

BRANCHED CHAIN AMINO ACIDS: REQUIREMENTS AND
ANTAGONISM IN THE MALE BROILER CHICK

by

Mohamad Talal Farran

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Advisory Committee:

Professor	Owen P. Thomas
Professor	Joseph H. Soares, Jr.
Professor	James L. Heath
Associate Professor	Larry W. Douglass
Associate Professor	Joseph Sampugna

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ABSTRACT

Title of Dissertation: Branched Chain Amino Acids:
Requirements and Antagonism in
the Male Broiler Chick

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Dissertation directed by: Dr. Owen P. Thomas
Professor
Department of Poultry Science

Experiments were conducted to study the effect of feeding the branched-chain amino acids (BCAA), leucine (Leu), isoleucine (Ile) and valine (Val) to 7-21 day old male broiler chicks. Using the central composite rotatable design, the results of response surface regression analysis showed that optimum body weight gain (BWG) and feed conversion values (FC) would be obtained with dietary levels of 1.16, 0.81 and 0.90% for Leu, Ile and Val respectively.

Chicks fed a Val deficient diet ad libitum exhibited a ricket-like condition which was characterized by a low Ca content in the bone. The value was 134 as compared to 156 and 172 mg/g dry bone for chicks fed a diet deficient in the three BCAAs and a Val supplemented diet respectively. Similar results were obtained when equal amounts of the three diets were given to the birds by using pair feeding and force feeding techniques. Although birds fed the Val deficient diet and those pair fed the BCAA deficient diet had similar BWG, bone measurements (bone

ash, dry bone, and bone Ca) were significantly lower ($p < 0.05$) for the Val deficient group. Serum Ca (mg/dl) was not significantly different. Excretion of urinary Ca, however, was enhanced by feeding a Val deficient diet as compared to the Val supplemented treatment suggesting that a proper ratio of the BCAAs may be required to form the bone matrix which serves as a base for mineral deposition.

Feeding a Val deficient diet significantly ($p < 0.05$) lowered the protein content of the feathers. The value was 82.7% as compared to 85.0 and 88.0% for all BCAA deficient and Val supplemented diets respectively. Valine deficiency also changed the pattern of feather amino acids by increasing the levels of aspartic acid, glutamic acid, methionine, tyrosine, histidine and lysine. Cysteine level, however, was decreased.

High dietary Leu ($\geq 3.0\%$) depressed BWG and FC of ad libitum fed birds. When Ile and Val were simultaneously added to the diet the growth rate and FC were similar to the controls. The ketogenic property of Leu was tested by measuring β -OH-butyrate level in the plasma (mg/l) and found to be nonsignificant.

DEDICATION

To my wife Sinan and my daughter Sarah.

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INTRODUCTION

Leucine, isoleucine and valine are commonly referred to as the branched-chain amino acids (BCAA's) due to the presence of an aliphatic chain branching off the main carbon chain of the amino acid residue. These neutral amino acids are essential for different species including chicken and humans. A nutritional antagonism among the BCAA's, however, has been demonstrated in chicken, rats, as well as other species. Under certain conditions, symptoms associated with a BCAA toxicity or deficiency may be the result of an antagonism.

Experiments conducted in the past to determine the chick's requirements for these amino acids considered either one or two of these amino acids at a time. Considering all three amino acids simultaneously is a better approach.

A complete understanding of the antagonism among the BCAA's is not only of academic interest but also of practical significance. Elucidating this antagonism provides useful information as to the value of feedstuffs that are either high or low in at least one of these amino acids. It, therefore, becomes important in the formulation of diets for poultry.

SELECTED LITERATURE REVIEW

Requirements

The leucine, isoleucine, and valine requirements of broilers have been studied by different research workers. Using a synthetic amino acid diet, Grau and Peterson (1946) reported that the requirements of young chicks aged between 14-24 days, for leucine, isoleucine and valine are 1.50, 0.50 and 0.70% respectively. They concluded that the DL-forms of valine and isoleucine compared with the L-forms, failed to support good body weight gain. Feeding an amino acid diet to one-week-old chicks for a period of 10 days, Dobson et al. (1964) determined the requirements of leucine, isoleucine and valine to be 1.30, 0.80 and 0.95%, respectively. Similarly, during the second week post-hatching, Dean and Scott (1965) found that the levels of these amino acids necessary for supporting maximum growth were 1.20, 0.80 and 0.82%, respectively. Hewitt and Lewis (1972) found that the requirements for the same amino acid for broiler chicks in the starter period were 1.34, 0.62 and 0.79%. The diet used in their experiment contained 18% protein, 4% of which was supplied in the form of synthetic amino acids. In 1974, D'Mello set the minimum requirements for leucine, isoleucine and valine for chicks at 0.98, 0.53 and 0.63% respectively. Based on 48-hour hydrolysis time of the diet, Thomas et al. (1981)

estimated chick requirement for isoleucine to be 0.78% for a diet containing a metabolizable energy value (ME) of 3200 kcal/kg diet. All other amino acids were maintained at levels of at least 105% of the recommended requirements. Mori and Okumura (1984) reported that the estimated requirements for leucine, isoleucine and valine in 8-18 day old White Leghorn male chicks were 1.2, 0.50 and 0.80% respectively for a 14.2 Mj/Kg diet (3390 kcal/kg). Their estimations were comparable to those obtained for broilers by Blair et al. (1972). Finally, the published NRC requirements (1984) were 1.35, 0.82 and 0.85% for leucine, isoleucine and valine, respectively, in the broiler starter diet.

Antagonism

1. Effect on Growth

Toxicity induced by feeding excessive amounts of leucine has been studied by many research workers over the last three decades. It was reported by Harper and co-workers (1955) that the growth of rats fed a low protein diet ad-libitum was retarded if 3% of L-leucine was added to the diet. They further showed that the addition of a small amount of isoleucine to such a diet alleviated the growth-retarding action of the excess leucine. Moreover, the addition of both isoleucine and valine to the above diet by Benton et al. in 1956, fully overcame the ill-effects of excess dietary leucine. These observations, along with others made by Spolter and Harper (1961) using

5% dietary leucine, suggested that an excess of dietary L-leucine acted as an antagonist of isoleucine and valine in the rat and thereby increased the requirements for these two amino acids. Excessive amounts of leucine supplements (7 - 10%) to a normal protein diet, failed to induce toxicity in rats as measured by growth rate (Daniel and Waisman, 1968).

Chick studies by Smith (1968) showed that arginine supplementation may counteract a wide range of toxicities caused by lysine, methionine, valine, isoleucine, histidine, glycine, tyrosine, cystine, threonine, and phenylalanine. Leucine toxicity, however, could not be corrected for by arginine supplementation. D'Mello and Lewis (1970a,b) fed graded levels of leucine, isoleucine and valine to chicks during the starter period and measured feed consumption and body weight gain. Results showed that surplus leucine (2.9%) in the presence of a low dietary isoleucine (0.58%) inhibited growth rate markedly compared to that obtained by feeding the same level of leucine with a higher level of isoleucine (0.65%). The authors concluded that the need for isoleucine cannot be estimated with any degree of precision unless "due attention" is given to the interaction between isoleucine and leucine. When these two amino acids were used in excess, impaired growth rate and feed conversion resulted. The authors suggested the presence of another type of interaction between leucine and valine. They

further demonstrated that both isoleucine and valine supplementations were needed for a complete reversal of the adverse effects of excess leucine in the diet. The authors suggested that both interactions are responsible for counteracting the adverse effects exerted by excess leucine in the same manner that lysine-arginine interaction operates. The presence of BCAA interactions in chicken was studied and confirmed by Boldizar et al. (1973), Smith and Austic (1978), Penz et al. (1984a,b) and Bray (1970) in a study with young pullets.

