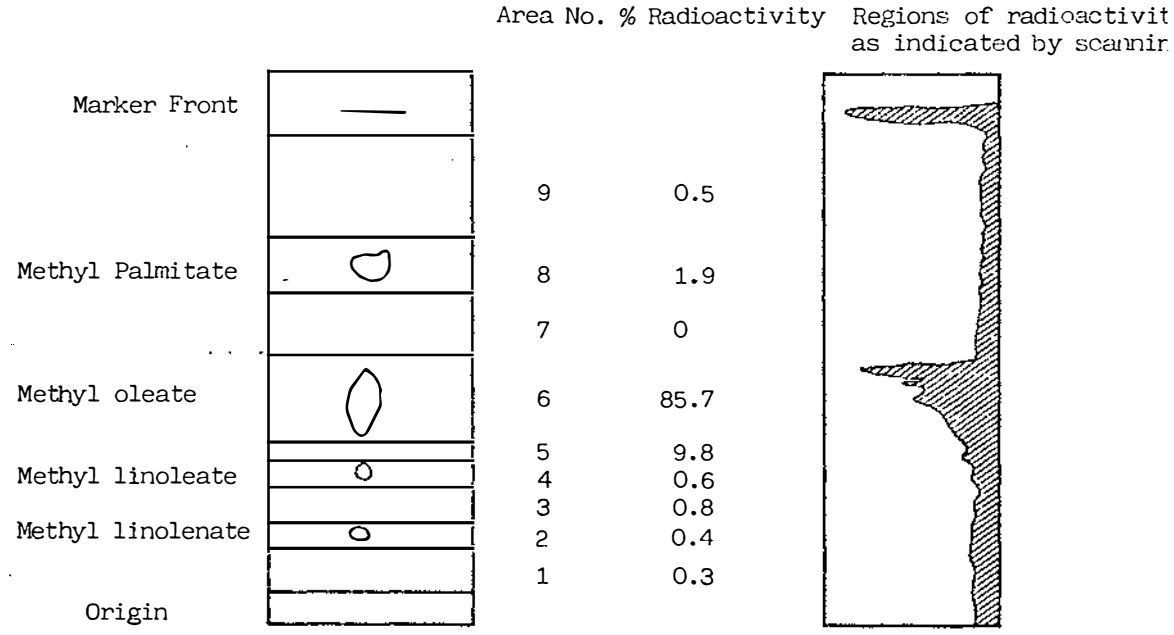
d) Test for degree of Saturation (TLC on $AgNO_3$ plates):

The methylated unknown compound was chromatographed on $AgNO_3$ TLC plates. The standards methyl palmitate, methyl oleate, methyl linoleate and methyl linolenate were prepared by the same method and also chromatographed with the unknown.

The chromatoplate was developed in chloroform:methanol (99.5:0.5 v/v). After spraying with 2',7'-dichlorofluorscein, the 9 areas as indicated by the position of standards (shown in Fig. 15) were then scraped off and extracted in ether:methanol (9:1 v/v). The silver was removed by adding 0.5 mls of 1% (w/v) NaCl in methanol:water (9:1 v/v). The extract was then counted in Toluene Scintillant. Another TLC plate was prepared by applying the standards on top of the unknown. This was then developed as above, scanned for radioactivity using a Packard Radiochromatogram Scanner Model 7200 and then sprayed with 2',7'-dichlorofluorscein to identify the areas.

Most of the radioactivity was present in the region corresponding to methyl oleate, so that the unknown appeared to be an unsaturated compound(s) with one double bond.

Fig. 15: The Movement of the Radioactivity (^{14}C) on Silver Nitrate TLC Plates



e) G.L.C. of Methyl Esters

(i)G.L.C. of methyl esters - LPC excreta extract

Gas liquid chromatography of the methylated unknown compound was carried out (as discussed on p 77) together with a series of methylated fatty acids to act as standards. The results, given in Table XVII showed that most of the radioactivity appeared in the area of the 18:2 fraction. Since this result differed from that using the $AgNO_3$ - TLC plate, the sample was mildly hydrogenated, so as to remove unsaturated fatty acids,and brominated as for identification of a cyclopropane fatty acid (Brian and Gardner, 1968).

Table XVII: The Distribution of Radioactivity after Methylation

Fractions Collected	Standard	% Radioactivity
1	Hexane	0.10
2	$C_{14:0}$	0.24
3		0.00
4	$C_{16:0}$	0.24
5	$C_{17:0}$	0.36
6		0.24
7	$C_{18:0}$	1.31
8	$C_{18:1}$	1.60
9	$C_{18:2}$	88.40
10		1.60
11	$C_{18:3}$	2.49
12		3.58

The two samples (a) hydrogenated sample and the
(b) brominated sample

where analysed consecutively on the Gas Liquid Chromatograph. The results are shown in Table XVIII.

Table XVIII: The Distribution of Radioactivity after Hydrogenation and Bromination

Fraction Collected	Standard	Hydrogenation % Radioactivity	Bromination % Radioactivity
1		0.3	0
2	$C_{14:0}$	0.4	0
3	$C_{16:0}$	0.4	0
4	$C_{17:0}$	0.4	0
5	$C_{18:0}$	0.5	1.3
6	$C_{18:1}$	0.5	0.5
7	$C_{18:2}$	19.8	0.8
8		76.5	1.7
9	$C_{18:3}$	1.2	0.9

After hydrogenation the area of radioactivity was more easily resolved due to the removal of 18:2 fatty acid by the hydrogenation process. The radioactivity did not appear to move from its original position. This indicated that the compound was saturated. The subsequent reaction with bromine slowed the movement of the radioactively marked compound(s) which confirmed that the compound(s) was a C_{19} cyclopropane fatty acid.

(ii) G.L.C. of Methyl Esters - SBM excreta extract

In a subsequent experiment, Experiment 2, a pooled excreta sample from SBM fed chickens was prepared for GLC analysis. In this experiment 9 chickens, which were being fed a 13% SBM diet, were intubated with 6.25 μCi L-(methyl ^{14}C) methionine. The excreta was collected for 3 x 24 hours. An excreta subsample from each chicken fed the 13% SBM diet was pooled and extracted in hot ethanol as described on p 84. The extract was then partitioned using chloroform:methanol:water (8:4:3 v/v/v). The lower fraction was methylated using the method described on p 77 . Gas liquid chromatography of the methylated unknown was carried out, as previously described, together with a series of methylated fatty acids to act as standards. This demonstrated that the majority of the radioactivity (70%) had a retention time in the area between 18:2 and 18:3 (area 8 in Table XVIII) which indicated that C_{19} cyclopropane fatty acid was also present in the lower phase extract from excreta of the chickens fed the 13% SBM diet.

(B) Upper Phase ("Water Fraction") Analysis

After the initial extraction of the excreta from LPC fed chickens in ethanol and subsequent partitioning with chloroform:methanol: water (65:35:3 v/v/v), the upper phase was retained as the "water fraction" (described on p 87).

Initially a two dimensional chromatogram (illustrated in Fig. 16) of the unhydrolysed water fraction was developed in the ascending direction using the solvent system (Feeny and Bostock, 1968):

a) 2% (v/v) acetic acid ("HAc") followed by
b) n-butanol/acetic acid/water (60:15:25 v/v/v) ("BAW").

The "water fraction" was also hydrolysed by refluxing the sample for 20 hours in 6 M HCl under nitrogen and then chromatographed using the two dimensional chromatography as above. The two dimensional chromatograms of the unhydrolysed and hydrolysed sample were compared by staining with prussian blue or ninhydrin, radioactive counting (Fig. 16) and sensitivity to U.V. light.

In the unhydrolysed sample, a ninhydrin and prussian blue stainable area and the radioactive area had the same R.f. value. Subsequent chromatography of the hydrolysed sample separated the ninhydrin and prussian blue areas (Fig. 16). The radioactivity moved to two separate areas (a prussian blue stained area and a ninhydrin stained area) which suggested that either:-

a) the "phenolic" and "protein" material moved in a similar fashion and that hydrolysis destroyed the structure of both the "protein" and "phenolic" compounds so that they moved differently after hydrolysis.

or b) The "phenolic" and "protein" material was associated together and hydrolysis destroyed this association so that they moved separately after hydrolysis.

A methionine standard which was chromatographed under the same conditions as above was found to have the same R.f. value as the radioactive-ninhydrin sensitive area of the hydrolysed "water fraction." This result was checked by using the following technique:-

Two dimensional development of the hydrolysed radioactive sample and the methionine standard was carried out using electrophoresis (3 kV in pH 2.1; 1 hour) followed by 17 hours chromatographic development using butanol:acetic acid: water (5:1:4 v/v/v) as the developing solvent. The results from the second technique confirmed that methonine moved with the radioactive ninhydrin fraction.

The movement of the radioactivity therefore suggested that

(i) phenolic compounds had been methylated and

(ii) radioactive methionine was probably present in the "water fraction."

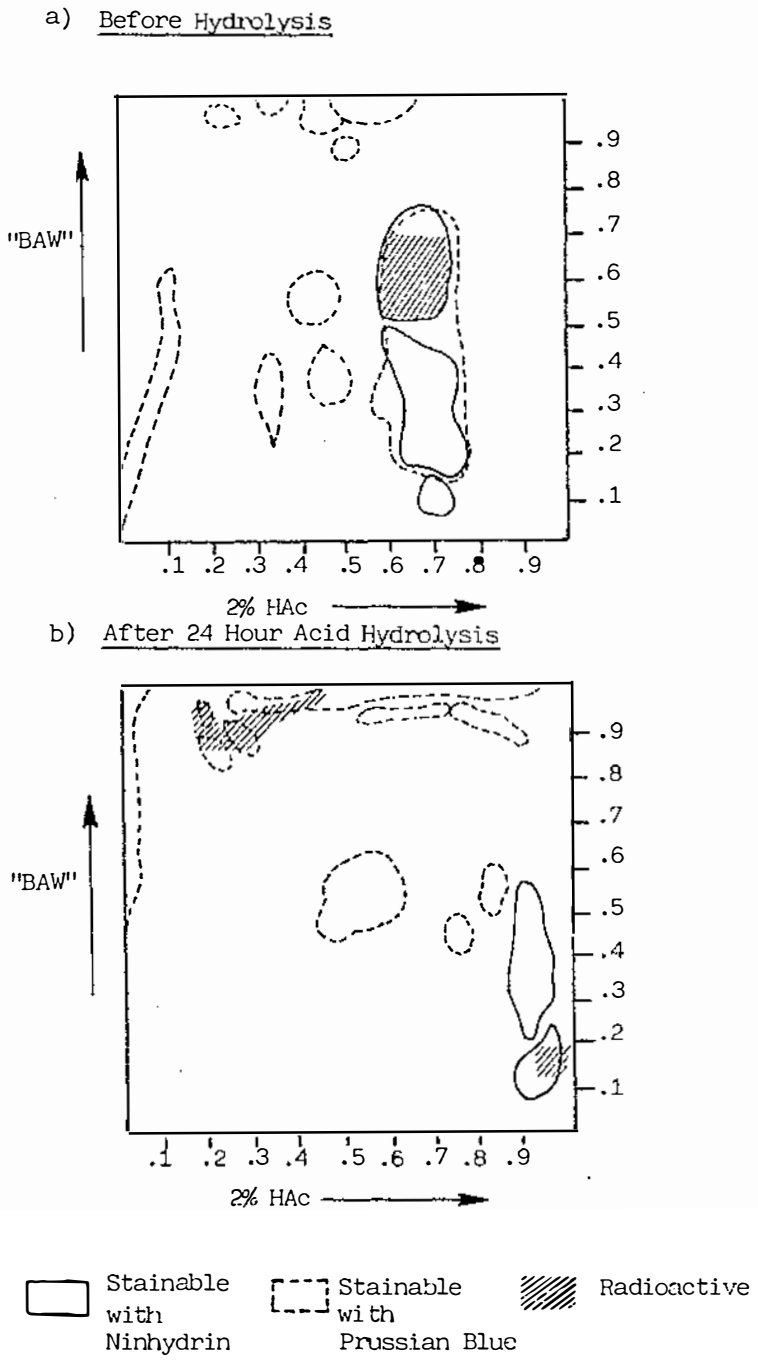
In order to determine whether the unhydrolysed samples of "phenolics" and "protein" were associated together, development of the unhydrolysed "water" fraction was carried out on a larger scale using reverse electrophoresis (3 kV in pH 2.1 buffer for 1 hour) initially. Four radioactive regions, as illustrated in Fig. 17 were formed during electrophoresis.

(i) One region moved towards the positive electrode i.e. has a negative charge at pH 2.1. This region was unstainable both with ninhydrin and prussian blue. It was not responsive to ultraviolet light. This region was unidentified.

(ii) The origin was stainable with prussian blue and faintly stained with ninhydrin.

(iii) Two regions moved towards the negative electrode - one region was stainable with prussian blue and the other with ninhydrin.

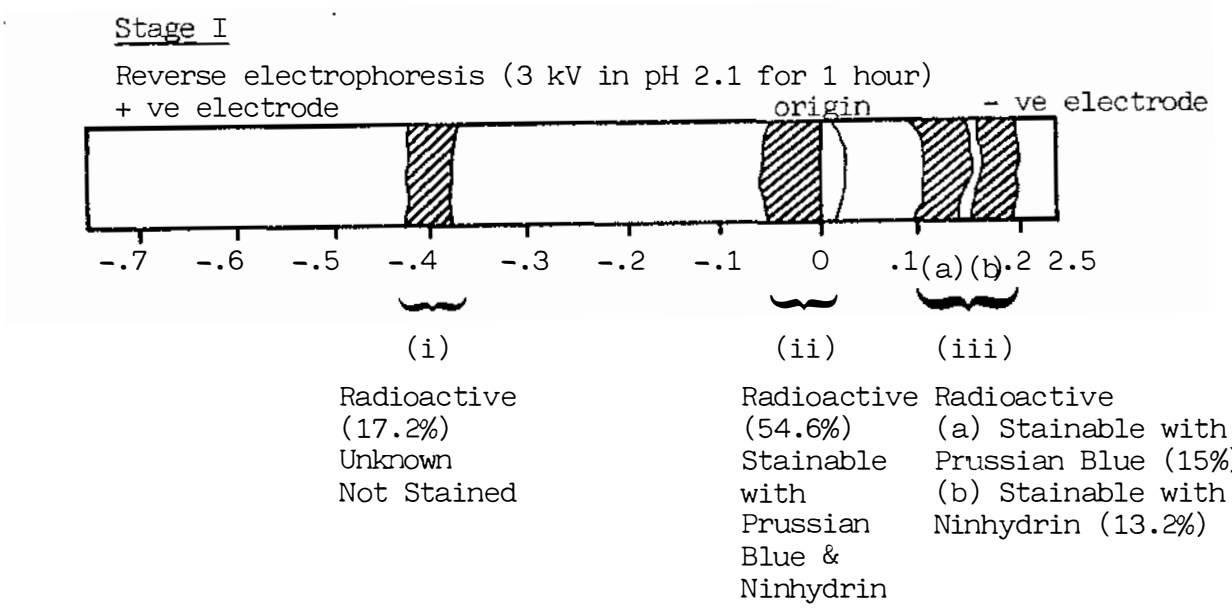
Fig. 16: Two Dimensional Development ("BAW"; 2% HAc) of the "Water Fraction" Before and After Acid Hydrolysis.

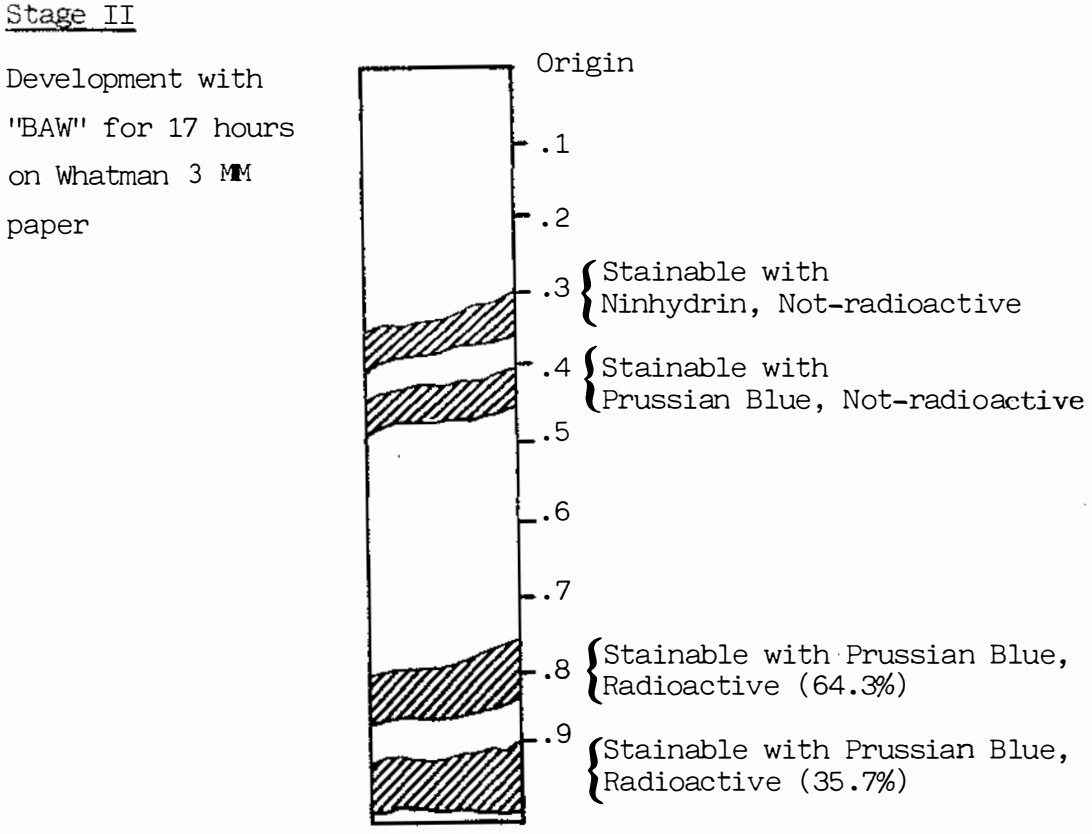


As the origin, area (ii), was stainable with both ninhydrin and prussian blue, this was cut out and sewn onto Whatman 3 MM and developed with "BAW" for 17 hours (Fig. 17). Four discrete stainable areas were found (Fig. 17). This suggested that there was probably no complex formation between the phenolic and protein compounds.

These results showed that there were at least 3 types of compounds in the "water fraction" which were radioactively labelled. After electrophoresis there were 2 regions of radioactivity which were sensitive to prussian blue and so were presumably phenolic in nature; these two regions accounted for nearly 70% of the total radioactivity in the "water fraction". In a second zone of radioactivity (containing 17% of the total "fraction" radioactivity) there was a very acidic compound(s), but this was not identified. The third radioactive compound was assumed to be methionine; this accounted for 13% of the total radioactivity in the "water fraction."

Fig. 17: The Results of the Two Stage Determination to Test Whether the Unhydrolysed "water fraction" contained Phenolic and Protein Material Associated Together. (% total radioactivity given in brackets)





Experiment 2: "Is the Excretion of the Methyl Group from Methionine higher due to feeding LPC?"

The purpose of Experiment 2 was to compare the total amount of ^{14}C from L-(methyl ^{14}C) methionine excreted and the distribution of the ^{14}C in the "lipid" and "water" fractions when the chickens were fed LPC or SBM containing diets.

Animals cannot synthesise cyclopropane fatty acids, as the enzyme cyclopropane synthetase does not appear to be present in animal tissue. However this enzyme is widely distributed among bacteria (Goldfine, 1972)and it is possible that the gut microflora may be influencing the experimental results. Hence the effect of including an antibiotic in these diets was also investigated.

Method:

a) Treatments

The following diets fed in Experiment 2 included the basal diet plus:-

A 13% SBM
B 13% SBM + antibiotic*
C 15% LPC
D 15% LPC + antibiotic*

* Neomix (Neomycin Sulphate-Neomycin activity 50% - Upjohn Pty. Ltd.) - Supplementation level 200 ppm at the expense of cornstarch.

The ingredient and nutrient composition of the experimental diets is summarised in Appendix A, Tables XXI and XXII.Batch 4 of both SBM and LPC was used in the Experiment (Tables III and IV).

Two groups of twenty-five chickens reared in a battery brooder, were fed a common diet for the first week and were then fed on either diet A or diet C for another week. At the end of the second week the chickens were individually weighed and two groups of 20 were selected on a weight basis and randomly placed in individual cages. Each group of 20 chickens was maintained on

the experimental diets A or C for a third week to enable them to adjust to their new environment. The surplus chickens were discarded.

After the third week the 20 chickens on diet A were divided into two groups of 9 and fed either diet A or diet B. Similarly the 20 chickens on diet C were divided into two groups of 9 which were fed on either diet C or diet D. The remaining 4 chickens were discarded. A day later 6.25 µCi of L-(methyl ^{14}C) methionine (Radiochemical Amsterdam, Australia Pty. Ltd.) was introduced into the crop of each chick. Three x 24 hour total excreta collections were made on individual trays. The excreta were frozen, freeze-dried and weighed. Analysis for the level of radioactivity and amino acids then followed.

b) Preparation of Excreta for Total ^{14}C Count:

Prior to scintillation counting the excreta were prepared using the Van Slyke wet combustion method (Fuchs and de Vries, 1972). Carbon dioxide was trapped in a 1:2 v/v ethanolamine:2 methoxyethanol mixture. Two millilitre aliquots of the mixture were pippetted into 20 mls Triton X-100 scintillant fluid in preparation for counting. The samples were allowed to stand overnight before counting began.

c) Fractionation:

After extraction of the excreta with hot ethanol as previously described (p 84), the extract was fractionated with chloroform: methanol:water (8:4:3 v/v/v) (p 90). The radioactive levels in the two fractions were counted using Triton X-100 toluene scintillation solvent for the upper fraction and toluene scintillation solvent for the lower fraction.

Results

The statistical analyses for Experiment 2 are summarised in Appendix B, Table XI.

The overall ^{14}C excretion level (Table XIX) was not significantly different for the chickens fed LPC diets compared to those fed SBM diets with or without antibiotic. After fractionation of the excreta with chloroform:methanol:water (8:4:3 v/v/v), the radioactive level in the "lipid fraction" of chickens fed the 15% LPC diet was significantly higher than that of the chickens fed the 13% SBM diet (2.28, 1.24 respectively). No significant differences in the radioactivity levels were apparent between the "water fractions" of both sets of excreta.

The addition of antibiotic slightly increased the excretion level of ^{14}C for chickens fed either the LPC or SBM diets but this was not statistically significant. The presence of antibiotic also did not significantly affect the percentage of radioactivity in the "lipid" and "water" fractions. However it is of interest that the inclusion of antibiotic affected these fractions in a converse manner. For example, the "lipid" fraction from the SBM fed chickens showed an increase in ^{14}C excretion due to the inclusion of antibiotic in the diet, whereas the corresponding fraction from the LPC fed chickens showed a decrease. The "water" fractions showed the reverse effect.

Table XIX: Comparison of the Effect of LPC and Antibiotic with that of SBM and Antibiotic on the Level of Methyl Group ^{14}C Excretion.

		Overall % Radioactivity Recovered (24 hours)	% Radioactivity in Fraction (Chloroform:Methanol:Water 8:4:3 v/v/v)	
			"Water Fraction" (upper)	"Lipid Fraction" (lower)
A	13% SBM	2.98 ± 0.54	1.69* ± 0.36	1.24^{a} ± 0.25
B	13% SBM + antibiotic	3.42 ± 1.19	1.61 ± 0.49	1.78^{ab} ± 0.59
C	15% LPC	3.67 ± 1.13	1.41 ± 0.34	2.28^{c} ± 0.54
D	15% LPC + antibiotic	3.81 ± 0.88	1.65 ± 0.58	2.11^{bc} ± 0.49
	SEM		0.15	0.16

* ± SEM - 9 Chickens/treatment

Values with common superscript letters do not differ significantly ($P < 0.05$)

Experiment 3: Measurement of the Ornithine Excretion Level due to feeding LPC and SBM

In addition to the possible detoxification of aromatic compounds by methylation, these compounds may also be detoxified by conjugation with ornithine (Williams, 1959). Burden et al. (1973) found that the feeding of 1% benzoic acid (an example of an aromatic acid which conjugates with ornithine) caused a 7% reduction in growth (significance $P < 0.1$) with approximately 42% of the benzoic acid administered being excreted in the form of ornithuric acid.

The purpose of this experiment was therefore to measure the level of ornithine excretion of LPC fed chickens compared to SBM fed chickens. As the chicken can only synthesise ornithine from arginine (Tamir and Ratner, 1963) the level of ornithine excreted was related to the chicken's intake of arginine.

Method

The four diets summarised below were those previously used in Trial 5 . These included the basal diet plus:-

A 13% SBM
B 15% LPC
C 15% LPC + 0.3% arginine
D 15% LPC + 0.3% methionine

Each diet was fed ad libitum to four sets of three individually housed adult cockerels (total = 12 cockerels) for five days prior to the start of the experiment. Individual total excreta collections (3 by 24 hours) were made into water baths containing mecuric chloride (to prevent bacterial growth - Baldwin et al.,1959). The excreta material was frozen and subsequently dried in a freeze drier. The total dry excreted material for each cockerel was weighed.

The excreta material collected over three days was pooled for each cockerel, sampled and prepared for amino acid analysis. The excreta samples were hydrolysed using the normal technique described earlier for proteins.

The nutrient and ingredient composition of the diets used in Experiment 3 is summarised in Appendix A, Tables XV and XVI.

Results

The results are presented in Table XX. The statistical analyses are summarised in Appendix B, Table XII. The total amount of ornithine excreted by SBM fed cockerels over the 3 day collection period tended to be higher than the amount excreted by the LPC fed cockerels. This difference, however, was not significant ($P < 0.05$). When the amount of ornithine excreted was related to arginine intake the excretion level of ornithine from cockerels fed the SBM diet was approximately 3% of the ingested arginine. The ornithine excretion level was slightly increased to 5.14% of the ingested arginine by feeding the cockerels the 15% LPC diet. This was not statistically significant at the 5% level. Supplementation of the LPC diet with either methionine or arginine also did not significantly alter the total amount of ornithine excreted nor the amount of ornithine excreted in relation to arginine intake.

Table XX: Summary of Analysis for Ornithine Excretion Level and the Effect of Methionine and Arginine Supplementation

Treatment	Total Ornithine Excreted (g/cockerel)	Ornithine %*	Apparent Arginine Availability %[1]
13% SBM diet	0.18	3.10 ± 0.77	86.17 ± 1.55^{cd}
15% LPC diet	0.11	5.14 ± 1.51	82.74 ± 1.67^{de}
15% LPC diet + 0.3% arginine	0.11	3.38 ± 1.77	86.62 ± 2.19^{c}
15% LPC diet + 0.3% methionine	0.10	3.59 ± 0.58	80.85 ± 2.17^{e}
Significance			$P < 0.05$

* $$\text{Ornithine \%} = \frac{\text{Ornithine Excreted (g)}}{\text{Apparent Arginine Available (g)}} \times \frac{100}{1}$$

Apparent Arginine Available = Apparent Arginine Availability % x Arginine Intake (g)

[1] $$\text{Apparent Arginine Availability \%} = \frac{\text{Arginine Intake (g)} - \text{Arginine Excreted (g)}}{\text{Arginine Intake (g)}} \times \frac{100}{1}$$

Values with common superscript letters do not differ significantly ($P < 0.05$)

DISCUSSION (Experiments 1 - 3)

Experiments 1-3 were designed to investigate whether arginine or methionine or both were required to any significant extent for the detoxification of compounds in LPC. Initially (Experiment 1) L-(methyl ^{14}C)methionine was fed with an LPC diet to an adult cockerel. The excreta of this cockerel was collected, extracted and partitioned into a "lipid" and a "water fraction"; radioactivity was present in these two fractions in a ratio of 1:2.3 ("water":"lipid" fraction).

Approximately 70% of the radioactivity in the "water fraction" appeared to be present in phenolic compounds. These compounds had presumably been methylated by either the chicken or the gut microflora. These phenolic compounds were not complexed with proteins in the excreta. Therefore the frequently made suggestion (e.g. Pirie,1978) that such an association may occur during the production of LPC and so reduce the digestibility of LPC is not supported by these results. Similarly, the results do not indicate that phenolics and proteins associate during digestion.

Methylated phenolic compounds similar to those found in the excreta of LPC fed chickens may have been present in the "water fraction" of excreta from chickens fed the SBM diet but this was not investigated. However, when L-(methyl ^{14}C) methionine was given to chickens fed either LPC or SBM diets (Experiment 2), there were no significant differences in the percentage total radioactivity which was recovered in the "water fraction" obtained from the excreta of these two groups of chickens. Therefore the requirement for methyl groups for the detoxification of aromatic compounds was apparently similar for chickens fed either the LPC or SBM diets.

In contrast, the percentage radioactivity recovered in the "lipid fraction" was significantly increased in the excreta of the chickens fed the LPC diet compared to those fed the SBM diet. As most of the radioactivity in this fraction was found to be in a

cyclopropane fatty acid (95% in the "lipid fraction" of the chickens fed the LPC diet and 70% in the "lipid fraction" of chickens fed the SBM diet), this suggested that the utilisation of methionine in the LPC diet was probably being influenced by the gut micro-organisms. This was also indicated by the results obtained when antibiotic was added to the diets. The addition of antibiotic to LPC diet fed to chickens tended to decrease the level of radioactivity in the "lipid fraction" whereas the radioactivity level increased in the "lipid fraction" of chickens fed the SBM + antibiotic diet. Such results, together with the significant difference between radioactive levels in the LPC and SBM "lipid fractions", indicate that differences in the diets can modify the activity and/or the population structure of the microflora; as was also suggested by Adams (1980).

Another possible indicator of detoxification was measurement of the amount of ornithine excreted by LPC and SBM fed cockerels. Compared to the results of Nesheim and Garlich (1963) who found a substantial effect due to feeding 2% (w/w) benzoic acid, the increase in ornithine excretion due to feeding a 15% LPC diet compared to a 15% SBM diet was small. Nesheim and Garlich (1963) calculated the arginine intake over a test period. They found that approximately 2% of the arginine eaten was excreted in the form of ornithine conjugates when chickens were fed a basal diet, but when 2% benzoic acid was introduced into the basal diet approximately 40% of the ingested arginine was excreted as ornithine conjugated products.

The low amount of ornithine excreted due to feeding LPC compared to SBM therefore, indicated that large amounts of arginine were not being required for ornithine synthesis and that the detoxification of aromatic compounds via ornithine was not a major contributing factor to the recorded growth depression due to feeding LPC.

In conclusion, therefore, the results of this study indicated that the detoxification of aromatic compounds by either methylation or conjugation with ornithine played an insignificant role in the poor growth response of chickens fed LPC diets. This conclusion reflected the results of Eggum and Christensen (1975) who found no significant effect on growth due to detoxification mechanisms operating in rats fed either barley and/or tannins. The feeding of LPC compared to SBM, however, increased the level of cyclopropane fatty acid in the excreta. This indicated that the gut microflora may be influencing the nutritional value of LPC and/or that LPC may be influencing the metabolism and/or structure of the gut microflora.

Experiment 4: Exhaustive Enzyme Digestion of "Pure" LPC and SBM

The *in vitro* exhaustive enzyme digestion was designed to investigate whether LPC protein could be hydrolysed by enzymes to a similar extent as SBM protein. The *in vitro* digestion was carried out over 4 days as the results of a shorter digestion period and/or low enzyme level may be masked by an inhibitory property inherent in LPC.

Method

(i) Enzymes: The enzyme digest mixture includes pancreatin (BDH Ltd., England) and pronase (Sigma Chemicals, U.S.A.). Pancreatin was included to provide lipase and amylase activity in order to remove any possible "shielding" effects due to the presence of fats and carbohydrates. The 10% (w/v) pancreatin solution was centrifuged (5,000 x g for 5 minutes) before use to remove insoluble matter.

Pronase is a mixture of several proteolytic enzymes including endopeptidases and exopeptidases which are produced by a strain of *Streptomyces griseus*. The enzymes present are neutral and alkaline proteases, amino peptidase and carboxypeptidase (Narahashi, 1970). The enzyme mixture provides the required enzyme activities without excessively increasing the amount of protein material.

(ii) Buffer: The pH 7.5 buffer solution used was 0.1 M sodium borate in 0.05 M hydrochloric acid. It also contained 0.01 M calcium chloride.

(iii) Exhaustive Digest: Exhaustive enzyme digests were carried out on 200 mg of LPC or SBM in 10 mls of buffer solution of pH 7.5. Two aliquots of each of the enzymes, pancreatin and pronase, were added at 24-36 hour intervals to give for each enzyme a final enzyme/substrate ratio of 1%(w/v). Pancreatin was added first followed by pronase with the pH of 7.5 being maintained

throughout by the addition of 0.1 M sodium hydroxide. The temperature of the digest was maintained at 37°C and the solution was continuously agitated. Toluene was added to all digests. Duplicate SBM and triplicate LPC samples were digested.

After centrifugation (10,000 x g for 5 minutes) the supernatants were analysed for amino acids. Enzyme blanks were also analysed so that the amino acid digestibility values could be corrected for the additional amino acids due to enzyme addition. The percentage of each amino acid digested was calculated from the following formula:-

$$\% \text{ Amino Acid Digested} = \frac{\text{Supernatant Amino Acid (g)}}{\text{Total Amino Acid (g)}} \times \frac{100}{1}$$

Results

The results given in Table XXI demonstrated that the *in vitro* mean digestion level of protein in LPC was lower by approximately 6% compared to SBM protein. There was some variability in LPC results, which may have resulted from the fine powdery nature of the product. This caused it to stick to the glassware while being agitated over the digestion period.

The results do show that none of the individual amino acid digestibility values for LPC were markedly lower than the mean amino acid digestibility value for LPC. These results, therefore, indicate that either the LPC protein can be almost hydrolysed as effectively as SBM by enzyme action, or that the method used here was unsuitable to detect any differences. However, because the *in vitro* data often differs from the *in vivo* results (Akeson and Stahman, 1965), these results must be viewed with caution.