Ueda et al. (1981) demonstrated that Single Comb White Leghorn chicks fed an isonitrogenous supplement of excessive amounts of L-leucine, L-lysine, L-phenylalanine or L-methionine (1.32, 0.92, 1.66 or 1.50 respectively) showed depressed growth rate when the diets were given ad libitum. Force-feeding to the level of control chicks, however, completely alleviated the leucine growth depression (100%), with only partial growth restoration in the case of lysine or phenylalanine (88% each). The latter effects were attributed to the depressed utilization of consumed nitrogen. Force-feeding a methionine-excess diet resulted in leg weakness, retarded crop emptying and finally death of all chicks before the end of the experiment which lasted for a period of 12 days. The results for leucine toxicity agree with those obtained by Spolter and Harper (1961). These workers, however, used 1 unit protamine zinc insulin injections to stimulate food intake

in rats fed the diet with a 5% excess of leucine. This feed intake stimulation resulted in a better rate of growth indicating that an excess of dietary leucine per se would not appear to be toxic. D'Mello and Lewis (1971) induced toxicity in one-week-old chicks by feeding a dietary excess of added lysine or leucine (1.5% each). In their experiments, values of daily body weight gain were 11.8, 5.0 and 16.3 g for leucine, lysine and control chicks, respectively. Data of these studies showed that, for both leucine and lysine toxicities, growth retardation preceded the depression in food intake, suggesting that the decrease in food intake was not the primary effect. The authors further confirmed their findings by using pair-feeding techniques in which growth rate of chicks fed both leucine and lysine was found to be less than for the control chicks. Subsequent work by Ueda et al. (1981) also showed that leucine toxicity was less severe than that of lysine.

The nutritional antagonism among BCAA's has also been demonstrated in a variety of species including turkeys (Tuttle and Balloun, 1976) and pigs (Oestemer et al., 1973). However, Mason and Ward (1979; 1981a) presented data showing that a diet containing a 5% excess of leucine did not affect the growth rate of the Japanese quail. Mason and Ward (1981b) confirmed their finding with a comparative study using chicks and quail raised under the same conditions. These results showed that differences

among species in their response to excess dietary amino acids exist.

Although attempts to induce valine and isoleucine toxicities have been made in chicks, the level of dietary leucine was not mentioned. While Smith (1968) succeeded in retarding growth of chicks by adding 2% of either valine or isoleucine to the diet, the results of Smith and Austic (1978) failed to show significant growth retardation by feeding 2 and 1.75%, added valine and isoleucine respectively.

Blair et al. (1976) published an amino acid diet for chicks that gave satisfactory growth. Okumura and Mori (1979) reported that feeding chicks an essential amino acid at 50% of the level recommended by Blair et al. (1972) in the starter period resulted in decreased food intake and growth rate. In comparing the BCAA's, the most severe effect was obtained with isoleucine, the least with leucine while the severity of the valine deficiency was intermediate.

Little has been reported on the BCAA deficiency-related antagonism. Kimura and Tahara (1971) showed that force-feeding rats with a valine-free diet resulted in an acute weakness accompanied by a 50% mortality rate. These symptoms were alleviated, however, by force-feeding a valine-leucine free diet. No similar experiments have been conducted with chicks. Some information, however, could be drawn from related studies. In a chick study by

Sugahara et al. (1969) it appeared that the effects of feeding a diet deficient in either valine or isoleucine (40%) were much more drastic than feeding a diet deficient in all of the essential amino acids. Birds fed the leucine deficient diet (40%) grew faster than the birds fed all the essential amino acid at the 40% level. These data would indicate that leucine has to be on a higher dietary plane than isoleucine and/or valine for growth rate to be severely retarded.

2. Effect on Blood Amino Acids

The concentration of BCAA's in the blood of chicks has been studied by many research workers. D'Mello and Lewis (1970a,b) and D'Mello (1974) reported, in general, that as the level of a single BCAA in the diet increased, there was a sharp increase in its plasma concentration accompanied by a consistent drop in the concentration of the other two amino acids. Similar findings were obtained by Hewitt and Lewis (1972), Smith and Austic (1978), Penz et al. (1984a), Tannous et al. (1965) (rat data), and Oestemer et al. (1973) (pig data).

In their study, Hewitt and Lewis (1972) added levels of 0.08% and 0.16% isoleucine and/or valine to a basal diet which was considered to be adequate for chick growth. Total plasma amino acid levels were reduced when 0.08% of isoleucine or valine was added to the diet from 852 to 669 and 687 Mmoles/100 ml, respectively. Further increase in the dietary level of either valine or isoleucine (0.16%)

led to an increase in total plasma amino acid levels. The authors gave no explanation for this phenomenon.

Smith and Austic (1978) observed an initial drop in blood isoleucine and valine following a dietary supplement of 2.25% leucine, but returned to normal in the last 6 days of the experiment which lasted for a period of 2 weeks. The authors concluded that chicks show an adaptive response to low dietary leucine supplements (up to 2.25%).

3. Feather Abnormality

Penz et al. (1984a) reported that a type of feather abnormality was noted in chicks fed diets with an excess of leucine (5.4%). The barbules and barbicels did not bend normally and as a result, feathers were easily broken. Supplementation with isoleucine and valine (0.64% each) prevented the disorder. Their descriptions of feather abnormalities agreed with those obtained with valine and isoleucine deficiencies by Anderson and Warnick (1967) and with valine deficiency by Robel (1977). In another study, Penz et al. (1984b) confirmed the effect of BCAA interaction on feather structure. The abnormality induced by feeding 4.05% excess leucine caused a reduction in feather protein accompanied with increased values of feather leucine, histidine, methionine, lysine and alanine. On the other hand, the levels of valine, isoleucine and cystine in the feather decreased. Supplementation with isoleucine and valine (0.64% each) prevented the

changes in feather composition and caused values comparable to those obtained with control chicks.

Causes of Antagonism

Experimental results on the mechanism by which excess leucine increases the requirements of isoleucine and valine are limited. It has been proposed that these changes are due to different factors.

Digestion, Absorption and Excretion

The effects of dietary BCAA excesses on their relative absorption and excretion have not been thoroughly examined. Based on the results of their studies with rats, Spolter and Harper (1961) suggested that the growth-retarding action of an excess dietary leucine (5%) was not associated with the processes of protein digestion. The ill-effect was obtained with a diet in which the protein was replaced by an amino acid mixture.

Competition among leucine, isoleucine and valine during intestinal absorption has been suggested by Hagihiro et al. (1960), as reported by Smith and Austic (1978). Results of their experiments in which rat intestinal segments were used to study amino acid absorption, supported the view that these amino acids compete with each other for absorption sites. Reiser and Christiansen (1962) also reported that intestinal absorption of L-valine was reduced by the presence of excesses of the L-isomers of leucine, isoleucine, tryptophan, methionine or phenylalanine. The results obtained by

Tannous et al. (1966) with intact rats, did not support this hypothesis. In their experiments, no abnormal accumulation of isoleucine or valine was observed in either the intestinal wall or in the intestinal contents of animals fed the three experimental diets: control, high leucine (5%) and high leucine plus isoleucine (0.33%) and DL-valine (0.3%). Liver free amino acids were not affected by any of these treatments and the pattern was similar to that found in the intestines. The blood BCAA pattern resembled that of the skeletal muscles though differing from that in liver and intestines. Based on these observations, the authors suggested that excess leucine either enhances the utilization or the catabolism of isoleucine and valine in the liver and/or muscles.

The conflicting reports in the literature concerning the requirements of the BCAA's for the broiler chicks might have resulted partially from the incomplete understanding of the relationship among these amino acids. Therefore a series of studies was designed to further elucidate this relationship at both suboptimal and toxic levels. Other studies were designed to estimate the requirements of chickens for the BCAA's varying the levels of the three amino acids at the same time.