Table XXI: Results of *in vitro* Exhaustive Digestion

	Mean % Amino Acid Digested[1]	
	"Pure" LPC*	"Pure" SBM+
Cystine	69	86
Threonine	79	80
Serine	78	79
Valine	79	86
Methionine	66	69
Isoleucine	76	86
Leucine	72	76
Tyrosine	80	90
Phenylalanine	69	76
Histidine	68	73
Lysine	79	75
Arginine	74	80
Overall Mean	74	80
Overall Mean Range	66 - 79	78 - 82

[1] % Amino Acid digested = $\frac{\text{Supernatant amino acid(g)}}{\text{Total amino acid (g)}} \times \frac{100}{1}$

* Triplicate - LPC batch number 5

\+ Duplicate - SBM batch number 4

Experiment 5: Comparison of the "Corrected (CAAA) and Apparent (ApAAA) Amino Acid Availability of LPC and SBM Ingredients and of diets containing LPC and SBM"

In this experiment "apparent" and "corrected" (corrected for endogenous secretion) amino acid availabilities were measured by analysis of the excreta from cockerels which had been fed either LPC or SBM or diets containing these protein concentrates. The method was based on the TME method (p 17).

Likuski and Dorrell (1978) were the first to publish amino acid availability results derived by the TME method. The terms "apparent" and "corrected" amino acid availability are defined by the equations given below in the Methods section.

The availability values of amino acids as determined by the excreta method can be over-estimated because of the activities of the microflora in the large intestine. The extent of the influence by the microflora on the availability estimate is unclear (McNab, 1980). However, microbial fermentation is considered to have less of an effect on protein degradation in poultry than in other monogastrics, e.g. pigs) because of the faster rate of digesta passage through the lower gut (Zebrowska, 1978). Other factors, such as variations in dietary protein level are believed to influence the amount of endogenous secretion (McNab, 1980) and so influence the amino acid availability estimates. As the protein levels of the diets used in this experiment were similar, it was assumed that any variation in endogenous output was a characteristic of the "test" ingredient as both protein sources were studied under comparable conditions.

Other factors, e.g. rates of digestion and/or absorption, which may effect the availability of dietary amino acids to growing chickens cannot be measured by this type of assay. The purpose of this study was therefore to compare two similar protein sources under comparable conditions.

Method

The ingredient and dietary amino acid availabilities were measured using the True Metabolisable Method (TME) described on p 17. The amino acid availability values were estimated in terms of the "apparent" amino acid availability (ApAAA) and the "corrected" amino acid availability (CAAA). These were calculated using the formulae outlined by Likuski and Dorrell (1978):-

$$\% \text{ Apparent Amino Acid Availability (ApAAA)} = \frac{\text{g amino acid fed} - \text{g amino acid excreted}}{\text{g amino acid fed}} \times \frac{100}{1}$$

$$\% \text{ "True" or "Corrected" Amino Acid Availability (CAAA)} = \frac{\text{g amino acid fed} - (\text{g amino acid excreted} - \text{g endogenous amino acid excreted})}{\text{g amino acid fed}} \times \frac{100}{1}$$

From the equations, three sets of amino acid analyses were required for the calculation of CAAA and ApAAA.

(i) The amino acid composition of the feed which is fed to the cockerel.

(ii) The amount of amino acid excreted by a fed cockerel.

(iii) The amount of amino acid excreted under starvation conditions to give a value for the endogenous excretion level.

Thirty-two adult Sykes cockerels were housed in single cockerel cages in an environmentally controlled room. All cockerels were maintained on a commercial 18% protein (N x 6.25) chick grower diet so that any possible carry-over effect of the maintenance diet to the subsequent availability estimates was considered to be equally distributed across all treatments. Two separate "feeding" periods of 24 hours each were carried out a week apart. Before each experimental "feeding" period each cockerel was starved for 24 hours.

In order to have an endogenous amino acid estimate for each individual cockerel a "cross-over" design was adopted. For each experimental feeding period, four groups of 4 cockerels were force-fed 16 g "pure" SBM or 14 g "pure" LPC or 25 g SBM diet or 25 g LPC diet. Similarly four groups of 4 cockerels were starved for a further 24 hours for the estimation of endogenous output. The excreta collection period was 24 hours after which the excreta were individually placed in pots and frozen.

The following week, during the experimental "feeding" period the order of the cockerels was reversed, i.e. the previously fed cockerels were now starved and the previously starved cockerels were force-fed with the relevant ingredient or diet. The excreta collection period was again, 24 hours. The excreta were frozen, freeze-dried, ground and weighed. The amino acid analyses were carried out as described on p 16 .

Results

The mean values for ApAAA and CAAA for the ingredients LPC and SBM are given in Table XXII for each of the essential amino acids. Comparable figures for the diets containing LPC or SBM are given in Table XXIII. The statistical analyses for Experiment 5 are summarised in Appendix B, Tables XIII and XIV.

The mean overall amino acid availability was 81.6% for "pure" LPC and 92.4% for "pure" SBM on a CAAA basis (a difference of 12%), or 70.4% and 85.3% for "pure" LPC and "pure" SBM respectively on an ApAAA basis (i.e. a difference of 15%). Cystine had the lowest availability value in "pure" LPC (51.2% CAAA and 11.9% ApAAA compared to 80.8% CAAA and 75.7% ApAAA in "pure" SBM).

The effect of the above differences in amino acid availability were brought into perspective when the diets containing LPC or SBM were compared using the same assay procedure (Table XXIII).

Table XXII: Summary of the Results of Experiment 5 - LPC and SBM Ingredient Amino Acid Availability using the TME Method

	"Pure" LPC ApAAA[1] %	"Pure" LPC CAAA[2] %	"Pure" SBM ApAAA %	"Pure" SBM CAAA %
Cystine	11.9 ± 3.5*	51.2 ± 2.0	74.7 ± 0.6	89.8 ± 0.6
Threonine	75.7 ± 1.0	84.9 ± 0.5	72.7 ± 0.6	79.4 ± 0.5
Serine	69.5 ± 1.2	82.0 ± 0.6	84.8 ± 0.4	92.2 ± 0.3
Valine	77.2 ± 0.9	85.2 ± 0.6	86.7 ± 0.4	96.1 ± 0.3
Methionine	80.4 ± 0.8	86.9 ± 0.6	87.4 ± 0.3	96.5 ± 0.3
Isoleucine	78.1 ± 0.9	84.5 ± 0.4	89.4 ± 0.3	94.4 ± 0.3
Leucine	80.6 ± 0.8	86.1 ± 0.4	89.2 ± 0.3	94.2 ± 0.3
Tyrosine	65.3 ± 1.4	76.3 ± 0.6	87.8 ± 0.3	94.0 ± 0.3
Phenylalanine	76.2 ± 1.0	83.8 ± 0.4	87.4 ± 0.3	94.0 ± 0.3
Histidine	73.3 ± 1.0	83.2 ± 0.5	88.6 ± 0.3	91.6 ± 0.3
Lysine	75.0 ± 1.0	85.5 ± 0.6	86.2 ± 0.4	92.9 ± 0.4
Arginine	80.8 ± 0.8	89.2 ± 0.5	89.4 ± 0.3	93.9 ± 0.3
$\bar{X}$	70.4	81.6	85.3	92.4

* SEM - 8 chickens/treatment

[1] Apparent Amino Acid Availability (ApAAA) = $\frac{\text{g. amino acid fed} - \text{g. amino acid excreted}}{\text{g. amino acid fed}} \times \frac{100}{1}$

[2] Corrected Amino Acid Availability (CAAA) = $\frac{\text{g. amino acid fed} - (\text{g. amino acid excreted} - \text{g. endogenous amino acid excreted})}{\text{g. amino acid fed}} \times \frac{100}{1}$

Table XXIII: Summary of the Results of Experiment 5 - LPC and SBM Dietary Amino Acid Availability using the TME Method

	LPC Diet		SBM Diet	
	ApAAA[1] %	CAAA[2] %	ApAAA %	CAAA %
Cystine	59.1 ± 1.0*	72.2 ± 1.0	69.7 ± 0.5	80.7 ± 0.9
Threonine	74.7 ± 0.6	81.6 ± 0.5	76.4 ± 0.4	85.7 ± 0.8
Serine	76.7 ± 0.6	83.4 ± 0.6	79.7 ± 0.4	87.8 ± 0.7
Valine	79.2 ± 0.6	83.5 ± 0.6	81.0 ± 0.3	86.5 ± 0.4
Methionine	82.1 ± 0.5	85.9 ± 0.5	82.2 ± 0.3	87.5 ± 0.4
Isoleucine	81.7 ± 0.5	85.7 ± 0.5	81.5 ± 0.3	86.9 ± 0.4
Leucine	85.3 ± 0.5	88.4 ± 0.3	85.0 ± 0.2	89.3 ± 0.3
Tyrosine	81.7 ± 0.5	89.8 ± 0.5	79.8 ± 1.4	87.0 ± 1.5
Phenylalanine	75.5 ± 0.6	81.3 ± 0.6	77.8 ± 0.4	89.5 ± 1.1
Histidine	69.6 ± 0.7	78.6 ± 0.7	80.9 ± 0.3	94.6 ± 1.0
Lysine	73.3 ± 0.6	82.2 ± 0.7	85.6 ± 0.2	91.2 ± 0.4
Arginine	83.9 ± 0.5	88.7 ± 0.5	84.3 ± 0.3	94.3 ± 0.9
$\bar{X}$	76.9	83.4	80.3	88.2

* SEM - 8 Chickens/treatment

[1] ApAAA - Apparent Amino Acid Availability

[2] CAAA - "Corrected" Amino Acid Availability

The difference in the mean amino acid availability between the diets was only 5% or 4%. Cystine still had the lowest availability value in the LPC diet but it was only 8-10% below that in the SBM diet.

Experiment 6: Comparison of Amino Acid Digestibility measured at the ileum with amino acid availability measured by excreta analysis

Since the TME assay approach may include a bias due to the effect of microflora in the lower gut, the apparent ileal digestibility and amino acid availability by excreta analysis of the LPC and SBM diets were compared. The purpose of the experiment was to determine whether or not digestion was being affected by the inclusion of LPC to a greater extent than that indicated by availability measurement by excreta analysis (i.e. to remove the influence of micro-organisms in the hind gut).

Method

The chickens used in this experiment were also used for the experiment studying pancreatic enzyme activity (Experiment 7).

Forty-eight Sykes strain cockerel day-old chicks were randomised into two groups, wingbanded, placed in battery brooders and fed a common diet for one week. At the end of the first week both groups of chickens were transferred in sub-groups of 4 to the modified battery brooders described on page 15 . The nutrient and ingredient composition of the diets is summarised in Appendix A, Tables XXIII and XXIV. The two treatments were:-

(i) 13% SBM (6 replicates of 4 chickens each)

and (ii) 15% LPC (6 replicates of 4 chickens each)

On the 24th day of the experiment 0.3% (w/w) chromic oxide was added to both diets and a 3 x 24 hour total excreta collection was made on all 6 replicates on days 24 - 26. The excreta samples were individually collected and frozen. At the end of 28 days 4 replicates on each diet were individually weighed and gassed with chloroform for the estimation of ileal digestibility (the other two replicates on each diet were used in Experiment 7).

The ileal contents were removed into a container by gentle squeezing of 10 cm of ileum (as measured from above the junction of the caeca). The ileal samples were frozen, freeze-dried and subsequently analysed for amino acids and for chromic oxide. The method outlined by Czanocki et al.(1961) was used for chromic oxide determination.

The values for apparent ileal amino acid digestibility were calculated using the following equation (Schneider and Flatt, 1975):-

$$\text{Ileal amino acid digestibility \% (ApAAD)} = 100 - 100 \times \frac{\text{\% indicator in the feed} \times \text{\% nutrient in the ileum}}{\text{\% indicator in the ileum} \times \text{\% nutrient in the feed}}$$

Results

The results are given in Table XXIV. The statistical analyses are summarised in Appendix B, Table XV.

The difference in the mean apparent amino acid ileal digestibility estimates for the LPC and SBM diets was 26%. In contrast, the mean apparent amino acid availability estimates, which were derived from analysis of the excreta from chickens fed either the SBM diet or the LPC diet, only varied by 8%. Again cystine was the amino acid most affected by the presence of LPC in the diet, with its digestibility-availability being reduced by 45% compared to the SBM diet according to the ileal approach and by 17.6% according to the excreta technique.

The two methods, (ileal assay and excreta analysis) gave availability - digestibility results for cystine which differed by 44% for the LPC diet and by 16.5% for the SBM diet. The fact that ileal ApAAD and the excreta ApAAA estimates for cystine were the lowest would indicate that cystine was probably the limiting amino acid in the LPC diet.

Table XXIV: Summary of the Results of Experiment 6 - Comparison of the Apparent Amino Acid Digestibility[1] (ApAAD)/Availability[2] (ApAAA) as Measured in the Ileum and Excreta.

	LPC Diet		SBM Diet	
	ApAAD%[3] (Ileum)	ApAAA%[4] (Excreta)	ApAAD% (Ileum)	ApAAA % (Excreta)
Cystine	18.5 ± 1.9[5]	62.2 ± 0.9	63.3 ± 1.8	79.8 ± 1.0
Threonine	49.9 ± 1.8	72.5 ± 0.6	69.5 ± 1.8	81.8 ± 0.9
Serine	45.6 ± 1.4	75.2 ± 0.8	71.3 ± 1.2	82.5 ± 0.9
Valine	53.3 ± 1.7	77.2 ± 0.5	76.8 ± 1.5	84.1 ± 0.8
Methionine	56.7 ± 2.7	86.6 ± 0.4	86.1 ± 1.9	86.0 ± 0.7
Isoleucine	55.1 ± 1.5	77.5 ± 0.5	80.4 ± 1.0	87.0 ± 0.8
Leucine	61.5 ± 2.0	80.9 ± 0.4	80.7 ± 0.7	87.1 ± 0.6
Phenylalanine	53.0 ± 1.8	74.3 ± 0.6	79.7 ± 0.5	83.4 ± 0.8
Histidine	48.6 ± 2.2	68.5 ± 0.6	74.2 ± 0.8	81.4 ± 0.9
Lysine	54.8 ± 1.4	73.1 ± 0.5	83.3 ± 0.8	85.9 ± 0.7
Arginine	62.6 ± 1.7	81.4 ± 0.4	82.9 ± 1.0	87.3 ± 0.6
Tyrosine	62.9 ± 0.9	82.3 ± 0.4	83.3 ± 0.4	86.9 ± 0.6
$\bar{X}$	51.3	75.5	77.5	83.9
Difference in $\bar{X}$	24.2		6.4	

[1] - 4 replicates

[2] - 6 replicates

[3] $$\text{ApAAD} = 100 - 100 \times \frac{\%\text{ indicator in the feed} \times \%\text{ nutrient in the ileum}}{\%\text{ indicator in the ileum} \times \%\text{ nutrient in the feed}}$$

[4] $$\text{ApAAA} = \frac{\text{g. amino acid fed} - \text{g. amino acid excreted}}{\text{g. amino acid fed}} \times \frac{100}{1}$$

[5] SEM

Comparison of the digestibility estimates (measured in the ileum) and the availability estimates (measured in the excreta) indicated that the gut microflora were influencing the availability estimates. This was particularly evident for the LPC diet as the digestibility-availability difference was 24.2% compared to only 6.4% for the SBM diet.

Experiment 7: Effect of LPC on pancreatic size and level of pancreatic proteolytic enzymes

An indication that trypsin inhibitors are present in the diet is an increase in pancreatic size (pancreatic hypertrophy) (Liener, 1979). A related indicator is an increase in the level of pancreatic enzymes. Therefore a comparison was made of pancreatic size and of trypsin and chymotrypsin activities when either LPC or SBM containing diets were fed.

Method

(i) Treatment: 15% LPC and 13% SBM diets were each fed to 6 replicate groups of 4 chicks as described previously in Experiment 6. On the 27th day of the experiment two replicate groups for each diet had their food removed for 18 hours before the end of the experiment. These are termed "fasted chicks". Fasting has been reported by Gertler and Nitsan (1970) to increase the levels of enzyme activity in the pancreas. The other four replicate groups for each diet were not fasted and are referred to as "fed". At the end of 28 days the chickens were individually weighed and then gassed. The ileal contents of these chicks were used for Experiment 6 and for the present Experiment 7. In Experiment 7 individual pancreases were removed, weighed and frozen within 15 minutes of the chickens' death. The enzyme activity in the pancreases was then determined.

(ii) Pancreatic Enzymes: For the determination of enzyme activities, the pancreases were homogenised in 10 volumes of water (Nitsan and Liener, 1976). Activity measurements were made on the supernatant remaining after centrifuging at 15000 x g for 20 minutes. The zymogens of the pancreatic homogenates were activated by incubation with an equal volume of 1%(w/v) enterokinase for 1 hour at 37°C (Gertler and Nitsan, 1970). Trypsin and chymotrypsin activities were measured using N benzoyl DL arginine-p-nitroanilide (Erlanger et al., 1961) and N glutaryl-L phenylalanine-p-nitroanilide (Erlanger et al., 1966) as the respective substrates.