MATERIALS AND METHODS

Study 1: Preliminary Study

Study 1 was designed to obtain a diet deficient in at least two of the three BCAA's, namely leucine or isoleucine.

Experimental procedure. One-day-old male broiler chicks were placed in Petersine brooders for a period of three weeks. The pens had wire floors and were artificially heated. Heat was thermostatically controlled to provide temperatures of 35, 32 and 29°C for the first, second and third week of the rearing period respectively. A basal diet (Table 1) was prepared and analyzed to contain 20.5% protein (A.O.A.C., 1975). Metabolizable energy (ME) was calculated to be 3200 kcal/kg diet. Amino acid analysis of the basal diet (Appendix 1) showed that BCAA levels were below the NRC required values (1984). The basal diet contained 1.16, 0.64 and 0.82% of leucine (Leu), isoleucine (Ile) and valine (Val)¹ respectively. One half percent of the synthetic L-forms were added to the basal diet such that 8 different test diets were prepared. Thus a 2x2x2 factorial treatment arrangement with complete randomized design (CRD) was used.

¹ This is the order by which the 3 BCAA's will always be cited thereafter unless otherwise stated.

Table 1. Basal diets.

Ingredient: (%)	Study	Study	Study	Study
	1	2	5	6
Wheat	49.350	27.00	38.4	67.7
Peanut meal (Exp. 50%)	22.880	23.00	16.7	17.3
Cerelose (dextrose)	11.490	35.20	18.8	-
Soybean meal	5.780	5.90	6.1	6.7
Yellow corn	-	-	10.5	-
Gelatin	3.000	-	-	-
Dicalcium phosphate 18.5/22	2.050	2.20	2.13	1.99
Limestone	0.950	0.87	0.97	0.97
L-Lysine *HCl 98%	0.738	1.03	1.03	0.93
Salt	0.549	0.49	0.55	0.50
L-Threonine	0.457	0.53	0.54	0.47
DL-Methionine 99%	0.377	0.45	0.46	0.36
L-Phenylalanine	0.219	0.42	0.49	0.31
Choline Chloride-50	0.145	0.220	0.21	0.16
L-Histidine	0.115	0.190	0.21	0.13
L-Glycine	-	0.230	0.39	0.17
L-Tryptophan	0.082	0.110	0.11	0.08
Vitamin premix	0.200 ¹	0.200 ¹	0.20 ¹	0.05 ²
Mineral premix ³	0.050	0.050	0.05	0.05
Cocciostat ⁴	0.050	0.050	0.05	0.05
Santoquin mix 5	0.016	0.016	0.016	0.016

¹ To supply the following per kilogram of diet: Vit. A 5510 I.U., Vit. D₃ 2205 I.U., Vit. E 4.4 I.U., riboflavin 8.8 mg, Vit. B₁₂ 180 mcg, niacin 53 mg, D-calcium pantothenate 24 mg, menadione sodium bisulfate 4.41 mg, folic acid 1.36 mg, pyridoxine 8.0 mg, thiamine 6.61 mg, biotin 45.4 mcg.

² To supply the following per kilogram of diet: Vit. A 5500 I.U., Vit. D₃ 2200 I.U., Vit. E 4.4 I.U., Vit. B₁₂ 13.2 mcg, riboflavin 6.6 mg, niacin 33 mg, D-calcium pantothenate 11 mg, menadione 1.45 mg, folic acid .22 mg, pyridoxine 1.1 mg.

³ To supply the following per kilogram of diet: Calcium 150 mg, manganese 165 mg, zinc 88 mg, iron 55 mg, copper 6.6 mg, iodine 1.65 mg.

⁴ Amprol Hi-E, MSD-AGVET, Rahway, N.J.

⁵ Monsanto Company, St. Louis, Mo.

The 8 treatments were made isonitrogenous by adding glutamic acid to the diets.

Chicks were fed the basal diet for the first week of the experiment. At the end of this period, birds were weighed and distributed to pens so that every pen had approximately the same range and mean. There were 8 birds per replicate and 4 replicates per treatment. The 256 birds were fed the 8 test diets for an experimental period of two weeks. Feed and water were given ad libitum and continuous artificial light was supplied. At the end of the experimental period, body weight gain (g) and feed conversion (kg diet consumed/kg gain) for each pen were measured. Data were analyzed using ANOVA procedure (SAS, 1982). The following model was used:

$$Y_{ijkl} = \mu + L_i + I_j + V_k + L_i I_j + L_i V_k + I_j V_k + L_i I_j V_k + E_{ijkl}. \text{ Where}$$

Y_{ijkl} = the observed value for the l^{th} replicate of the i^{th} , j^{th} and k^{th} levels of the three fixed treatments leucine, isoleucine, and valine respectively.

μ = the grand mean

- L_i = the fixed effect of leucine for its i^{th} level.
- I_j = the fixed effect of isoleucine for its j^{th} level.
- V_k = the fixed effect of valine for its k^{th} level.
- $L_i I_j$ = the first order interaction effect for the i^{th} level of leucine and the j^{th} level of isoleucine.
- $L_i V_k$ = the first order interaction effect for the i^{th} level of leucine and the k^{th} level of valine.
- $I_j V_k$ = the first order interaction effect for the j^{th} level of isoleucine and the k^{th} level of valine.
- $L_i I_j V_k$ = the second order interaction effect for the i^{th} level of leucine, the j^{th} level of isoleucine and the k^{th} level of valine.
- E_{ijkl} = the random error associated with Y_{ijkl} experimental unit.

Study 2

This study was designed to make the basal diet more deficient in the 3 BCAA's. Also to investigate the relationship among the BCAA's at suboptimal levels.

Experimental procedure. Using a 3x3x3 factorial treatment arrangement with randomized complete block design (RCBD), the location of the batteries being the blocking factor in this experiment. Two batteries were near the main door, 2 others were close to the heaters and the remaining two were in the middle of the room. Therefore each block constituted 2 batteries and contained the 27 different treatment combinations. Study 2 was conducted maintaining all experimental conditions described in study 1, except for the total number of birds (648) and the number of replicates per treatment (3). A semi-purified basal diet was prepared (Table 1) and analyzed to contain 0.96, 0.52 and 0.65% of Leu, Ile and Val respectively. All other amino acids were at least at 104% of their requirements. The following factor levels (% of the diet) were used:

Leu	0.96	1.21	1.46
Ile	0.52	0.67	0.82
Val	0.65	0.80	0.95

Body weight gain and feed conversion (BWG and FC) were measured and birds were examined for physical

appearance. Data were analyzed by the ANOVA procedure (SAS Institute, Inc., 1982).

Determination of BCAA Requirements

Study 3. Study 3 was initiated to determine the minimum requirements of male broilers for the BCAA's during the starter period.

Experimental procedure. Experimental conditions were similar to those of experiment 2 except that 288 birds were used in a 3x4 factorial treatment arrangement with RCBD. The basal diet was similar to that of experiment 2 except that the level of Leu was increased to 1.04% and held constant (an intermediate value between the 2 levels of Leu in the basal diets used in studies 1 and 2). Ile and Val were added to the basal diet by increments of 0.09 and 0.05% respectively. The factor levels (% of the diet) were:

Ile	0.58	0.67	0.76	
Val	0.65	0.70	0.75	0.80

Body weight gain and FC values were recorded as previously described and data were analyzed by using the General Linear Model Procedure (SAS, 1982).

Response surface studies (4, 5, and 6). In the previous experiment, Leu level was kept constant while those of Ile and Val were varied. This phenomenon could be reversed or done differently, i.e, keeping constant the levels of Ile or Val and varying the levels of the other remaining factors. Determining the requirements for 3

interrelated amino acids may be accomplished by using response surface regression (Cochran and Cox, 1957). The factorial approach in which the 3 factors are handled at the same time and at the same level of importance is recommended. However, the use of 3 levels of each factor leads to 27 treatment combinations. Therefore, there is a need for an efficient test such as the central composite rotatable design (Cochran and Cox, 1957) which gives similar results with less treatments used. The objectives of studies 4, 5 and 6 were then to determine the requirements of the chicks for the BCAA's based on obtaining the optimum combinations of these amino acids.

Experimental procedures. Experimental conditions in studies 4, 5 and 6 were similar to those described previously. The number of birds was 360 in study 4 where 15 treatments and 3 replicates per treatment were used. Five hundred seventy-six birds were used in studies 5 and 6 (18 treatments with 4 replicates per treatment). Tables 2 and 3 summarize the factor levels and the treatment combinations respectively. In studies 5 and 6 the emphasis was made on the central treatment in which the coded levels of the 3 BCAA's were zero.

The basal diet used in study 4 was similar to that of study 2. In study 5 a portion of the cerelose content was substituted by yellow corn (10.5%). The basal (Table 1)

Table 2. Levels of the 3 BCAA's used in studies 4, 5 and 6.

	Coded levels	-1.682	-1	0	+1	+1.682
Study 4						
	Leu	0.96	1.00	1.06	1.12	1.16
	Ile	0.52	0.56	0.62	0.68	0.72
	Val	0.65	0.69	0.75	0.81	0.85
Study 5						
	Leu	1.00	1.08	1.20	1.32	1.40
	Ile	0.51	0.59	0.71	0.83	0.91
	Val	0.63	0.72	0.83	0.95	1.03
Study 6						
	Leu	1.12	1.20	1.32	1.44	1.52
	Ile	0.61	0.67	0.76	0.85	0.91
	Val	0.75	0.79	0.85	0.91	0.95

Table 3. Treatment combinations used in studies 4¹, 5 and 6 (Cochran and Cox, 1957).

Treatment	Coded Combinations		
	Leu	Ile	Val
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	-1.682	0	0
10	+1.682	0	0
11	0	-1.682	0
12	0	+1.628	0
13	0	0	-1.682
14	0	0	+1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0

¹ The first 15 treatments were used in study 4.

contained 18.4% protein. Calculated ME value was 3200 kcal/kg diet. Maintaining the same level of ME, a control diet containing 23% protein was used to be compared with the test diets low in protein.

Wheat was substituted for cerelese in study 6 (Table 1) which increased the protein content to 20.6%. An isocaloric positive control diet containing 18.1% protein was also prepared.

The growth parameters were recorded and data were analyzed using the GLM procedure (SAS, 1982). A polynomial regression equation was generated in order to obtain the optimum response for both BWG and FC. A GCONTOUR procedure was then performed and data were plotted on contour graphics. The shape of the response surface was drawn in a three dimensional plot using the G3D procedure (SAS, 1985).

The polynomial regression equation has the following form:

$$\begin{aligned} \hat{Y} = & a + b_1 \text{ Leu} + b_2 \text{ Ile} + b_3 \text{ Val} \\ & + b_4 \text{ Leu}^2 + b_5 \text{ Ile}^2 + b_6 \text{ Val}^2 \\ & + b_7 \text{ Leu Ile} + b_8 \text{ Leu Val} + b_9 \text{ Ile Val} \end{aligned}$$

where:

a = intercept

b₁, b₂ b₉ = coefficients for polynomial regression.

Antagonism Studies

Antagonism at Suboptimal Levels

Study 7. The objectives of study 7 were to investigate the effects of feeding a Val deficient diet, a diet

deficient in all BCAA's or Val supplemented diets, on amino acid composition of feathers and on the Ca content of the bone. Another objective was to determine whether or not the choline level of the diet was adequate.

Experimental procedure. All the experimental conditions were similar to those described previously with 144 birds used in a 2x3 factorial treatment arrangement with CRD.

The basal diet used in this study was similar to that of study 2 except that it was adjusted to have adequate levels of Leu and Ile (1.37 and 0.82% respectively). Choline chloride (50%) was added to the basal by an increment of 0.25%. Valine was supplemented in an increment of 0.20%. The 6 treatment combinations were arranged as follows:

	Dietary Added Choline Chloride, 50%		
Dietary Val%	1.00	1.25	1.50
0.63	1	2	3
0.83	4	5	6

A seventh treatment (negative control) corresponding to the basal of study 2 was also used. This diet was found to be suboptimal in all the BCAA's. It will be referred to as the BCAA deficient diet. Diets 1 and 4 will be referred to as Val deficient and Val supplemented, respectively.

Body weight gain and FC values were recorded for birds fed the seven treatments. Moreover, birds from Val

supplemented, Val deficient and BCAA deficient treatments were used for feather amino acid analysis and bone Ca determination.

Five primary feathers were taken from each bird, pooled together within each pen, cut and analyzed for protein (A.O.A.C., 1975) and amino acid content (Appendix 1). Two tibias were collected from birds representing the average weight of each pen (6/treatment). Bones were cleaned, placed in 16 x 100 mm borosilicate screwcap test tubes and dried in an oven for 24 hours at 100°C. Deionized water and concentrated nitric acid were added on the basis of 1:1 (v/v) and tubes were stood at room temperature for 24 to 48 hours until the digestion was complete. Tubes were placed in a heating block set at 80°C. Aliquots of hydrogen peroxide 50% (0.2 ml) were added every five minutes until the solution was clear. During this step tubes were frequently vortexed and vented. Finally the tubes were cooled to room temperature and solutions were filtered into graded tubes using Whatman #2 papers. The filtrates were brought up to 14 ml by adding deionized water. Samples were then diluted (1/2500) using a 0.5% solution of lanthanum oxide. A serum standard¹ was similarly diluted. Working standards with the concentrations of 0.5, 1, 2, 4 and 6 ppm of Ca were also prepared starting from a calcium atomic absorption standard¹ solution.

¹ Sigma Chemical Co., St. Louis, MO.

The absorbances of the working standards and serum standard as well as those of the samples were obtained using a Perkin-Elmer 503 Atomic Absorption Spectrophotometer. Absorbances of the working standards were plotted against their concentrations and the following regression line was obtained:

$$Y = ax + b \text{ where}$$

$$y = \text{absorbance}$$

$$x = \text{concentration}$$

$$a = \text{slope}$$

$$b = \text{intercept}$$

The concentrations of the samples were obtained as follows:

$$\hat{x} = \frac{y - b}{a} \quad \text{In ppm or } \mu\text{g/ml}$$

Ca concentration (mg/g dry bone) =

$$\hat{X} \cdot \text{dilution factor} \cdot \frac{\text{volume of the digestion mixture}}{\text{weight of dry bone}}$$

$$= \frac{\mu\text{g}}{\text{ml}} \cdot 2500 \cdot \frac{1 \text{ mg}}{1000 \mu\text{g}} \cdot \frac{14 \text{ ml}}{\text{g dry bone}}$$

Data were analyzed by the ANOVA procedure with means compared by the SNK method where appropriate (SAS, 1982).

Studies 8 and 9

These studies were designed to investigate the problem of leg abnormality associated with feeding a Val deficient diet as compared to a diet deficient in all BCAA's or a control diet, under equal feed intakes.

Experimental procedure. Force feeding and pair-feeding techniques were performed in two separate experiments. The two studies were conducted at the same time on chicks from the same hatch and raised similarly throughout the first week of age.

Study 8. Force-Feeding Trial.

The experimental conditions were similar to those described previously with the following exception: Fifty-four birds were used in a complete randomized design. The Val supplemented diet was given ad libitum to the assigned 3 replicates. The average amount of feed consumed (g/day) was force-fed to chicks assigned either the Val deficient or the BCAA deficient diets. Feed was given to birds by the means of 250 cc syringe attached to a plastic tubing. A slurry consisting of 2 parts water to 1 part feed was prepared and given to birds three times a day (8:00, 15:00 and 21:00 hr). The duration of this experiment was 11 days because of the high rate of mortality that occurred in valine deficient birds.