Table XXV: Effect of feeding LPC or SBM on pancreatic size and level of pancreatic proteolytic enzymes (Experiment 7)

Treatment	Mean Pancreas Weight g/100 g bodyweight	Mean Enzyme Level[d]			
		"Fasted Chickens"		"Fed Chickens"	
		Chymotrypsin	Trypsin	Chymotrypsin	Trypsin
LPC Diet	0.4581[a] ± .0132[e]	19.47 ± 1.65	11.50 ± 2.22	20.38[b] ± 2.98	6.48[c] ± 0.8
SMB Diet	0.3809[a] ± .0100	16.08 ± 0.78	8.28 ± 1.12	13.67[b] ± 0.88	4.64[c] ± 0.4

Significance of difference between means :

a $P < 0.01$
b $P < 0.05$
c $P < 0.1$
d Enzyme levels are expressed in absorbance units per g. weight of pancreas per 100 gm body weight

[e] S.E.M.

Results

The statistical analyses for Experiment 7 are summarised in Appendix B, Table XVI. The data for pancreatic weights along with pancreatic enzyme levels are given in Table XXV. The chickens fed the LPC diet had significantly larger ($P < 0.01$) pancreases per unit of body weight than those fed the SBM diet (0.458 g versus 0.381 g per 100 g body weight respectively).

The chicks fed with the LPC diet also appeared to have higher activities of chymotrypsin and trypsin in the pancreas (Table XXV) compared with those fed SBM, irrespective of whether the chickens were subsequently fasted or fed. The difference was statistically significant in fed chickens (chymotrypsin $P < 0.05$; trypsin $P < 0.1$) but not in fasted chickens. The differences in the chymotrypsin and trypsin activity levels in the pancreases of the "fasted" chickens may be statistically less sensitive due to the lower number of chickens/treatment used. However, the results indicated that trypsin inhibitors were present in the LPC.

Experiment 8: Effect of antibiotics on the apparent amino acid availability in LPC or SBM diets as measured in the excreta.

The objectives of this experiment were to study whether chickens fed with the LPC diet would respond to antibiotic supplementation and investigate if antibiotic feeding had any effect on apparent amino acid availability.

Method

The chicks were fed the following diets which consisted of basal plus:-

A 13% SBM
B 13% SBM + antibiotic*
C 15% LPC
D 15% LPC + antibiotic*

* 200 ppm Neomix at expense of cornstarch

Chicks (total = 36) were individually housed (as described on page 15) and reared as described in Experiment 2 on the experimental diets A and C containing 15% LPC or 13% SBM, to 21 days of age. The experimental period, when all four diets A to D were fed to the chickens, was from 21 to 24 days (i.e. 4 periods of 24 hours). Total excreta collections were made over the last three 24-hour periods. The excreta were analysed for amino acids as described earlier. The apparent amino acid availability was calculated as in Experiment 6.

Results

The statistical analyses are summarised in Appendix B, Tables XVII and XVIII. The results given in Table XXVI show that no change in growth rate or in food utilisation was obtained when the SBM diet was supplemented with antibiotic. In contrast, chicks fed LPC diets with antibiotic supplementation had

Table XXVI: Experiment 8: Effect of Antibiotic (Neomix) Supplementation on the Growth of Chickens Fed LPC or SBM Diets

	Weight Gain (g) G	Food Intake (g) F	Food Utilisation F/G
13% SBM	72.9^{a}	182.25^{a}	2.50^{a}
13% SBM + antibiotic*	72.7^{a}	181.75^{a}	2.50^{a}
15% LPC	52.1^{c}	157.34^{c}	3.02^{b}
15% LPC + antibiotic*	62.9^{b}	162.91^{b}	2.59^{ab}
SEM+	2.5	0.96	0.09

+ SEM (9 chicks per treatment)- 4 day experimental period

* Antibiotic Neomix - 200 ppm

Values with common superscript letters do not differ significantly ($P < 0.01$).

Table XXVII: The Effect Of Antibiotics On Apparent Amino Acid Availability (Total Collection of Excreta in Experiment 8) in LPC or SBM containing Diets.

Amino Acid	ApAAA%			
	15% LPC Diet	15% LPC + Antibiotic	13% SBM Diet	13% SBM + Antibiotic
Cystine	64.2 ± 1.4	69.8 ± 0.7	85.7 ± 0.6	85.6 ± 0.6
Threonine	73.7 ± 1.0	81.6 ± 0.6	83.3 ± 0.5	83.1 ± 0.5
Serine	76.1 ± 1.0	83.6 ± 0.6	85.0 ± 0.4	84.1 ± 0.5
Valine	77.2 ± 0.9	84.1 ± 0.5	87.4 ± 0.6	86.9 ± 0.4
Methionine	80.7 ± 0.7	88.8 ± 0.4	91.8 ± 0.8	90.5 ± 0.3
Isoleucine	77.6 ± 0.9	85.3 ± 0.5	90.1 ± 0.3	89.7 ± 0.4
Leucine	82.4 ± 0.6	87.6 ± 0.4	88.8 ± 0.4	89.1 ± 0.4
Tyrosine*	69.5 ± 1.2	95.4 ± 0.7	78.7 ± 0.6	75.6 ± 0.8
Phenylalanine	78.5 ± 0.8	89.2 ± 0.5	86.1 ± 0.4	84.6 ± 0.5
Histidine	75.9 ± 0.9	82.2 ± 0.6	87.9 ± 0.4	86.7 ± 0.5
Lysine	76.5 ± 0.9	84.7 ± 0.5	87.9 ± 0.4	93.6 ± 0.3
Arginine	82.2 ± 0.7	87.9 ± 0.4	89.5 ± 0.3	89.3 ± 0.7
$\bar{X}$	76.2	83.2	86.9	86.5

SEM - 9 Chickens/treatment

$$\text{ApAAA\%} = \frac{\text{g amino acid fed} - \text{g amino acid excreted}}{\text{g amino acid fed}} \times \frac{100}{1}$$

* Difficulties were experienced in the analysis of tyrosine in excreta samples so that the reliability of the values at times was difficult to ascertain.

significantly improved growth rates ($P < 0.01$) and tended to have improved food utilisation compared to the chickens fed the unsupplemented LPC diet.

The mean apparent amino acid availability estimates of the diets shown in Table XXVII reflected the growth of the chickens to the antibiotic supplementation i.e. whereas the mean apparent amino acid availability estimate of the SBM diet was unaffected by antibiotic supplementation, the mean apparent amino acid availability in the LPC diet was improved by approximately 7%. The results, therefore, indicated that the gut microflora were influencing the nutritional value of the LPC diets.

The availability estimate for cystine in the LPC diet was only improved by approximately 5%. The apparent availability of cystine in the LPC plus antibiotic diet was still lower than the apparent amino availability estimates of the other LPC + antibiotic dietary amino acids. This result indicated that a change in microfloral activity due to feeding the LPC diet was probably not the only factor reducing the availability of the cystine in the LPC diet and that cystine was the first limiting amino acid in the LPC diet when antibiotic was added.

Discussion (Experiments 4 - 8)

An interum conclusion in this study was that either methionine and/or cystine supplementation was capable of relieving the growth depressing effect of feeding a 15% LPC diet to chickens compared to feeding an equivalent SBM diet. In an attempt to explain these results a variety of studies have been presented.

(i) Digestibility and/or Availability Studies

The mean values for the amino acid availability-digestibility estimates of "pure" LPC were similar. These were 74% for the *in vitro* digestibility estimates (Experiment 4) and 70.4% ApAAA and 81.6% CAAA (Experiment 5). Also the apparent amino acid availability estimates of the diets did not greatly differ in three different experiments (Experiments 5, 6 and 8). The mean apparent amino acid availability estimates for the 15% LPC diet were 76.9%, 75.5% or 76.2% and 80.3%, 83.9% or 86.9% for the SBM diet (Experiments 5, 6 and 8 respectively). The difference in the means between the two diets therefore varied between 3.4 and 10.7%. The mean of the corrected amino acid availability estimates for the diets containing LPC and SBM in Experiment 5 were also within the above range, differing by about 5%.

By contrast, the mean apparent amino acid digestibility estimate obtained from analysis of the ileal contents showed a much greater difference between the digestibilities of the LPC and SBM diets. The difference was 26%.

In all the *in vivo* experiments, cystine had lowest amino acid availability-digestibility estimates in "pure" LPC and in the LPC diet. These were also always lower than those in the "pure" SBM or in the SBM diet. The availability of cystine in "pure" LPC as measured in the excreta (Experiment 5) was low (11.9% ApAAA, 51.2% CAAA) compared to both the mean amino acid availability estimate for "pure" LPC (70% ApAAA, 82% CAAA) and the availability of

cystine in "pure" SBM (74.7% ApAAA, 89.8% CAAA). The low availability value for cystine in LPC was less evident when the amino acid availabilities of the diets (15% LPC and 13% SBM) were compared using the TME-type method (Experiment 5). The cystine availability in these diets differed by only 10%.

The difference in the apparent cystine availability estimates between the two diets were greater in Experiments 6 and 8. The difference was approximately 20%. This larger difference may be due to a change in methodology. In Experiments 6 and 8 feeding was ad libitum whereas the chickens in Experiment 5 were force-fed. Also the young age of the chickens in Experiments 6 and 8 compared to the age of the adult cockerels used in Experiment 5 may have influenced the availability estimates.

Again, in contrast to the above, the analysis of the ileal contents (Experiment 6) indicated that the digestibility of cystine in the diet containing LPC was much lower than for the diet containing SBM. The values were 18.5% and 63.3% for the LPC and SBM diets respectively; a 45% difference.

The lower digestibility values obtained in the ileum compared to the values obtained by analysis of the excreta indicate

a) that the activity of the gut microflora in the hind gut were influencing the estimates of amino acid availability. For example, in Experiment 6 the apparent ileal digestibility estimate for cystine in the diet containing LPC was 18.5% compared to cystine availability estimate of 62.2% as measured in the excreta; a difference of 44%.

b) That the LPC diet was being digested and/or absorbed at a slower rate relative to the SBM diet. The mean apparent amino acid ileal digestibility difference between two diets was 26.2%.

c) That the volume of endogenous secretions were possibly increased due to feeding the diet containing LPC and/or that the recycling of these secretions to the animal was reduced. Pancreatic secretions are "rich" in cystine (Neurath, 1961) so that the cystine digestibility estimates may reflect the level of endogenous proteins present in the ileum.

Much more cystine was present in the ileal contents of chickens fed the diet containing LPC compared to those fed the diet containing SBM according to the digestibility estimates. The apparent cystine ileal digestibility of the LPC diet was 18.5% compared to the corresponding estimate for the SBM diet of 63.3%. The results obtained in Experiment 5 also indicate that there was a large endogenous loss of cystine. The ApAAA of cystine (11.9%) in the LPC diet was much lower than the CAAA value (51.2%) whereas there was little difference between the corresponding values for the SBM diet (74.7% ApAAA and 89.8% CAAA).

(ii) Pancreatic Hypertrophy and Enzyme Levels

In this study (Experiment 7) the size of the pancreases and level of enzyme activity in the pancreases varied with the type of diet fed. Chickens fed the LPC diets had larger pancreases (relative to chicken body weight) than those chickens fed the SBM diet. The level of trypsin and chymotrypsin activity in the pancreases of the chickens fed the LPC diet also increased compared to the enzyme activity in the pancreases of the chickens fed the diet containing SBM.

Pancreatic hypertrophy is associated with the presence of trypsin inhibitors (Liener, 1979). Enlargement of the pancreas has also been associated with increased secretory activity (Gertler et al., 1967) which requires increased enzyme synthesis (Neurath, 1961; Liener and Kakade, 1969; Rakis, 1974).

(iii) Contributory Effect of the Gut Microflora:

The addition of antibiotic to the 15% LPC diet (Experiment 8) improved the growth of LPC fed chickens. The growth of the SBM + antibiotic fed birds was unaffected. This suggested that the gut microflora were influencing the nutritional value of diets in which LPC was included. This conclusion was supported by the observation that the mean amino acid availability of the LPC diet as measured in the excreta (Table XXVI) increased by about 7% in the presence of antibiotic.

In line with the growth results, the amino acid availability of the SBM diet was unchanged by antibiotic addition. The presence of LPC in the diet therefore probably influenced either the structure and/or the metabolism of the gut microflora. This change in the microflora probably reduced either the digestion and/or absorption of the amino acids in the LPC diet and/or the recycling of the endogenous proteins. Carrol et al. (1953) reported somewhat similar results. They found that when an antibiotic was fed with raw soybean, the cystine content of the small intestine increased. They suggested that the antibiotic protected against bacterial degradation of cystine.

From the above Experiments 4-8 several characteristic changes follow as a result of the feeding of LPC to chickens, namely:-

(i) pancreatic hypertrophy;
(ii) increased levels of proteolytic enzymes present in the pancreas;
(iii) retarded digestion and/or absorption and/or increased endogenous secretion levels;
(iv) altered gut microfloral activity.

CHAPTER 8: DISCUSSION

Although Leaf Protein Concentrate has not yet reached the stage of standard commercial production in New Zealand, it may be commercially produced in the near future.

There are some economic and agronomic complications, in the grassland farming approach in N.Z., for example in the timing of pasture harvesting with grazing and in the usage of the pressed residue. Consequently, development of LPC production rests on factors other than its nutritional efficiency. Should these wider problems be overcome and LPC be made available in volume in N.Z., the work carried out towards this thesis, indicates that despite some nutritional limitations, these could be surmounted and LPC usefully used as a component of diets for chickens.

It was shown initially that LPC containing diets require additional methionine and/or cystine when fed to chickens in order to achieve growth levels comparable to those achieved by chickens fed an equivalent SBM containing diet. It is known that methionine can be used by the chicken for the synthesis of cystine. As cystine was found to be as effective as methionine in relieving the growth depression due to feeding LPC diets, it was concluded that cystine was the first limiting amino acid in the Ryegrass-White Clover LPC diets.

In the growth trials it was found that the LPC containing diets required approximately 0.2% additional methionine, which was higher than the amount of methionine contributed by the LPC to the diet (0.12%). Similar results were obtained with the addition of an equivalent amount of cystine (0.16%) in place of the 0.2% methionine added to the LPC containing diets. Lesser amounts of cystine may have sufficed but this was not investigated. Since the contribution of cystine by the LPC to the diet was only 0.06%, these results suggested that the inclusion of LPC reduced the

availability of the cystine in the other dietary ingredients. Therefore, the growth depression due to the inclusion of LPC in the diets, was unlikely to be solely due to a low digestibility of the LPC protein.

The above conclusion was supported by the in vitro digestibility results and, in part, by the in vivo availability-digestibility results. The in vitro digestibility results demonstrated that the majority of the LPC protein could be hydrolysed by enzymes.

The mean estimates derived from the in vivo amino acid availability-digestibility methods varied according to the method used. The mean amino acid availability estimates for the ingredients LPC and SBM (fed as a sole dietary source) were 70.4% ApAAA, 81.6% CAAA and 85.3% ApAAA, 92.4% CAAA respectively. The differences in the means were approximately 15% and 11%; ApAAA and CAAA respectively. The corresponding results for the diets (15% LPC and 13% SBM) were 76.9% ApAAA and 83.4% CAAA for the LPC diet and 80.3% ApAAA and 88.2% CAAA for the SBM diet. The inclusion of the test proteins (LPC and SBM) into diets reduced the difference in the mean amino acid availability estimates from 15% to approximately 5%. However, the mean amino acid ileal digestibility estimates for the two diets (15% LPC and 13% SBM) differed by approximately 26%.

The estimates for the in vivo methionine availability-digestibility followed a similar pattern. The methionine availability estimates for the ingredients (LPC and SBM) differed by 7-10% whereas the corresponding estimates for the diets differed by no more than 2%. Again, however, the methionine ileal digestibility estimates differed by a larger amount; the difference was approximately 29%.

The availability of cystine in the ingredient LPC (11.9% ApAAA, 51.2% CAAA) measured by analysis of excreta (Experiment 5), was

low compared to corresponding cystine availability of the ingredient SBM (74.7% ApAAA, 89.8% CAAA). Such a result supports the findings of Donnelly (1980) who reported that the availability of cystine in Ryegrass-White Clover LPC was only 10%. The low cystine availability of LPC was, however, less evident when the cystine availabilities of the diets containing LPC or SBM were compared. These estimates differed by approximately 10% using the TME method and by approximately 17% ApAAA when the chickens were fed ad libitum.

By contrast, the ileal cystine digestibility estimate for the LPC diet was only 18.5% compared to 63.3% for the SBM diet i.e. a difference of 45% (Table XXIV). Such a difference could not be explained by the low digestibility-availability of cystine in LPC alone as 150 g LPC contributed only 0.06% cystine to the LPC diet or 16% of the total cystine in the 15% LPC diet. The ileal digestibility results therefore confirm the earlier suggestion arising from the growth trials that the cystine digestibility-availability of the whole diet was being reduced and that a low digestibility of the LPC alone could not explain the observed results.