Blood sampling. One hour following a meal (post-prandial), a five to six ml blood sample was taken from each bird and divided equally into two tubes where one contained 200 μ l of heparin. Birds were then euthanized by cervical dislocation. Blood samples were centrifuged at 3500 rpm for 15 minutes, and both sera and plasma were obtained and kept frozen until analyzed.

Bone Ca and ash%. Both tibias were taken from each bird, cleaned and stored frozen until analyzed for bone calcium and bone ash. The procedure for bone Ca determination was performed on left tibias in similar manner to that described in study 7.

Right tibias were placed in crucibles and dried in an oven at 100°C until constant weights were obtained. Bone samples were then ashed in a muffle furnace at a temperature of 600°C for 6-7 hours. Percent ash was obtained by the following formula:

$$\text{Ash \% of dry matter} = \frac{\text{Ash weight}}{\text{Dry bone weight}} \times 100$$

Serum Ca determination. Serum samples were thawed, diluted 1/50 with a solution of lanthanum oxide 5%. The other steps performed were similar to those described for bone samples. The following regression line was used to calculate the concentration of Ca in the serum.

$$y = ax + b$$

$$\hat{x} = \frac{y - b}{a} \quad \text{ppm or mg/l}$$

$$\begin{aligned} \text{Ca concentration (mg/dl)} &= X \cdot \text{dilution factor} \\ &= \frac{\text{mg}}{\text{l}} \cdot 50 \cdot \frac{0.1 \text{ l}}{\text{dl}} \end{aligned}$$

Plasma amino acid analysis. Plasma samples were thawed and amino acid analysis was performed (Appendix 1).

Study 9. Pair-feeding trial. In this experiment the number of birds, statistical design and test diets were

similar to those used in the force feeding study. Val supplemented birds and those assigned to the BCAA deficient treatment were given the same amount of diet consumed by the valine deficient chicks on a daily basis. The diet was presented twice a day with 12 hour intervals. Criteria measured were similar to those of the force feeding study.

Data from studies 8 and 9 were analyzed using the GLM procedure and means were separated by the LSD method where appropriate (SAS, 1982).

Urine collection procedure. Urine was collected from birds fed ad libitum the Val supplemented diet in study 8 and the valine deficient diet in study 9. The closest 2 birds to the mean body weight of the pen (6/treatment) were selected for the urine collection procedure.

Birds were anesthetized through the brachial vein with a pentobarbitol solution (1.8 ml/kg body weight). Once the bird was anesthetized the femoral vein and artery were each surgically isolated, catheterized, and surgically tied so that a tight seal existed around the catheter. Initial blood samples were collected after catheterizing the femoral vein. The ureters were then surgically isolated by making a deep incision in the ventral pelvic area, where the ureters have a close relationship with the bursa of fabricius. Both ureters were catheterized and joined by tubing leading to a glass tube. An infusion pump was attached to the anchored venule catheter and a

constant administration (0.4 ml/min) of warmed 10% mannitol, .25% inulin, and saline solution was administered throughout the surgical procedure. The catheterization procedure was completed in 15 minutes and an additional 15 minutes were allowed for the kidneys to equilibrate to the infusion rate. Overall, a 30-minute infusion period was given to each bird before urine samples were collected. Following the equilibration period, urine was collected for 20 minutes in a blood tube. Blood samples were taken at 10 minutes or at the midpoint of the urine collection. They were immediately cooled on ice, centrifuged and stored in a freezer along with urine samples. Birds were euthanized with an overdose of the anesthetic at the end of the procedure.

Inulin determination. Plasma and urine inulin was determined following the method of Waugh (1977) (Appendix 2).

Urinary calcium determination. Calcium in urine was assessed in a manner similar to that performed on serum Ca with the only exception that urine was diluted 1/20 instead of 1/50 for serum.

Urine amino acid analysis. Urine samples were thawed and amino acid analysis was performed (Appendix 1).

Kidney function measurements. The glomerular filtration rate (GFR) was determined as inulin clearance (C_{in}) (Smith, 1956) from the following formula:

$$\text{GFR (ml/min)} = C_{in} = \frac{U_{in} \times V}{P_{in}} \quad \text{where}$$

U_{in} = Urinary inulin (mg/ml)

P_{in} = Plasma inulin (mg/ml)

V = Urine volume (ml/min)

Calcium clearance was calculated as follows:

$$C_{Ca}(\text{ml/min}) = \frac{U_{Ca} \times V}{P_{Ca}} \quad \text{where}$$

C_{Ca} = Ca clearance (ml/min)

U_{Ca} = Urinary Ca (mg/ml)

P_{Ca} = Plasma Ca (mg/ml)

V = Urinary volume (ml/min)

Fractional excretion of Ca (FE_{Ca}) was calculated as a percentage of GFR.

$$FE_{Ca} \% = \frac{C_{Ca}}{\text{GFR}} \times 100$$

Data for all the above criteria were analyzed using a T test procedure (SAS, 1982).

Antagonism at High Levels of Leucine

Study 10. This experiment was designed to test whether or not the toxicity associated with leucine is alleviated by the addition of Ile and Val; and if the leucine toxicity is accompanied by ketosis.

Experimental procedure. All the experimental conditions were similar to those described previously. A 2x2x2 factorial treatment arrangement with CRD was used. The seventy-two birds were fed the 8 test diets for an

experimental period of two weeks. There were 3 birds per replicate and 3 replicates per treatment. The basal diet was similar to that used in study 1, but adjusted to contain 1.36, 0.83 and 0.86% Leu, Ile and Val respectively. It was analyzed to contain 21.8% protein. The calculated ME was 3190 kcal/kg diet. Amounts of 1.70% Leu, 0.33% Ile and 0.34% Val were added to the basal diet, making the factor levels used in the test diets as follows:

Leu	1.36	3.06
Ile	0.83	1.16
Val	0.86	1.20

In addition to the measurement of weight gain and feed conversion, plasma β -OH butyrate (mg/l) was enzymatically assessed using the method suggested by Hultman (1974) (Appendix 3). Data were analyzed using the ANOVA procedure (SAS, 1982).

Study 11

Corn gluten meal contains a high level of leucine (10.1%). Therefore this study was designed to test whether or not a diet containing a high level of corn gluten meal would have a negative effect on broiler performance.

Experimental procedure. Using a 2x2x4 factorial arrangement with CRD, study 11 was conducted maintaining all experimental conditions previously described except

for the total number of birds. There were 544 birds with 4 replicates and 8 birds/replicate. The basal diet (Table 4) was analyzed to contain 25.5% protein and calculated ME was 3100 kcal/kg diet. Levels of BCAA's in the basal diet were 2.75, 1.00 and 1.20% for Leu, Ile and Val respectively. Increments of 1.25% Leu and 0.40% Ile were added to the basal diet. Valine levels varied between 1.20 and 1.80% with increments of 0.20. An isonitrogenous, isocaloric positive control diet containing 8% corn gluten meal was prepared and fed to 32 birds (4 replicates with 8 birds/replicate). Data for BWG and FC values were analyzed by the ANOVA procedure (SAS, 1982).

Table 4. Basal diet used in Study 11.

Ingredient	%
Wheat	37.400
Yellow corn	23.960
Corn gluten meal	19.900
Soybean meal (49%)	6.560
Peanut meal (50%)	4.960
Dicalcium phosphate (18.5/22)	1.840
Limestone	1.150
L-lysine * HCl	0.661
Soybean oil	0.500
Salt	0.490
Choline chloride 50	0.178
Vitamin premix ¹	0.050
Mineral Premix ²	0.050
Coccidiostat ³	0.050
L-Tryptophan	0.037
Santoquin mix 6 ⁴	0.016

¹ To supply the following per kilogram of diet: Vit. A 5500 I.U., Vit. D₃ 2200 I.U., Vit. E 4.4 I.U., Vit. B₁₂ 13.2 mcg, riboflavin 6.6 mg, niacin 33 mg, D-calcium pantothenate 11 mg, menadione 1.45 mg, folic acid .22 mg, pyridoxine 1.1 mg.

² To supply the following per kilogram of diet: Calcium 150 mg, manganese 165 mg, zinc 88 mg, iron 55 mg, copper 6.6 mg, iodine 1.65 mg.

³ Amprol Hi-E, MSD-AGVET, Rahway, N.J.

⁴ Monsanto Company, St. Louis, MO.

RESULTS AND DISCUSSION

Study 1

The analysis of variance of the data revealed that the effect of the highest order of interaction (Leu*Ile*Val) for both weight gain and feed conversion was significant ($p < 0.05$). It follows that the mean comparison for the main effects was inappropriate. Compared to the basal which contained 1.16, 0.64 and 0.82% of the 3 BCAA's, individual addition (0.50%) of synthetic forms of Leu, Ile or Val did not improve weight gain values (418 vs. 409, 409 and 424 g respectively, Table 5). The results of feed conversion (Table 6) followed a similar

Table 5. Study 1. Weight gain of chicks fed diets containing different levels of leucine, isoleucine and valine.

		Leucine %			
		1.16		1.66	
		Isoleucine %		Isoleucine %	
		0.64	1.14	0.64	1.14
Valine %	0.82	418	409	409	387
	1.32	424	457	388	459

SEM = 10.3.

Except for Leu*Ile and Leu*Val, all main factors and interactions were significant ($p < 0.05$).

trend. The basal diet, however, should not be considered as adequate because of the synergistic effect of Ile and Val when both were added to the diet simultaneously. In fact, for both levels of Leu, 0.50% added levels of Ile and Val were required in order to elicit a response for both criteria measured. The magnitude of the synergism for weight gain, compared to the basal diet, was 39 and 41 g (Table 4). Although the levels of Leu (1.16%) and Ile (0.64%) in the basal diet were below the 1984 NRC recommended levels (1.36, 0.82% respectively), it was concluded that the basal diet was not deficient enough to elicit large responses. Therefore it was decided to formulate a diet with lower levels of Leu, Ile and Val.

Table 6. Study 1. Feed conversion of chicks fed diets containing different levels of leucine, isoleucine and valine.

		Leucine %			
		1.16		1.66	
		Isoleucine %		Isoleucine %	
		0.64	1.14	0.64	1.14
Valine %	0.82	1.47	1.42	1.52	1.47
	1.32	1.45	1.37	1.51	1.37

SEM = 0.012

Except for Leu*Val, all main factors and interactions were significant ($p < 0.05$).

Study 2

The results of study 2 are shown in Tables 7. All the interactions and the main factors for both weight gain and feed conversion were significant ($p < 0.05$). The basal diet corresponding to the lowest combination of the 3 BCAA's (Table 7) yielded a weight gain value of 344 g which was not improved by increasing dietary valine. Supplementing the basal with isoleucine (0.67 and 0.82%) decreased weight gain to the values of 275 and 284 g respectively. Fortification of the basal with synthetic leucine to give levels of 1.21 and 1.46 decreased the weight gain of the chicks (344 vs. 300 and 281 g). The feed conversion value obtained by feeding the basal diet was 1.59. It was not improved by adding either valine or isoleucine. When both amino acids, however, were added simultaneously, feed conversion values decreased by 0.12 - 0.14 units (Table 7). Similar trends were obtained for both criteria measured with the higher levels of leucine (1.21 and 1.46%). The highest observed response for both weight gain (442 g) and FC (1.42) was obtained by the addition of the 3 BCAA's simultaneously to the basal diet (Table 7).

Chicks fed the low valine diets (0.65%), in combination with added isoleucine and leucine, had low weight gains associated with a high incidence of feather and leg abnormalities.

Table 7. Study 2. Body weight gain (g) and feed conversion of 3-week-old male broilers fed a diet containing graded levels of leucine.

Ile %	Val %					
	0.65		0.80		0.95	
	BWG	F.C.	BWG	F.C.	BWG	F.C.
<u>0.96% leucine:</u>						
0.52	344	1.59	341	1.58	335	1.58
0.67	275	1.61	380	1.47	384	1.45
0.82	284	1.60	370	1.47	374	1.46
<u>1.21% leucine:</u>						
0.52	300	1.71	321	1.72	312	1.61
0.67	273	1.72	442	1.42	440	1.41
0.82	266	1.68	417	1.42	431	1.47
<u>1.46% leucine:</u>						
0.52	281	1.64	300	1.63	277	1.61
0.67	260	1.64	421	1.49	425	1.45
0.82	247	1.68	413	1.46	435	1.41

- SEM for: BWG = 10.3; FC = 0.027.
- All main factors and interactions were significant $p < 0.05$.

The outstanding feature of the feather abnormality was the concave structure of the feathers as they bend away from the body. The rachises of the valine deficient chicks were marginally broader and more pliable than those of the chicks fed the higher levels of valine. The feather abnormality associated with the valine deficiency was similar to that described by Anderson and Warnick (1967) and Robel (1977). It is also similar to the description of feather abnormality obtained by Penz et al. (1984a,b) with toxic levels of dietary leucine.

During the first week of the experiment, the valine deficient birds started to exhibit a squatting stance. This increased in severity towards the end of the study. The birds at this stage were lying down on either side avoiding movement other than to eat and drink. A valine associated leg abnormality has not been observed in chickens. Kimura and Tahara (1971) reported that force feeding rats with a valine-free diet resulted in an acute weakness accompanied by 50% mortality rate. These symptoms were alleviated, however, by force feeding a valine-leucine-free diet.

The results of study 2 clearly indicated that the basal diet was deficient in all BCAA's. They also showed that lowering the level of dietary valine was more detrimental than lowering the 3 BCAA's simultaneously, as reflected by the physical appearance of birds. Therefore, it was decided to use this diet as a basal for further

experiments in order to determine the requirements of the BCAA's for starter chicks, and to elucidate the antagonism among the BCAA's at suboptimal levels.

Determination of BCAA Requirements

Study 3

The analysis of variance performed on weight gain data in study 3 showed that the Ile*Val interaction along with the Ile effects were not significant ($p > 0.05$). Body weight gain responded only to valine supplementation. The requirements of broiler chicks for valine could be estimated to be 0.74% when isoleucine and leucine levels were 0.58 and 1.04% respectively (Fig. 1). This estimated value can be located at the intersection of the regression line that fits the data of the first three levels of valine and the line parallel to the X axis. The latter passes through the average weight gain value of the last 2 levels of valine. The ANOVA applied to feed conversion data resulted in a significant interaction ($p < 0.05$) and significant main factors as well. For all levels of isoleucine, feed conversion values improved by increasing dietary levels of valine (Table 8). Increasing the levels of isoleucine, however, elicited a beneficial response with the two highest levels of valine (0.75 and 0.81%).

Figure 1. Study 3. Effect of feeding graded levels of valine on weight gain of male broilers in the starter period.