Tamir et al. (1974) presented some-what similar results. They found the when a diet containing both SBM and radioactively labelled casein was fed to chickens, the level of undigested casein was 3-4 times higher in the ileum of chickens fed with raw soybean, compared to the level in chickens fed with heat-treated SBM. The digestibility difference, therefore was not confined to the soybean but extended to the other protein sources in the diet.

Because of the differing methionine and cystine availability-digestibility results, it is of interest to use these results to predict the amount of additional methionine required. The predicted methionine and cystine availability-digestibility differences of the two diets (LPC and SBM) are summarised in

Table XXVIII: Availability of Sulphur Amino Acids (Cystine and Methionine) in LPC and SBM and in diets containing LPC and SBM as determined by several Methods.

Method		Available Cystine (g/kg diet)	Available Methionine (g/kg diet)	Total Sulphur Amino Acid Available (g/kg diet)	Difference in Sulphur Amino Acid availability (a - b) in diets (g/kg)
TME method CAAA-ingredients (from Table XXII)	SBM[1]	3.91	2.37	6.28(a)	0.23
	LPC[1]	3.41	2.64	6.05(b)	
TME method ApAAA-ingredients (from Table XXII)	SBM	3.77	2.30	6.07(a)	
	LPC	3.17	2.56	5.73(b)	0.34
TME method CAAA-diets (from Table XXIII)	SBM Diet[1]	3.23	2.10	5.33(a)	
	LPC Diet[1]	2.67	2.40	5.07(b)	0.26
TME method ApAAA-diets (from Table XXIII)	SBM Diet	2.79	1.97	4.76(a)	
	LPC Diet	2.19	2.30	4.49(b)	0.27
Ileal method ApAAD-diets (from Table XXIV)	SBM Diet	2.53	2.10	4.63(a)	
	LPC Diet	0.68	1.60	2.28(b)	2.35
Total Excreta Collection in chickens ApAAA diets (from Table XXIV)	SBM Diet	3.19	2.06	5.25(a)	
	LPC Diet	2.30	2.42	4.72(b)	0.53

[1] The level of cystine and methionine contributed to the SBM or LPC diets by each major component

Component	Cystine (g/kg)	Methionine (g/kg)
150 g LPC/ kg diet	0.6	1.2
130 g SBM/kg diet	0.9	0.8
800 g basal/kg diet	3.1	1.6

* For calculation of available cystine and methionine from the amino acid availability estimates for the "pure" ingredients, it was assumed that the amino acid availability of the basal diet was 100%.

Table XXVIII.

The predicted differences using the methionine + cystine availability estimates, ranged from 0.023% to 0.034%. In contrast, if the ileal digestibility results are used, the predicted difference was 0.235%. This compared favourably with the results of the growth trials. Approximately 0.2% methionine was required to be added to the 15% LPC diet to achieve the greatest growth improvement. The amount of methionine and cystine present in the lower region of the ileum was therefore a better indicator of the quantity of methionine + cystine unavailable to the chickens fed the LPC diets, than the estimates derived from analysis of the excreta.

The low ileal digestibility values of the LPC diet may be due to a lowered level of digestion and/or absorption. In addition, these low estimates may have been due to increased amounts of endogenous protein secretion. This latter suggestion was supported by the observation that the chickens fed the LPC diet developed enlarged pancreases. Pancreatic hypertrophy has been shown to be associated with increased secretion of pancreatic enzymes (Gertler et al., 1967).

In the present study, the activities of trypsin and chymotrypsin in the pancreases of the chickens fed the LPC diet were higher than in those fed the SBM diet. According to Neurath (1961) this indicates an increase in the synthesis of the pancreatic enzymes. As the pancreatic enzymes chymotrypsin and trypsin are "rich" in cystine (Neurath, 1961), an increased secretion level and subsequent endogenous loss of the enzymes may contribute to methionine and/or cystine unavailability (Barnes et al., 1965 a, b; Booth et al., 1960) to the chicken.

A number of workers have demonstrated that pancreatic hypertrophy and increased protease secretion can result from the presence of trypsin inhibitors in a diet (Liener, 1979). Rakis et al. (1975)

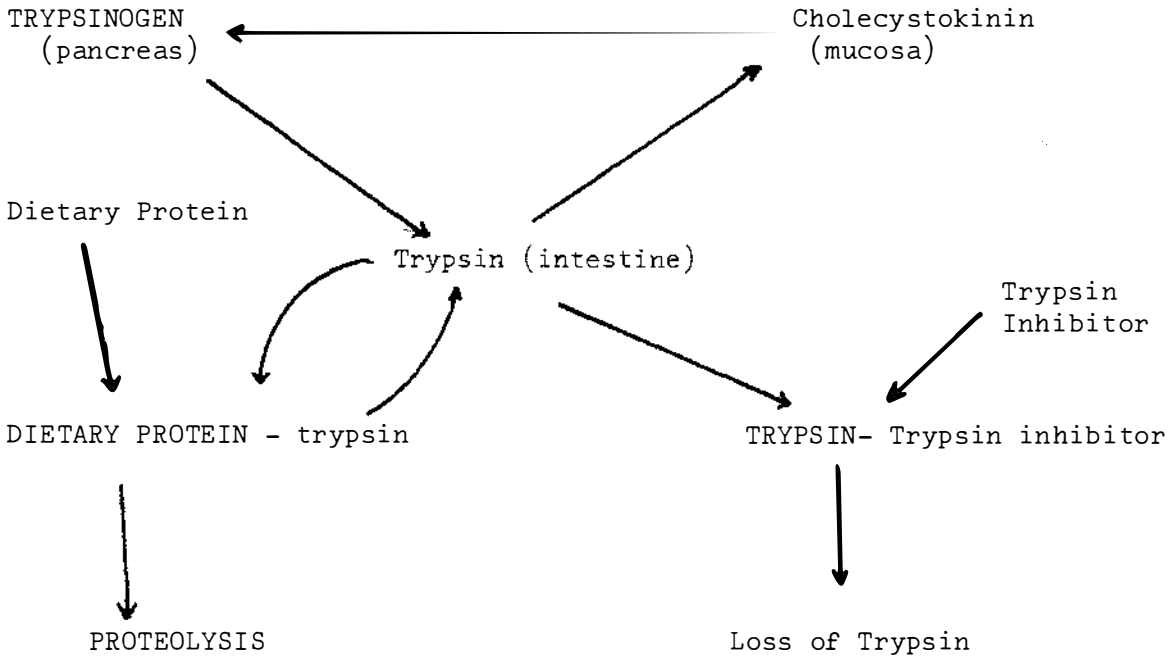
have reported that in rats that, as trypsin inhibitor activity decreased, weight gain, the protein efficiency ratio and nitrogen digestibility, all rapidly increased while pancreatic hypertrophy decreased. Extracts of soybean trypsin inhibitor isolated by affinity chromatography and added to diets have also been shown to cause both growth depression and pancreatic hypertrophy in rats (Kakade et al., 1973).

Trypsin inhibitors form inactive complexes with the pancreatic proteolytic enzymes (Liener, 1979). This lowers the level of active proteolytic enzymes in the intestine. The level of proteolytic enzymes in the intestine is probably controlled by a negative feed-back system under the influence of the hormone cholecystokinin, so that a lowering in active enzyme level in the intestine ultimately influences the level of enzyme secretion; this is shown in Fig. 18. The digestion of dietary protein is believed to be under the influence of the same mechanism without the protein forming inactive complexes with the enzymes (Schneeman et al., 1979). Inactivation of the proteloytic enzymes therefore increases the animals' requirement for "cystine-rich" proteolytic enzymes, increases the endogenous cystine loss via the complexed proteolytic enzymes and presumably slows the digestion rate.

The inactivation of enzymes by trypsin inhibitors, may explain the digestibility results. Digestion as measured in the ileal contents, was reduced by approximately 26% due to feeding LPC in a diet, compared to feeding SBM in a diet. This reduction in ileal digestion was similar to the results reported by Bielorai et al. (1973), who reported that the feeding of raw soybean meal to chickens reduced the net absorption of nitrogen by at least 20% compared to the feeding of heat-treated soybean.

Bielorai et al.(1977) obtained a correlation coefficient of 0.87 ($P < 0.01$) when comparing the amino acid absorption values for raw and heat-treated SBM in the duodenum. This indicated to

Fig. 18: Regulation of Trypsin Secretion (Adapted from Anderson et al., 1979)



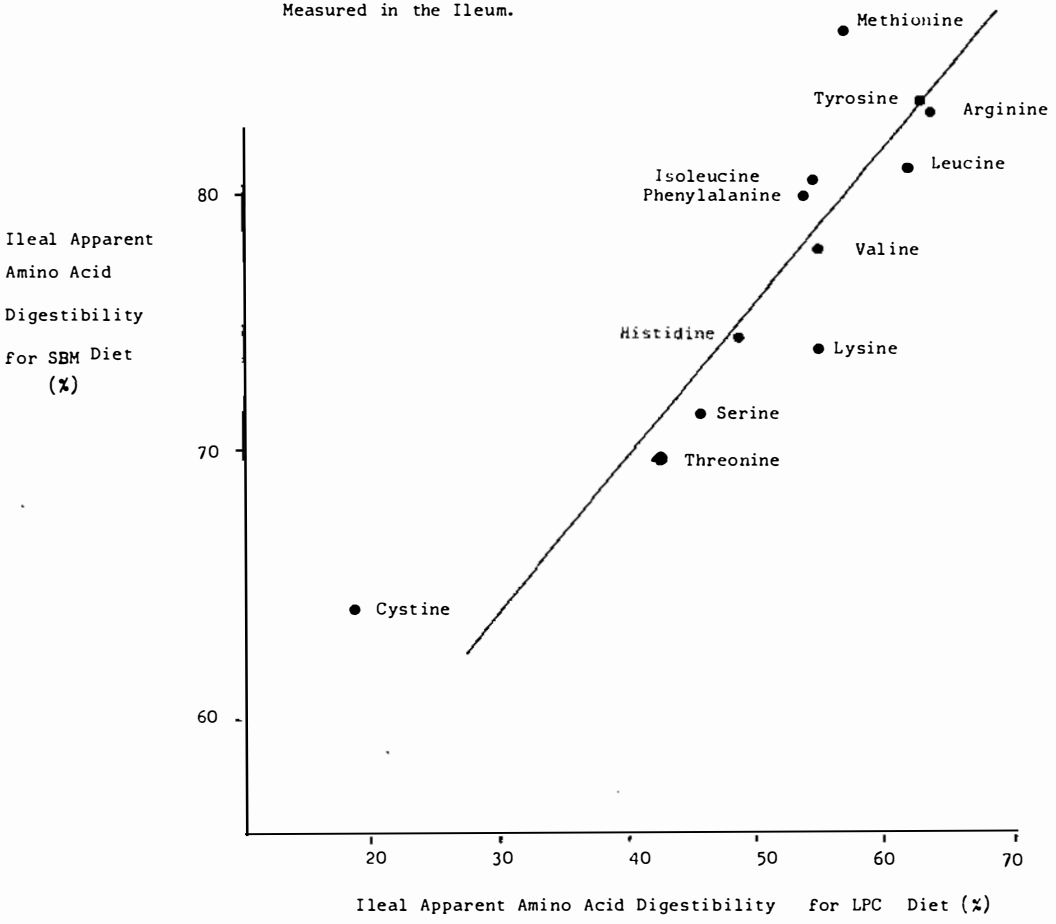
them a high relationship between the levels of endogenous nitrogen secretion (i.e. 76% of the variation accounted for) when comparing the effect of treated and untreated SBM. The correlation coefficient became 0.69 ($P < 0.1$) when calculated from the published results at the ileum (i.e. 48% of the variation accounted for). In this study, comparison of the amino acid ileal digestibility (LPC/SBM) yielded a correlation coefficient of 0.91 ($P < 0.01$) (83% of the variation accounted for) as illustrated in Fig. 19.

This relationship may be due to a lower digestion rate of the LPC diet or it may reflect a high endogenous protein secretion level. The second option was supported by the high level of cystine in the ileal contents of chickens fed the LPC diet. The apparent digestibility of cystine in the LPC diet was reduced by 45% compared to apparent digestibility of cystine in the SBM diet. This result suggests that endogenous protein breakdown was slow until the area of the caecum was reached. At the caecum, microfloral activity presumably reduced the amino acid levels. This was reflected by the higher availability values determined by excreta analysis, compared to the ileal digestibility values.

The recycling of endogenous proteins and/or digestion of the protein material in the LPC diet was also influenced by the activity of the gut microflora. This was indicated by an improvement in both the amino acid availability estimates and the growth of chickens fed LPC when the LPC diet was supplemented with antibiotic. The addition of antibiotic to the diet improved the mean ApAAA value by 7%. Similar observations have been reported in the literature.

Miller and Coates (1966) reported that germ-free chicks grew better on raw soybean diets than conventional chicks. These results were confirmed by Coates et al. (1970) who concluded that "the gut microflora exacerbated the effect on chicks of raw

Fig. 19 : Relationship between the SBM and LPC Dietary Amino Acid Digestibility as Measured in the Ileum.



soybean meal." Other workers have also shown that the supplementation with antibiotics of diets containing raw soybean partially alleviates the growth depression in rats (Barnes et al., 1965 c), chicks (Braham et al., 1959) and poults (Linerode et al., 1961).

There have been other similar reports on the effects of microflora, by Gracey et al. (1971), Ruff (1978) and Turk (1978). Ruff and Edgar (1982) reported impaired intestinal absorption of glucose and methionine when birds were innoculated with coccidial oocytes. The absorption rate was lowered by 52% and the severity was directly proportional to the dose level of oocytes.

Although it is possible that microflora may produce toxic substances which may in turn adversely affect bird growth, (Rerat, 1978) the results presented in this study did not confirm this. Increasing the intake levels of the LPC diet by pelleting (Trials 3 and 7) did not decrease the growth rate. If toxicity was a contributing factor the reverse would be expected.

The nature and/or activity of the microfloral population, however, may have been altered due to feeding the LPC diet compared to the SBM diet. This was indicated by the difference in the amount of cyclopropane fatty acid excreted by chickens fed the two diets and by the different effect of added antibiotic on the growth of chickens fed either diet and on the estimates of availability of the amino acids.

The conditions for a change in the gut micro-organisms may have been created by the reduction in ileal protein digestion and/or absorption of the LPC diet. Jayne-Williams and Coates (1969) have suggested that reduced protein digestibility measured at the ileum, when raw soybean is fed, may affect the balance of the microflora in the intestinal tract which will in turn reduce the absorption of nutrients.

Some other possible factors, apart from trypsin inhibitor activity, which may reduce the protein digestibility of the whole diet were, as mentioned in Chapter 5, the interaction of protein material with other compounds after ingestion and the detoxification of phenolic compounds. No evidence for the presence of either factor was found in this study. No protein-phenolic complexes were found in the excreta of chickens fed the LPC diet. Also the percentage radioactivity recovered in the excreta was not significantly different for chickens fed either the LPC or SBM diets after they had been intubated with L-(methyl ^{14}C)methionine.

Such a result contrasts with the findings of Elkin et al. (1978). They reported that when chickens were fed L-(methyl ^{14}C)methionine with either high-tannin sorghum or low-tannin sorghum that the level of radioactivity recovered in the excreta of chickens fed high-tannin sorghum was significantly higher the level recovered in the excreta of chickens fed low-tannin sorghum. Elkin et al. (1978) postulated that methionine was preferentially bound by tannins during the digestion process. Lack of variation in the radioactive excretion levels therefore indicates that the digestibility of methionine in the LPC diet was not being reduced due to the formation of complexes during the digestion process. The relatively high estimates for methionine availability in the LPC diet also supports such a conclusion. In addition, these results did not indicate that significant quantities of methionine were required for the detoxification of phenolic compounds. The overall amount of radioactivity excreted by chickens fed either the LPC or SBM diet did not differ signficantly. The corresponding radioactivity levels in the excreta "water fractions" did not differ significantly either. The possible role of ornithine, which is derived from arginine, for the detoxification of phenolic compounds, was also investigated. Once again, there was no significant difference in the amount of ornithine excreted by chickens fed diets containing LPC or SBM.

From the results of this study it therefore appears that the feeding of LPC to chickens in comparison to feeding SBM

(i) required the addition of relatively large amounts of methionine and/or cystine to the diet

(ii) caused pancreatic hypertrophy

(iii) increased the level of proteolytic enzymes present in the pancreas

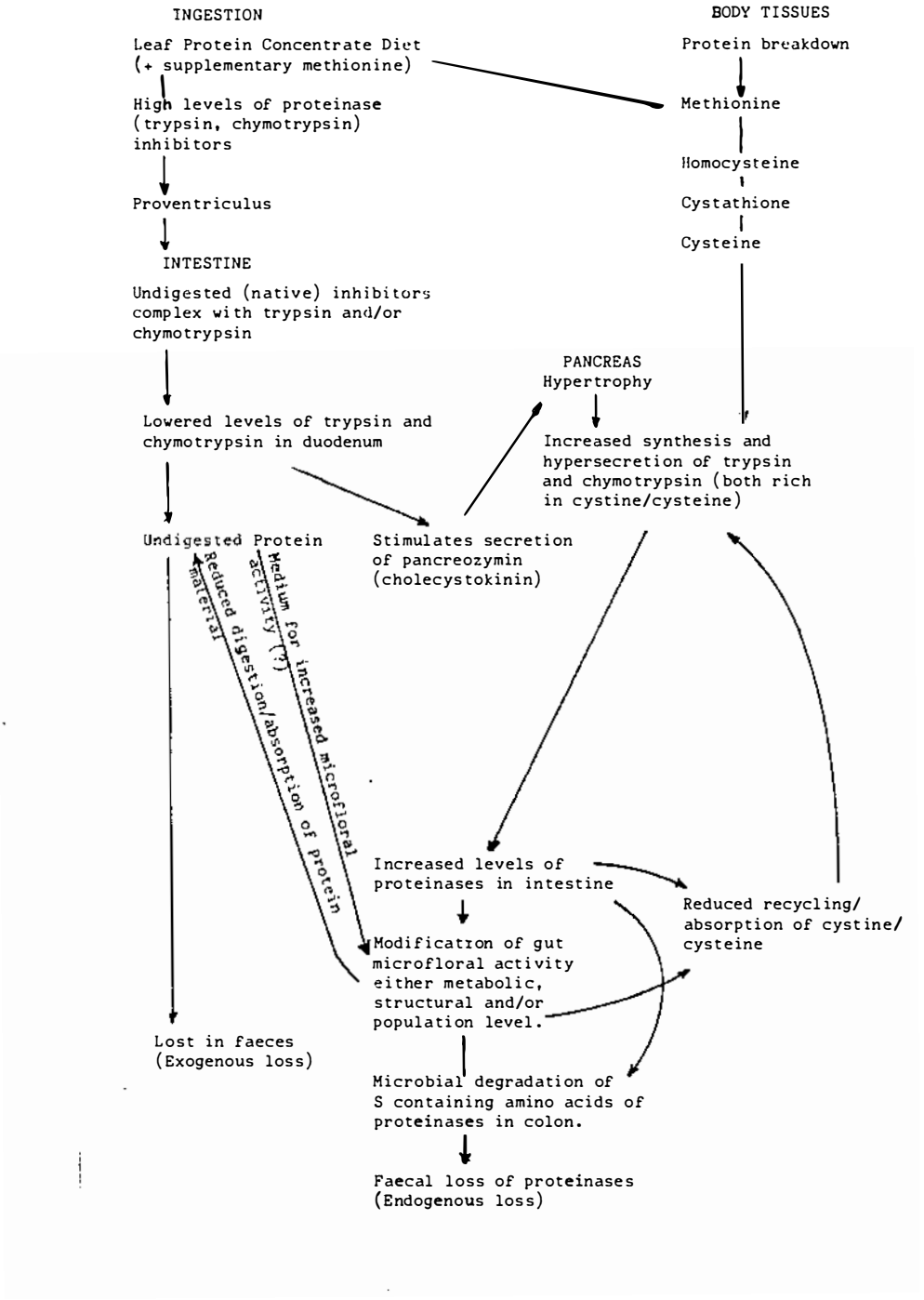
(iv) retarded digestion and/or absorption as measured in the ileal contents

(v) influenced the gut microfloral nature and/or activity.

Each of these physiological and metabolic effects have been reported as being characteristic of chickens fed raw soybean or trypsin inhibitors. Thus, it was concluded that it was necessary to add methionine and/or cystine to the LPC diets because of the presence of trypsin inhibitors in the LPC. A diagram showing the suggested general effects of feeding LPC is shown in Fig. 20.

The actual identity of the trypsin inhibitors requires further investigation. However it is of interest to speculate their nature from the present work and that of others. Subba Rau et al.(1972) from work with various LPC's, reported that a linear relationship exists between weight gain and the amount of organic sulphur or cystine sulphur in LPC and that an inverse relationship exists between weight gain and phenolic content of LPC. Recalculation of the data (shown in Figs. 21 and 22) demonstrated a high correlation between both weight gain and organic sulphur/phenolic content and weight gain and cystine sulphur/phenolic content (correlation coefficients 0.935 and 0.971 with 10 d.f. respectively or 87% and 94% of the variation accounted for respectively). Such a relationship may explain the variation in nutritional value of the different LPC's studied. Subba

Fig. 20 : Scheme to explain the possible deleterious effect of proteinase inhibitors on the nutritive value of proteins. (Modification from Richardson, 1981)



Rau's et al. (1972) data is in line with the findings of this study i.e. not only is there a relationship between cystine and/or methionine and growth but there is also anti-nutritional "activity" present in some LPC's e.g. phenolics in the case of Subba Rau et al. (1972) quoted above.

Humphries (1980), reported that protease inhibitors in Italian Ryegrass could be removed by PVP treatment before heat precipitation and that the inhibitor(s) was heat stable. Both characteristics apply to most phenolic compounds. Donnelly (1980) showed that sodium metabisulphite treatment during processing improved the nutritional availability of the sulphur amino acids in LPC, with greatest improvement occurring in the Ryegrass-White Clover LPC. Pierpoint (1971) noted that bisulphite treatment reduced the formation of o-quinones from polyphenolics and, as already mentioned, quinones readily interact with proteins. Therefore it appears probable that polyphenolics were affecting the nutritional quality of LPC, not by causing the product to have a lower digestibility due to the formation of indigestible complexes as suggested by Donnelly (1980), but by interfering with the digestion process via trypsin-inhibitor mechanisms. Preliminary work has shown that feeding the 13% SBM diet supplemented with only 0.07%(w/w) benzoquinone to chickens produced a significant increase ($P<0.05$) in pancreatic size, (Johns, D.C., unpublished results). This result supported the hypothesis that phenolics may act as trypsin inhibitors.

Despite not identifying the specific chemical compound(s) adversely affecting the growth of chickens fed the LPC diet, the research has proceeded to the point where sufficient is known to enable LPC to be successfully used as a component of diets for feeding to chickens.

Fig. 21 : Relationship Between Weight Gain and Organic Sulphur/Phenolic Intake (data calculated from Subba Rau et al., 1972)

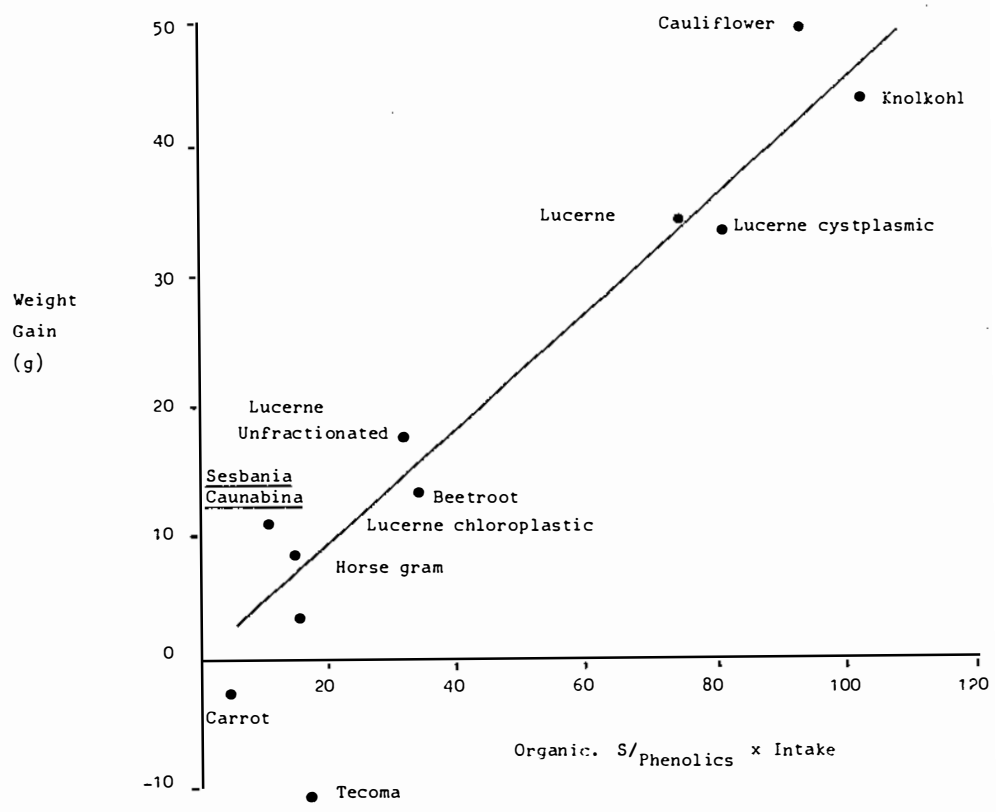
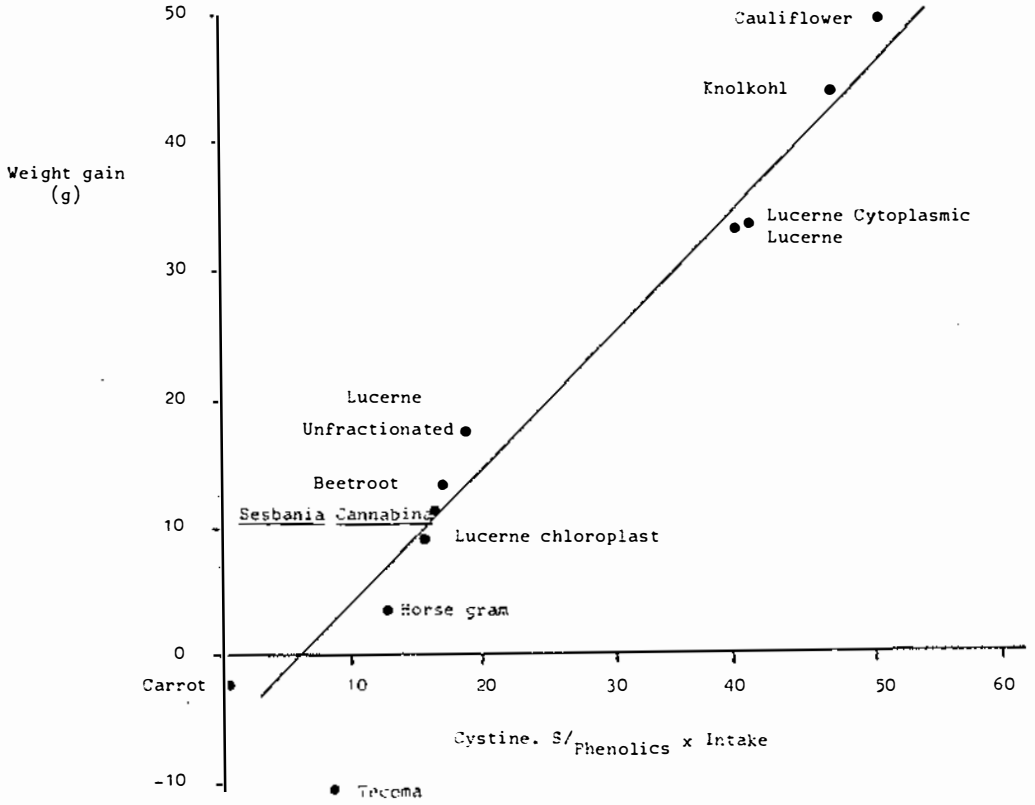


Fig. 22 : Relationship Between Weight Gain and Cystine Sulphur/Phenolic Intake (data calculated from Subba Rau et al., 1972)



APPENDIX A :

Sources of Dietary Ingredients Used in the Following Tables: I-XXIV

Leaf Protein Concentrate (LPC)

LPC was produced at Ruakura Animal Research Centre as described in Chapter 2.

Soybean Meal (SBM)

SBM (50%) was supplied by the Feed Processing Centre, Massey University. All other major ingredients were also supplied by the Feed Processing Centre, Massey University.

Vitamin and Mineral Premix

The premix suitable for growing egg-type stock was obtained from Northern Roller Mills, Auckland.

Cornstarch

The cornstarch was purchased from N.Z. Starch Products Ltd., Auckland.

Amino Acids

(i) L-Lysine.HCl (98%) was purchased from Kyowa Hakko Kogyo Co. Ltd., Japan.

(ii) DL-methionine was purchased from Nippon Kyaku Co. Ltd., Tokoyo, Japan.

(iii) L-cystine, L-arginine and L-histidine were purchased from Sigama Chemicals, U.S.A.

Chromic Oxide

Chromic oxide was purchased from BDH Ltd., England.

Antibiotic

The antibiotic, Neomix (equivalent to Neomycin activity 50%), was obtained from Upjohn Pty. Ltd., Auckland.

APPENDIX A: Trial 1 - Acceptability of LPC to Chickens

Table I: Constraints used in the Formulation of Diets for Trial 1.

		"80% NRC" Diets	"70% NRC" Diets
Available Phosphorus	% minimum	0.50 - 0.60	
Calcium	" "	0.90 - 1.0	
Potassium	" "	0.20	
Sodium	" "	0.13	
Chloride	" "	0.13	
Arginine	" "	1.12	0.98
Histidine	" "	0.46	0.32
Isoleucine	" "	0.67	0.60
Leucine	" "	1.28	1.12
Glysine + Serine	" "	0.92	0.81
Phenylalanine + Tyrosine	% minimum	1.20	1.05
Methionine + Cystine	% minimum	0.69	0.60
Threonine	" "	0.64	0.56
Valine	" "	0.80	0.70
Tryptophan	" "	0.18	0.16
Lysine	" "	1.00	0.88
Premix	equality	0.25	
Crude Protein	free		
Energy kJ/g	equality	11.71 (raised to 12.13 in diets C & D)	
Tallow	Maximum %	3.5	
Soyabean	" "	25.0	
Maize	" "	60.0	
Wheat	" "	60.0	
Barley	" "	60.0	
Pollard	" "	20.0	
Bran	" "	5.0	
Livermeal	" "	3.0	
Lupin	" "	10.0	
Peas	" "	10.0	
Blood	" "	2.0	
Brewers Grains	" "	5.0	
Meat & Bone	" "	25.0	
LPC	" "	25.0	

APPENDIX A:

Table II: The Ingredient Composition of the Experimental Diets used in Trial 1

	"80% NRC"		"70% NRC" + 2% Tallow	
	Control	LPC	Control	LPC
Ingredients	A	B	C	D
LPC	-	25.00	-	25.00
SBM	25.00	-	25.00	-
Maize	47.22	24.60	46.57	33.64
Wheat	-	25.19	-	14.90
Pollard	20.00	20.00	20.00	20.00
Bran	-	0.14	-	-
Brewers Grains	4.12	1.93	2.83	2.03
Bloodmeal	-	0.66	-	-
Tallow	-	-	2.00	2.00
Boneflour	1.98	-	2.01	-
Limestone	0.98	1.85	0.97	1.86
Salt	0.29	0.24	0.30	0.25
DL-Methionine	0.16	0.14	0.07	0.07
Premix	0.25	0.25	0.25	0.25

APPENDIX A:

Table III: The Nutrient Compostion of the Experimental Diets Used in Trial 1

Composition	A	B	C	D
Energy kJ/g	11.71	11.71	12.13	12.13
Crude Protein %	20.58	20.91	20.23	19.84
Calcium %	1.0	0.90	1.00	0.90
Available Phosphorus %	0.50	0.50	0.50	0.50
Total Phosphorus %	0.72	0.97	0.72	0.95
Chlorine %	0.21	0.19	0.21	0.20
Sodium %	0.13	0.13	0.13	0.13
Potassium %	0.84	0.41	0.83	0.38
Fat %	3.08	2.81	4.98	4.98
Ash %	4.95	6.54	4.90	6.47
Arginine %	1.32	1.12	1.32	1.08
Histidine %	0.50	0.48	0.50	0.45
Methionine + Cystine %	0.69	0.69	0.60	0.60
Lysine %	1.06	1.00	1.04	0.95
Isoleucine %	0.80	0.78	0.78	0.76
Leucine %	1.74	1.77	1.71	1.71
Threonine %	0.73	0.81	0.72	0.77
Phenylalanine + Tyrosine %	1.58	1.78	1.55	1.71
Tryptophan %	0.25	0.18	0.24	0.16
Valine %	0.88	1.09	0.87	1.02

APPENDIX A: Trial 2 - Lysine & Methionine Supplementation

Table IV: Constraints Used in the Formulation of Diets for Trial 2

Available Phosphorus	% minimum	0.5 - 0.6
Calcium	" "	0.9 - 1.0
Potassium	" "	0.2
Sodium	" "	0.13
Chlorine	" "	0.13
Arginine	" "	0.98
Histidine	" "	0.32
Isoleucine	" "	0.60
Leucine	" "	1.12
Glysine + Serine	" "	0.81
Phenylalanine + Tyrosine	% minimum	1.05
Methionine + Cystine	% minimum	0.64
Threonine	" "	0.56
Valine	" "	0.70
Tryptophan	" "	0.16
Lysine	" "	0.88
Premix	equality	0.25
Crude Protein	Free	
Energy kJ/g	equality	12.13
Tallow	"	2.00
SBM	"	15.00
LPC	"	15.00 (released to include additional 5%)
Maize	Maximum %	60.0
Wheat	" "	60.0
Barley	" "	60.0
Pollard	" "	20.0
Bran	" "	5.0
Livermeal	" "	3.0
Lupins	" "	10.0
Peas	" "	10.0
Bloodmeal	" "	2.0
Brewers Grain	" "	5.0
Meat & Bone Meal	" "	25.0

APPENDIX A:

Table V: Ingredient Composition of the Experimental Diets - Trial 2

	A	B	C	D
Maize	50.52	47.77	47.77	60.00
Pollard	20.00	20.00	20.00	5.85
Bran	2.18	5.00	5.00	1.58
Meat & Bone Meal	-	2.36	2.36	2.36
Brewers Grains	5.00	3.18	3.18	3.18
Bloodmeal	1.57	0.38	0.38	0.83
Livermeal	-	1.97	1.97	1.97
LPC	-	15.00	15.00	20.00
SBM	15.00	-	-	-
Tallow	2.00	2.00	2.00	2.00
Limestone	0.65	1.76	1.62	1.64
Salt	0.28	0.22	0.22	0.23
DL - Methionine	0.16	0.11	0.15	0.11
L-Lysine.HCl	-	-	0.1	-
Boneflour	2.39	-	-	-
Premix	0.25	0.25	0.25	0.25

(Additional synthetic methionine was added to ensure an increased level of 0.04% rather than computer formulation).