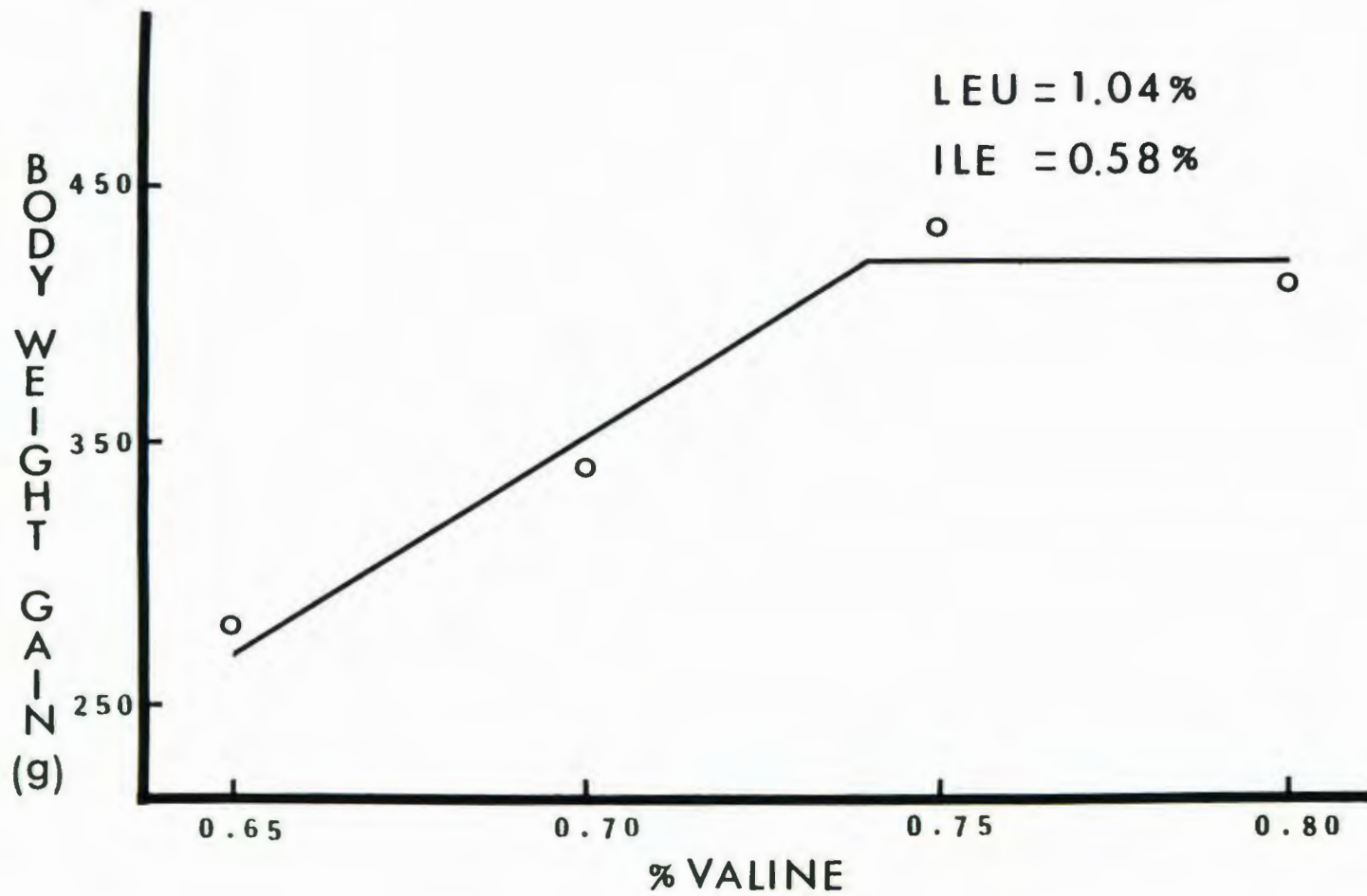


Table 8. Study 3. Feed conversion of 3-week-old broilers fed diets containing 1.04% Leu and varying levels of Val and Ile.

Ile %	Val %			
	0.65	0.69	0.75	0.81
0.58	1.64	1.54	1.50	1.49
0.67	1.66	1.57	1.44	1.42
0.76	1.65	1.54	1.44	1.42

SEM = 0.015.

Main factors and interactions were significant ($p < 0.05$).

The requirements of chickens for BCAA's estimated in study 3 were different from those found in the literature. The values of 0.58 and 0.74% for isoleucine and valine, respectively, were lower than those of Dobson *et al.* (1964), Dean and Scott (1965), and the NRC values (1984) (Table 9). They were close to the values of Hewitt and Lewis (1972) and higher than those of D'Mello (1974). The level of 1.04 for leucine was lower than all the values in Table 9 except for that of D'Mello (1974).

Most of the results presented in Table 9 were obtained by conducting studies in which one or two factors were varied and the other(s) remained fixed. Thomas *et al.* (1981) used graded levels of isoleucine keeping leucine and valine constant. D'Mello (1974) estimated chicken requirements for BCAA's using an approach similar to that

used in study 3 (2 factor factorial). This approach, however, does not suffice to estimate the requirements of three interrelated factors and response surface designs would be more appropriate.

Table 9. Branched-chain amino acid requirements proposed by different authors.

<u>Authors</u>	<u>Leu</u>	<u>Ile</u>	<u>Val</u>
Dobson <u>et al.</u> (1964)	1.30	0.80	0.95
Dean and Scott (1965)	1.20	0.80	0.82
Hewitt and Lewis (1972)	1.34	0.62	0.79
D'Mello (1974)	0.98	0.53	0.63
Thomas <u>et al.</u> (1981)	-	0.78	-
NRC (1984)	1.35	0.82	0.85
Current values	1.04	0.58	0.74