APPENDIX A:

Table VI: The Nutrient Composition of Rations Used in Trial 2

	A	B	C	D
Energy kJ/g	12.13	12.13	12.13	12.13
Crude Protein %	17.68	18.92		20.19
Calcium %	1.00	0.90		1.00
Available Phosphorus %	0.50	0.50		0.54
Chlorine %	0.13	0.17		0.13
Sodium %	0.20	0.13		0.18
Potassium %	0.68	0.42		0.40
Fat %	5.30	6.01		7.62
Ash %	4.65	5.61		5.86
Arginine %	1.02	0.98		0.98
Histidine %	0.46	0.43		0.41
Methionine + Cystine %	0.64	0.64	0.67	0.65
Lysine %	0.88	0.89	0.99	0.82
Isoleucine %	0.62	0.65		0.68
Threonine %	0.63	0.70		1.68
Phenylalanine + Tyrosine %	1.37	1.53		1.68
Tryptophan %	0.21	0.16		0.14
Valine %	0.82	0.93		1.01

APPENDIX A: Trial 3 - Lysine and Methionine Supplementation

Table VII: Constraints Used in the Formulation of Diets for Trial 3

Available Phosphorus	% Minimum	0.5 - 0.6
Calcium	"	0.9 - 1.0
Potassium	"	0.2
Sodium	"	0.13
Chlorine	"	0.13
Arginine	"	0.98
Histidine	"	0.32
Isoleucine	"	0.60
Leucine	"	1.12
Glycine + Serine	"	0.81
Phenylalanine + Tyrosine	"	1.05
Methionine + Cystine	"	0.64
Threonine	"	0.56
Valine	"	0.70
Tryptophan	"	0.16
Lysine	"	0.88
Premix	Equality	0.25
Crude Protein %	Free	-
Energy kJ/g	Equality	12.13
Tallow	"	2.00
SBM	"	15.00
LPC	"	15.00
Maize	Maximum %	60.00
Wheat	"	60.00
Barley	"	60.00
Pollard	"	20.00
Bran	"	5.00
Livermeal	"	3.00
Lupins	"	10.00
Peas	"	10.00
Bloodmeal	"	2.00
Brewers Grains	"	5.00
Meat & Bone Meal	"	25.00

APPENDIX A :

Table VIII: Ingredient Composition of Experimental Diets, Trial 3.

	A	B	C	D	E	F
Maize	50.52	47.77	41.77	47.77	47.77	60.00
Pollard	20.00	20.00	20.00	20.00	20.00	5.85
Bran	2.18	5.00	5.00	5.00	5.00	1.58
Meat & Bone Meal	-	2.36	2.36	2.36	2.36	2.36
Brewers Grains	5.00	3.18	3.18	3.18	3.18	3.18
Bloodmeal	1.57	0.38	0.38	0.38	0.38	0.83
Livermeal	-	1.97	1.97	1.97	1.97	1.97
LPC	-	15.00	15.00	15.00	15.00	20.00
SBM	15.00	-	5.00	-	-	-
Tallow	2.00	2.00	3.00	2.00	2.00	2.00
Limestone	0.65	1.76	1.76	1.56	1.76	1.64
Salt	0.28	0.22	0.22	0.22	0.22	0.23
DL-Methionine	0.16	0.11	0.11	0.16	0.11	0.11
L-Lysine HCl	-	-	-	0.15	-	-
Boneflour	2.39	-	-	-	-	-
Premix	0.25	0.25	0.25	0.25	0.25	0.25

APPENDIX A :

Table IX: The Nutrient Composition of Rations* Used in Trial 3

	A	B	C	D	E	F
Energy kJ/g	12.13	12.13	12.13	12.13	12.13	12.13
Crude Protein %	17.68	18.92	20.06	18.92	18.92	18.74
Calcium %	1.00	0.90	0.90	1.00	0.90	1.00
Average Phosphorus %	0.50	0.50	0.50	0.50	0.50	0.54
T[1]. Phosphorus %	0.75	0.89	0.94	0.89	0.89	0.85
Chloride %	0.20	0.17	0.17	0.17	0.17	0.18
Sodium %	0.13	0.13	0.13	0.13	0.13	0.13
Potassium %	0.68	0.42	0.60	0.42	0.42	0.40
Fat %	5.30	6.01	8.31	6.01	6.01	7.62
Ash %	4.65	5.61	5.78	5.61	5.61	5.86
Arginine %	1.08	0.98	1.09	0.98	0.98	1.02
Histidine %	0.48	0.43	0.49	0.45	0.45	0.44
Methionine + Cystine %	0.64	0.64	0.69	0.69	0.64	0.67
Lysine %	0.86	0.89	1.02	1.04	0.89	0.89
Isoleucine %	0.64	0.65	0.72	0.65	0.65	0.72
Threonine %	0.71	0.70	0.74	0.70	0.70	0.80
Phenylalanine + Tyrosine %	1.63	1.53	1.58	1.53	1.53	1.72
Tryptophan %	0.21	0.16	0.32	0.16	0.16	0.14
Valine %	0.95	0.93	0.95	0.93	0.93	1.03

* Amino acid analysis was carried out on each diet

[1] T^1. = Total

APPENDIX A : Effect of Additional Lysine, Arginine and/or Methionine

Table X: Constraints Used in the Formulation of the Basal for Trials 4-7

Available Phosphorus	% minimum	0.66 - 0.81
Calcium	"	1.08 - 1.25
Sodium	"	0.15 - 0.17
Chloride	"	0.15 - 0.30
Potassium	"	0.20
Arginine	"	0.60
Leucine	"	0.62 - 1.00
Isoleucine	"	0.39
Glycine + Serine	"	0.48
Lysine	"	0.49
Histidine	"	0.13
Methionine + Cystine	"	0.39
Tyrosine + Phenylalanine	% minimum	0.55
Threonine	% minimum	0.37
Valine	"	0.58
Tryptophan	"	0.07
Premix	Equality	
Crude Protein	Free	
Energy kJ/g		11.71
Tallow	Maximum %	3.50
Pollard	"	20.00
Bran	"	5.00
Brewers Grain	"	8.00
Maize	"	60.00
Barley	"	60.00
Soybean Meal	"	60.00
Wheat	"	60.00

APPENDIX A :

Table XI: The Calculated Composition of the Basal Used in Trials 4-7

Energy kJ/g	11.71
Calcium %	1.24
Available Phosphorus %	0.66
Total Phosphorus %	0.93
Sodium %	0.15
Chloride %	0.26
Potassium %	0.46
Crude Fibre %	2.91
Ash %	4.74
Fat %	5.61
Crude Protein %	12.09
Arginine %	0.60
Leucine %	1.00
Isoleucine %	0.39
Glycine + Serine %	0.77
Lysine %	0.49
Histidine %	0.27
Methionine + Cystine %	0.39
Tyrosine + Phenylalanine %	0.92
Threonine %	0.38
Valine %	0.58
Tryptophan %	0.15

APPENDIX A :

Table XII: The Ingredient Composition of the Basal* Used in Trials 4-7.

Pollard	20.00
Brewers Grain	8.00
Maize	43.49
Barley	18.48
SBM	2.91
Tallow	2.23
L-Lysine. HCl	0.05
Boneflour	4.18
Salt	0.34
Premix	0.32

* The above ingredient composition was mixed separately and then incorporated into the overall basal diet at the 80% level with variable levels of cornstarch. The level of cornstarch depended on the level of "test" protein in the overall test diet.

APPENDIX A :

Table XIII: Ingredient Composition of Diets Used in Trial 4

Diet	Basal %	Test	Cornstarch
A	80	15% SBM + 0.08% DL - methionine	4.92
B	80	15% LPC	5.00
C	80	15% LPC + 0.1% L-arginine	4.90
D	80	15% LPC + 0.2% L-arginine	4.80
E	80	15% LPC + 0.2% L-lysine	4.80
F	80	15% LPC + 0.6% L-lysine	4.40
G	80	15% LPC + 0.2% L-lysine + 0.2% arginine	4.60
H	80	15% LPC + 0.6% L-lysine + 0.6% arginine	3.80
I	80	15% LPC + 0.1% DL -methionine	4.90
J	80	15% LPC + 0.1% DL -methionine + 0.1% L-arginine	4.80
K	80	15% LPC + 0.1% DL -methionine + 0.2% L-arginine	4.70
L	80	15% LPC + 0.2% L-arginine + 0.2% L-lysine + 0.2% DL-methionine + 0.2% L-histidine	4.2. 4.40

APPENDIX A:

Table XIV: The Calculated Nutrient Composition of Diets Used In Trial 4

	A	B	C	D	E	F	G	H	I	J	K	L
Energy kJ/g	11.68	11.81										
Calcium %	1.03	1.13										
Available Phosphorus %	0.63	0.77										
Sodium %	0.13	0.13										
Chloride %	0.20	0.20										
Potassium %	0.67	0.73										
Arginine %	1.04	0.95	1.05	1.15			1.15	1.43		1.05	1.15	1.15
Leucine %	1.36	1.40										
Isoleucine %	0.64	0.63										
Lysine %	0.82	0.83			1.03	1.43	1.03	1.55				1.03
Histidine %	0.37	0.35										0.55
Methionine + Cystine %	0.75	0.65							0.75	0.75	0.75	0.85
Tyrosine + Phenylalanine %	1.40	1.44										
Valine %	0.51	0.56										

Amino acid analysis was carried out on the basal and ingredients SBM and LPC. The diet composition was calculated from the analysis results.

APPENDIX A : Trial 5: Methionine and Arginine Supplementation

Table XV: Ingredient Composition of Diets Used in Trial 5

Diet	Basal %	Test	Cornstarch %
A(44)	80	13% SBM + 0.03% DL-methionine	4.97
B(00)	80	15% LPC	5.00
C(01)	80	15% LPC + 0.1% L-arginine	4.90
D(02)	80	15% LPC + 0.2% L-arginine	4.80
E(03)	80	15% LPC + 0.3% L-arginine	4.70
F(10)	80	15% LPC + 0.1% DL-methionine	4.90
G(11)	80	" + 0.1% " " "	4.80
H(12)	80	" + 0.2% " " "	4.70
I(13)	80	" + 0.3% " " "	4.60
J(20)	80	" + 0.2% "	4.80
K(21)	80	" + 0.1% " " "	4.70
L(22)	80	" + 0.2% " " "	4.60
M(23)	80	" + 0.3% " " "	4.50
N(30)	80	" + 0.3% "	4.70
O(31)	80	" + 0.1% " " "	4.60
P(32)	80	" + 0.2% " " "	4.50
Q(33)	80	" + 0.3% " " "	4.40

The diets A, B, I and N were used in the evaluation of ornithine excretion level- Experiment 3.

Diet B was used in Experiment 1.

APPENDIX A :

Table XVI: The Calculated Nutrient* Composition of the Diets Used in Trial 5

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Energy kJ/g	11.48	11.81															
Calcium %	1.03	1.13															
Available Phosphorus %	0.61	0.77															
Sodium %	0.13	0.13															
Chloride %	0.20	0.20															
Potassium	0.63	0.73															
Crude Protein%	16.12	15.97															
Arginine %	0.97	00.88	0.98	1.08	1.18	0.88	0.98	1.08	1.18	0.88	0.98	1.08	1.18	0.88	0.98	1.08	1.13
Leucine %	1.29	1.34															
Isoleucine %	0.60	0.60															
Lysine %	0.75	0.74															
Histidine %	0.36	0.33															
Methionine + Cystine %	0.66	0.65	0.65	0.65	0.65	0.75	0.75	0.75	0.75	0.85	0.85	0.85	0.85	0.95	0.95	0.95	0.95
Tyrosine + Phenylalanine %	1.32	1.38															
Valine %	0.46	0.50															

* The ingredients LPC and SBM and basal were individually analysed for amino acids. The nutrient composition was calculated from the results.

APPENDIX A : Trial 6 : Methionine and Cystine Supplementation

Table XVII: Ingredient Composition of Diets Used in Trial 6

Diet	Basal %	Test	Cornstarch %
A	80	15% LPC	5.00
B	80	13% SBM	7.00
C	80	15% LPC + 0.2% DL-methionine	4.80
D	80	15% LPC + 0.16% L-cystine	4.84
E	80	15% LPC + 0.12% L-cystine + 0.05% DL-methionine	4.83
F	80	15% LPC + 0.08% L-cystine + 0.10% DL-methionine	4.82
G	80	15% LPC + 0.04% L-cystine + 0.15% DL-methionine	4.81

Table XVIII: The Calculated* Nutrient Composition of Diets Used In Trial 6

	A	B	C	D	E	F	G
Energy kJ/g	11.48	11.81					
Calcium %	1.03	1.13					
Available Phosphorus %	0.61	0.77					
Sodium %	0.13	0.13					
Chloride %	0.20	0.20					
Potassium %	0.63	0.73					
Crude Protein %	16.12	15.97					
Arginine %	0.97	0.88					
Leucine %	1.29	1.34					
Isoleucine %	0.60	0.60					
Lysine %	0.75	0.74					
Histidine %	0.36	0.33					
Methionine + Cystine %	0.66	0.65	0:85	0.81	0.82	0.83	0.84
Tyrosine + Phenylalanine %	1.32	1.38					
Valine %	0.46	0.50					

* **Amino acids calculated from analysis of results of the LPC and SBM ingredients and basal.**

APPENDIX A : Trial 7: Effect of Pelleting and Methionine Supplementation On Increasing Levels of LPC (15%, 20%) on Chick Growth

Table XIX: Ingredient Composition of Diets Used in Trial 7

Diet	Basal %	Test	Cornstarch %
A	75	13% SBM* (mash)	12
B	75	13% SBM (pelleted)	12
C	75	15% LPC (mash)	10
D	75	15% LPC + 0.2% DL-methionine (mash)	9.8
E	75	15% LPC (pelleted)	10
F	75	15% LPC + 0.2% DL-methionine (pelleted)	9.8
G	75	17.3% SBM* (mash)	7.7
H	75	17.3% SBM (pelleted)	7.7
I	75	20% LPC (mash)	5.0
J	75	20% LPC + 0.27% DL-methionine (mash)	4.7
K	75	20% LPC (pelleted)	5.0
L	75	20% LPC + 0.27% DL-methionine (pelleted)	4.73

* Equivalent amino acid values are calculated on an equal isoleucine value. This allows for a more direct comparison to be made rather than using crude protein values for equalising the test levels.

APPENDIX A :

Table XX: The Nutrient Composition of Diets* Used in Trial 7

Diets	A-B	C-E	G-H	I-K
Energy kJ/g	12.01	12.02	11.76	11.36
Calcium %	0.96	1.07	0.98	1.12
Available Phosphorus %	0.57	0.73	0.60	0.81
Sodium %	0.11	0.11	0.11	0.11
Chloride %	0.20	0.20	0.20	0.20
Potassium %	0.60	0.47	0.68	0.52
Crude Protein %	15.57	15.31	17.72	17.40
Arginine %	0.91	0.82	1.05	0.94
Leucine %	1.25	1.30	1.42	1.49
Isoleucine %	0.57	0.56	0.66	0.65
Lysine %	0.75	0.73	0.87	0.86
Histidine %	0.36	0.33	0.41	0.38
Methionine + Cystine %	0.46	0.47	0.51	0.53
Tyrosine + Phenylalanine %	1.25	1.31	1.44	1.52
Threonine %	0.52	0.56	0.59	0.65
Valine %	0.73	0.77	0.83	0.88

Diets D & F = Diets (C) + 0.2% methionine

Diets J & L = Diets (I) + 0.27% methionine

* The amino acid composition of the diets was calculated from the amino acid analysis of the basal, SBM and LPC.

APPENDIX A: Experiment 2: Measurement of the Excretion Level of the Methionine Methyl Group (^{14}C) Due to Feeding LPC.

Table XXI: Ingredient Composition of Diets Used in Experiment 2

Diets	Antibiotic	Basal	Cornstarch
13% SBM		80	7%
13% SBM + antibiotic	200 ppm	80	7%
15% LPC		80	5%
15% LPC + antibiotic	200 ppm	80	5%

Table XXII: Amino Acid Composition of the LPC and SBM Diets used in Experiment 2

	SBM Diet	LPC Diet
Crude Protein %	14.86	15.18
Arginine %	0.98	0.95
Leucine %	1.25	1.40
Isoleucine %	0.56	0.62
Lysine %	0.72	0.75
Histidine %	0.38	0.44
Methionine + Cystine %	0.59	0.54
Tyrosine + Phenylalanine %	1.26	1.43
Valine %	0.69	0.83
Threonine %	0.55	0.63

APPENDIX A: Experiment 5 - Comparison of "Corrected (CAAA) and Apparent Amino Acid (ApAAA) Digestibility of LPC and SBM Ingredients and the Diets Containing LPC and SBM.

Table XXIII: Ingredient Composition of Diets Used in Experiment 5

	Test Protein	Basal	Cornstarch
A	15% LPC	80%	5%
B	13% SBM	80%	7%

Table XXIV: Amino Acid Composition of Diets Used in Experiment 5

	A	B
Crude Protein %	15.30	15.90
Arginine %	0.96	0.95
Leucine %	1.42	1.34
Isoleucine %	0.64	0.69
Lysine %	0.75	0.90
Histidine %	0.38	0.44
Methionine + Cystine	0.53	0.51
Tyrosine + Phenylalanine %	1.43	1.49
Valine %	0.87	0.86
Glycine + Serine %	1.63	1.56
Threonine %	0.66	0.65

These diets were also used for the determination of enzyme activity levels and ileal digestion (Experiments 6 and 7).

APPENDIX B:

Statistics

a) Mean

The mean of a sample is the sum of measurements of all the items divided by the number of items:

$$\bar{X} = \frac{\Sigma x}{n}$$

x = sum of measurements of all items

n = number of items

b) Measure of Variability

The extent to which the individual observation are around a mean is measured by the estimate of variance. The symbol used for this estimate is "S^2" where:

$$S^2 = \frac{\Sigma (x - \bar{x})^2}{n - 1}$$

n = number of measurements in the sample

Operationally S^2 can be calculated as:-

$$S^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n - 1}$$

If the square root of the variance is taken a statistic called the "standard deviation" is obtained where:-

$$S = \sqrt{S^2}$$

i.e. the individual variation of the item in a set of data may be expressed by the deviations of the items from centrally located sample mean (Snedecor and Cochrane, 1976).

For the comparison of variances in more than two groups or treatments the statistical technique used is the Analysis of Variance as discussed in Snedecor and Cochrane (1976). Treatments and replicates

were assumed to be fixed for the two-way analysis of variance. To test the probability that the variances are estimates of the same population variance, the ratio between two estimates on the variance ratio is calculated and is designated as F. To ascertain whether the value of F is significant or not it is compared with tabulated figures in the relevant F tables (Snedecor and Cochrane, 1976).

c) Duncan's Multiple Range Test

In testing the significance of mean comparisons it is necessary that the testing procedure consider the probability of detecting real differences when they exist. It is generally preferred that the procedure assure the highest probability of rejecting the significance of mean differences when, in fact the differences are not real i.e. not to accept a null hypothesis when it is in fact wrong (Hills, 1966). The Duncan's Multiple Range Test includes protection against such an error and Hills (1966) recommended that where a mean comparison test is appropriate, the Duncan's Multiple Range Test should be used.

d)

***	denotes	$P < 0.01$
**	denotes	$P < 0.05$
*	denotes	$P < 0.1$

e) Amino Acid Availability - Digestibility

Analysis of variance examples are given for cystine, methionine, isoleucine, lysine and arginine as these are considered to be the more "important" amino acids and to also reflect the overall conclusions made for all amino acids.

APPENDIX B :

Table I: Analysis of Variance - Trial 1

Body Weights

Source of Variation	Degrees of Freedom	Mean Squares	F
Treatments	3	175046.036	125.20***
Replicates	3	918.40	0.66
Between compartments	9	2004.59	1.43
Within Compartments	208	1398.12	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	3	16137.72	43.31***
Replicates	3	400.17	1.08
Error	9	372.66	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	3	0.2296	328.00***
Replicates	3	0.0022	3.15
Error	9	0.0007	

APPENDIX B:

Table II: Analysis of Variance - Trial I
Comparative Amino Acid Intakes - from Table VI

Methionine + Cystine

Source of Variation	d.f.	M.S.	F
Treatment	4	0.8189	51.2***
Replicates	3	0.0231	1.44
Error	10	0.0160	

Lysine

Source of Variation	d.f.	M.S.	F
Treatment	4	2.6771	66.1***
Replicates	3	0.0626	1.5
Error	10	0.0405	

Arginine

Source of Variation	d.f.	M.S.	F
Treatments	4	7.2442	131.71***
Replicates	3	0.0835	1.52
Error	12	0.0555	

APPENDIX B:

Table III: Analysis of Variance - Trial 2

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	2	63186.29	374.10***
Replicates	3	3922.35	21.50***
Between Compartments	6	212.88	1.17
Within Compartments	156	182.04	

Feed Intake

Source of Variation	d.f.	M.S.	F
Treatments	2	7243.20	56.80***
Replicates	3	303.95	2.38
Error	6	127.51	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatment	3	0.1636	100.1***
Replicate	2	.0048	0.3
Error	6	.0162	

APPENDIX B:

Table IV: Analysis of Variance - Trial 2
Amino Acid Intake Comparison from Table VIII

Methionine + Cystine

Source of Variation	d.f.	M.S.	F
Treatments	4	0.17239	33.34***
Replicate	2	0.01085	2.09
Error	8	0.00517	

Arginine

Source of Variation	d.f.	M.S.	F
Treatments	4	0.4766	40.73***
Replicates	2	0.0420	3.59
Error	8	0.0117	

Histidine

Source of Variation	d.f.	M.S.	F
Treatments	4	0.57934	279.87***
Replicates	2	0.00739	3.57
Error	8	0.00207	

Lysine

Source of Variation	d.f.	M.S.	F
Treatments	4	1.5650	168.28***
Replicates	2	0.0339	3.64
Error	8	0.0093	

Table V: Analysis of Variance - Trial 3

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	5	29253.7	7.61***
Replicates	2	2025.7	0.53
Between Compartments	7	3841.8	5.5***
Within Compartments	237	698.5	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	5	2448.9	5.6**
Replicates	2	1006.0	2.3
Error	10	435.6	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	5	0.0287	19.2***
Replicates	2	0.0014	0.9
Error	10	0.0015	

APPENDIX B :

Table VI: Analysis of Variance - Trial 3
Comparative Amino Acid Intakes - Table X

Arginine

Source of Variation	d.f.	M.S.	F
Treatments	6	0.4528	13.05***
Replicates	2	0.0412	1.18
Error	12	0.0347	

Histidine

Source of Variation	d.f.	M.S.	F
Treatments	6	0.3914	60.21***
Replicates	2	0.0075	1.15
Error	12	0.0065	

Table VII: Analysis of Variance - Trial 4

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	11	34968.96	24.63***
Replicates	2	1105.12	0.78
Between Compartments	22	1419.73	2.39***
Within Compartments	468	594.03	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	11	1257.29	2.68
Replicates	2	968.01	2.07
Error	22	467.64	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	11	0.1958	44.50***
Replicates	2	0.0034	0.78
Error	22	0.0044	

APPENDIX B :

Table VIII: Analysis of Variance - Trial 5

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	15	15491.75	15.91***
Subclass : Arginine	3	3169.84	3.25***
Methionine	3	68292.64	70.14***
Arginine x Methionine	9	1998.97	2.05**
Replicates	2	2134.02	2.19
Between Compartments	30	1130.37	1.16
Within Compartments	624	983.62	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	15	735.89	2.56**
Replicates	2	1872.85	6.26***
Error	30	286.94	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	15	0.1041	2.4**
Replicates	2	0.0008	0.02
Error	20	0.0043	

APPENDIX B:

Table IX: Analysis of Variance - Trial 6

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	6	4173.57	7.46***
Replicates	2	3965.73	7.09***
Between Compartments	12	653.64	1.17
Within Compartments	273	559.08	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	6	369.68	1.88
Replicates	2	809.21	4.11**
Error	12	196.58	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	6	0.0229	5.33***
Replicates	2	0.0075	1.74
Error	12	0.0043	

APPENDIX B :

Table X : Analysis of Variance - Trial 7 (Diets A - F)

Body Weight

Source of Variation	d.f.	M.S.	F
Treatments	5	38645.77	42.50***
Replicates	2	3038.78	3.34**
Between Compartments	10	1066.23	1.17
Within Compartments	234	909.39	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	5	6009.11	32.69***
Replicates	2	183.79	0.49
Error	10	374.84	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	5	0.1699	31.88***
Replicates	2	0.0141	2.65
Error	10	0.0053	

APPENDIX B :

Table X continued: Analysis of Variance - Trial 7(Diets G - L)

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	5	42465.65	49.63***
Replicates	2	1165.26	1.36
Between Compartments	10	1669.58	1.95
Within Compartments	234	855.63	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	5	2378.92	6.44***
Replicates	2	564.78	1.52
Error	10	369.30	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	5	0.2271	53.81***
Replicates	2	0.0258	6.11**
Error	10	0.0042	

APPENDIX B :

Table XI: Analysis of Variance - Experiment 2

Measurement of the Excretion Level of the Methionine Methyl Group (^{14}C) due to Feeding LPC.

(i) Total Radioactive Count in Excreta - 24 Hours

Source of Variation	d.f.	M.S.	F
Treatments	3	1.158	1.33
Error	32	0.872	

(ii) Radioactive Counts in Upper "Water Fraction"

Source of Variation	d.f.	M.S.	F
Treatments	3	0.213	1.18
Error	32	0.180	

(iii) Radioactive Counts in Lower "Lipid Fraction"

Source of Variation	d.f.	M.S.	F
Treatments	3	1.902	7.81***
Error	32	0.243	

APPENDIX B :

Table XII: Analysis of Variance - Experiment 3

Measurement of the Ornithine Level Due to Feeding LPC or SBM and the Effect of Arginine or Methionine Supplementation

(i) Comparison of Ornithine % Excretion Levels

Source of Variation	d.f.	M.S.	F
Treatments	3	2.64	2.3
Error	8	1.14	

(ii) Comparison of Apparent Arginine Availability

Source of Variation	d.f.	M.S.	F
Treatments	3	23.03	6.26**
Replicates	8	3.68	

APPENDIX B :

Table XIII: Analysis of Variance - Experiment 5

Comparison of LPC and SBM Ingredient Amino Acid Availability shown in Table XXII.

Apparent Amino Acid Availability

(i) Cystine

Source of Variation	d.f.	M.S.	F
Treatments	1	13493.4	236.83***
Error	12	57.0	

(ii) Methionine

Source of Variation	d.f.	M.S.	F
Treatments	1	169.40	56.66***
Error	12	2.99	

(iii) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	1	441.77	119.61***
Error	12	3.69	

(iv) Lysine

Source of Variation	d.f.	M.S.	F
Treatments	1	432.07	87.77***
Error	12	4.92	

(v) Arginine

Source of Variation	d.f.	M.S.	F
Treatments	1	254.63	88.13***
Error	12	2.89	

APPENDIX B :

Table XIII continued: Analysis of Variance - Experiment 5

Comparison of LPC and SBM Ingredient Amino Acid Availability shown in Table XXII

"Corrected" Amino Acid Availability

(i) Cystine

Source of Variation	d.f.	M.S.	F
Treatments	1	5110.3	253.52***
Error	12	20.2	

(ii) Methionine

Source of Variation	d.f.	M.S.	F
Treatments	1	320.464	412.80***
Error	12	0.776	

(iii) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	1	254.930	301.54***
Error	12	0.845	

(iv) Lysine

Source of Variation	d.f.	M.S.	F
Treatments	1	173.67	107.66***
Error	12	1.61	

(v) Arginine

Source of Variation	d.f.	M.S.	F
Treatments	1	76.748	83.48***
Error	12	0.920	

APPENDIX B :

Table XIV: Analysis of Variance - Experiment 5

Comparison of LPC and SBM Diet Amino Acid Availability Shown in Table XXIII.

	Apparent Amino Acid Availability for Diets		
(i) Cystine			
Source of Variation	d.f.	M.S.	F
Treatments	1	396.55	85.92***
Error	12	4.62	
(ii) Methionine			
Source of Variation	d.f.	M.S.	F
Treatments	1	0.036	0.04
Error	12	0.913	
(iii) Isoleucine			
Treatments	1	0.063	0.07
Error	12	0.957	
(iv) Lysine			
Source of Variation	d.f.	M.S.	F
Treatments	1	202.31	111.22***
Error	12	1.83	
(v) Arginine			
Source of Variation	d.f.	M.S.	F
Treatments	1	9.545	13.54***
Error	12	0.705	

APPENDIX B :

Table XIV continued: Analysis of Variance - Experiment 5

"Corrected" Amino Acid Availability for Diets

(i) Cystine

Source of Variation	d.f.	M.S.	F
Treatments	1	249.91	48.78***
Error	12	5.12	

(ii) Methionine

Source of Variation	d.f.	M.S.	F
Treatments	1	8.992	10.13***
Error	12	0.888	

(iii) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	1	4.068	5.28**
Error	12	0.940	

(iv) Lysine

Source of Variation	d.f.	M.S.	F
Treatments	1	311.90	115.43***
Error	12	2.70	

(v) Arginine

Source of Variation	d.f.	M.S.	F
Treatments	1	21.526	26.53***
Error	12	0.811	

APPENDIX B :

Table XV: Analysis of Variance - Experiment 6

Comparison of Apparent Amino Acid Digestibility Measured at the Ileum With the Amino Acid Availability Measured in the Excreta.

(i) Cystine

Source of Variation	d.f.	M.S.	F
Treatments	3	2615.56	392.23***
Error	16	6.67	

(ii) Methionine

Source of Variation	d.f.	M.S.	F
Treatments	3	692.95	94.98***
Error	16	7.30	

(iii) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	3	853.69	326.45***
Error	16	2.62	

(iv) Lysine

Source of Variation	d.f.	M.S.	F
Treatments	3	858.07	404.39***
Error	16	2.12	

(v) Arginine

Source of Variation	d.f.	M.S.	F
Treatments	3	526.14	164.62***
Error	16	3.20	

APPENDIX B :

Table XVI: Analysis of Variance - Experiment 7

Effect of LPC on Pancreatic Size and Level of Pancreatic Proteolytic Enzymes

(i) Comparison of Pancreas Weights

Source of Variation	d.f.	M.S.	F
Treatments	1	0.0476	21.38***
Error	30	0.0022	

(ii) Comparison of Pancreatic Chymotrypsinogen Levels in "Fed" Chicks

Source of Variation	d.f.	M.S.	F
Treatments	1	304.5	5.30**
Error	22	57.4	

(iii) Comparison of Pancreatic Trypsinogen Levels in "Fed" Chicks

Source of Variation	d.f.	M.S.	F
Treatments	1	20.18	3.71*
Error	22	5.43	

(iv) Comparison of Pancreatic Chymotrypsinogen Levels in "Starved" Chicks

Source of Variation	d.f.	M.S.	F
Treatments	1	28.70	3.44
Error	8	8.35	

(v) Comparison of Pancreatic Trypsinogen Levels in "Starved" Chicks

Source of Variation	d.f.	M.S.	F
Treatment	1	32.0	2.58
Error	10	12.4	

APPENDIX B :

Table XVII: Analysis of Variance - Experiment 8

Effect of Antibiotics on the Apparent Amino Acid Availability in LPC and SBM as Measured in the Excreta

Body Weights

Source of Variation	d.f.	M.S.	F
Treatments	3	874.54	15.76***
Replicates	2	94.36	1.70
Treatment x Replicates	6	55.32	0.99
Error	24	55.50	

Food Intake

Source of Variation	d.f.	M.S.	F
Treatments	3	92.09	11.08***
Replicates	2	0.98	0.12
Treatment x Replicates	6	5.07	0.61
Error	24	8.31	

Food Utilisation

Source of Variation	d.f.	M.S.	F
Treatments	3	0.68	9.71***
Replicates	2	0.14	2.00
Treatments x Replicates	6	0.19	2.71
Error	24	0.07	

APPENDIX B :

Table XVIII: Analysis of Variance - Experiment 8

Comparison of Amino Acid Availability as Measured in the Excreta Using LPC/SBM Diets Unsupplemented and Supplemented with Antibiotic (Neomix) in Table XXVII.

(i) Cystine

Source of Variation	d.f.	M.S.	F
Treatments	3	1087.08	149.21***
Error	32	7.29	

(ii) Methionine

Source of Variation	d.f.	M.S.	F
Treatments	3	224.57	114.99***
Error	32	1.95	

(iii) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	3	300.28	108.93***
Error	32	2.76	

(iv) Lysine

Source of Variation	d.f.	M.S.	F
Treatments	3	461.77	154.05***
Error	32	3.00	

(v) Isoleucine

Source of Variation	d.f.	M.S.	F
Treatments	3	300.28	108.93***
Error	32	2.76	

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