Response Surface Studies

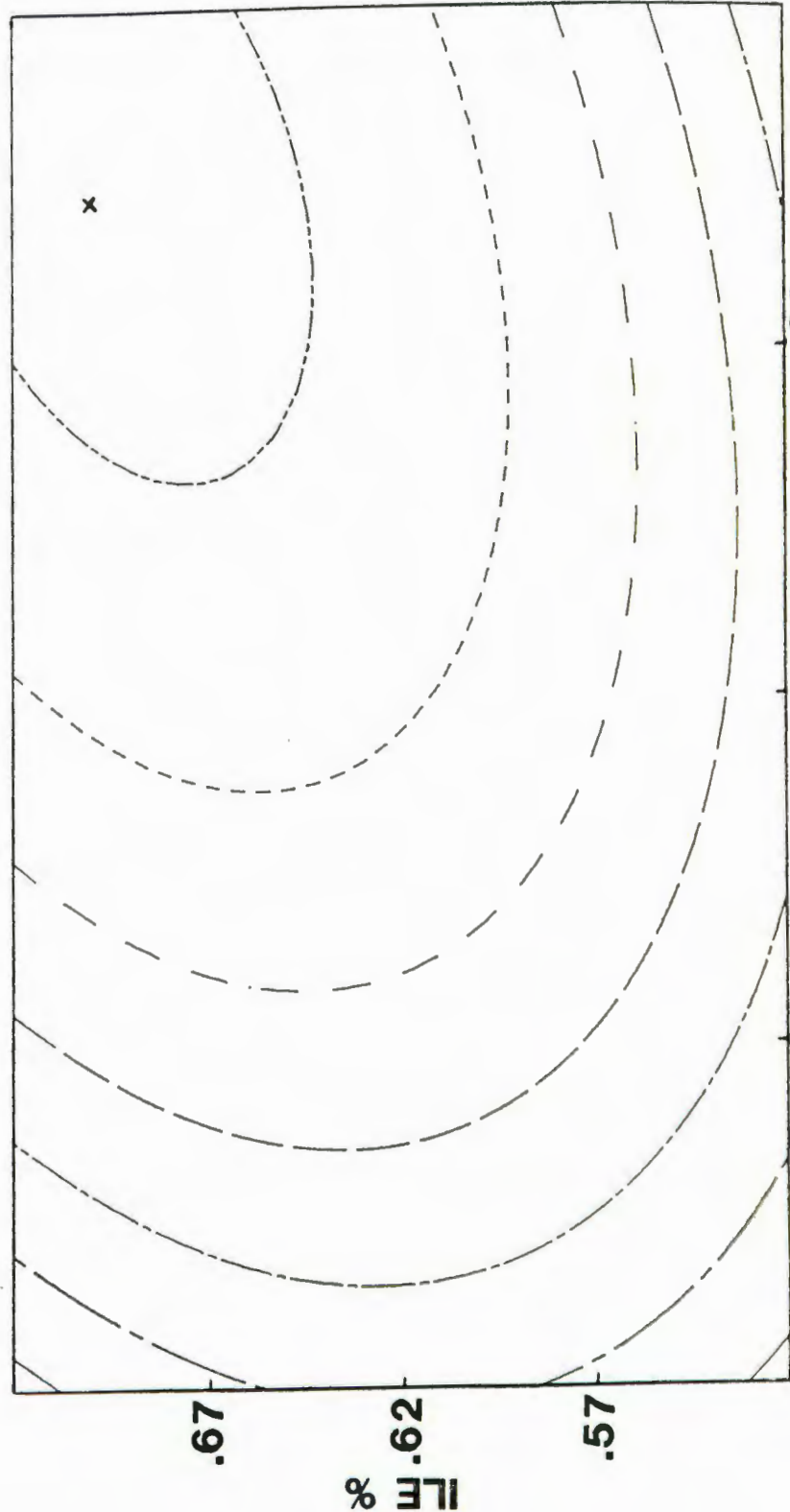
Studies 4, 5 and 6

Response surface regression analysis applied to study 4 data showed that optimum body weight gain would be obtained with dietary levels of 1.12, 0.70 and 0.82% for leucine, isoleucine, and valine respectively. The optimum response to feed conversion would be obtained by feeding the combination of 1.08, 0.71 and 0.84%. Plotting these results on contour graphics resulted in Figs. 2 and 3, where leucine level was fixed at its optimum values found in the analysis. Isoleucine and valine varied on the X and Y axes respectively. It should be noted that leucine was arbitrarily picked up to represent the fixed factor. If isoleucine or valine were chosen as the fixed variable

Figure 2. Study 4. Weight gain of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.12%.

$$\sqrt{\text{MSE}} = 26.76$$

$$R^2 = 0.807$$



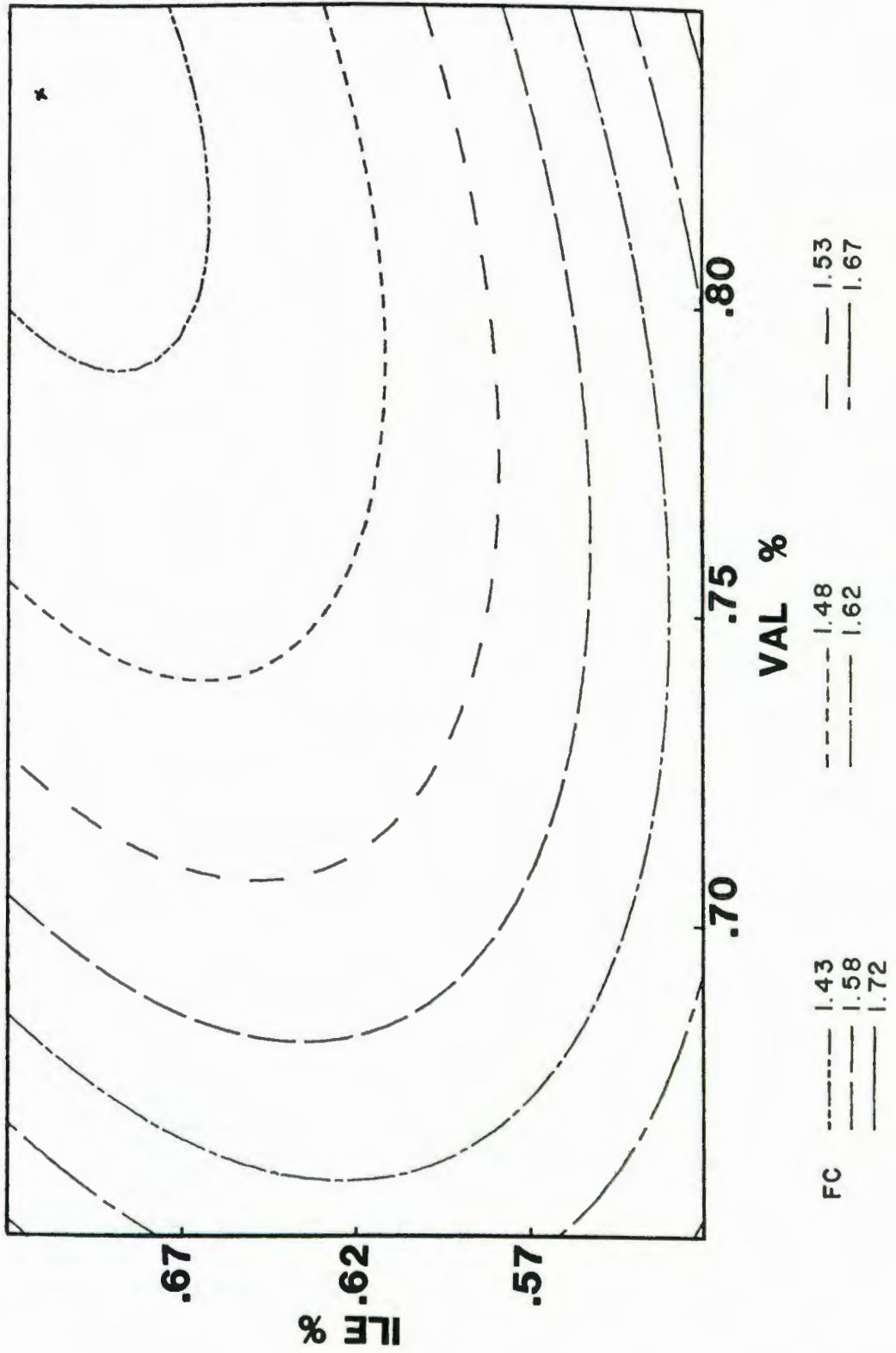
WT ——— 263
 ——— 377
 - - - 490

VAL % - - - 301
 - - - 415
 - - - 339
 - - - 452

Figure 3. Study 4. Feed conversion of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.08%.

$$\sqrt{\text{MSE}} = 0.0412$$

$$R^2 = 0.783$$



the remaining factor along with leucine would be the varied variables and different figures might result from these different combinations.

The contour lines in Figs. 2 and 3 correspond to the response of the different combinations possible of isoleucine and valine with the fixed values of leucine. The levels of the contour lines are presented in the legend of the figures. The optimum response, maximum in the case of weight gain and minimum for feed conversion, can be located in the upper right corner of Figs. 2 and 3. The shapes of the response surface for both criteria measured are presented in Appendices 4 and 5. The requirements of chickens for leucine, isoleucine, and valine (both criteria measured) were 1.12, 0.71 and 0.84% respectively.

Although the estimated values fall within the range of the actual levels for all the factors used, it may be argued that these ranges were not large enough to cover the requirements of the birds. Furthermore, test diets contained a high percentage of cerelose (35.2%) and low protein content (18.7%). A positive control treatment was also absent from study 4. All these facts were taken into consideration in study 5 (cerelose 18.8%).

The results of study 5 (Figs. 4 and 5, Appendices 6 and 7) indicated that the optimum values of both weight gain and feed conversion (combined) would be obtained by feeding the levels of 1.16, 0.77 and 0.90% for leucine,

Figure 4. Study 5. Weight gain of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.12%.

$$\sqrt{\text{MSE}} = 21.78$$

$$R^2 = 0.909$$

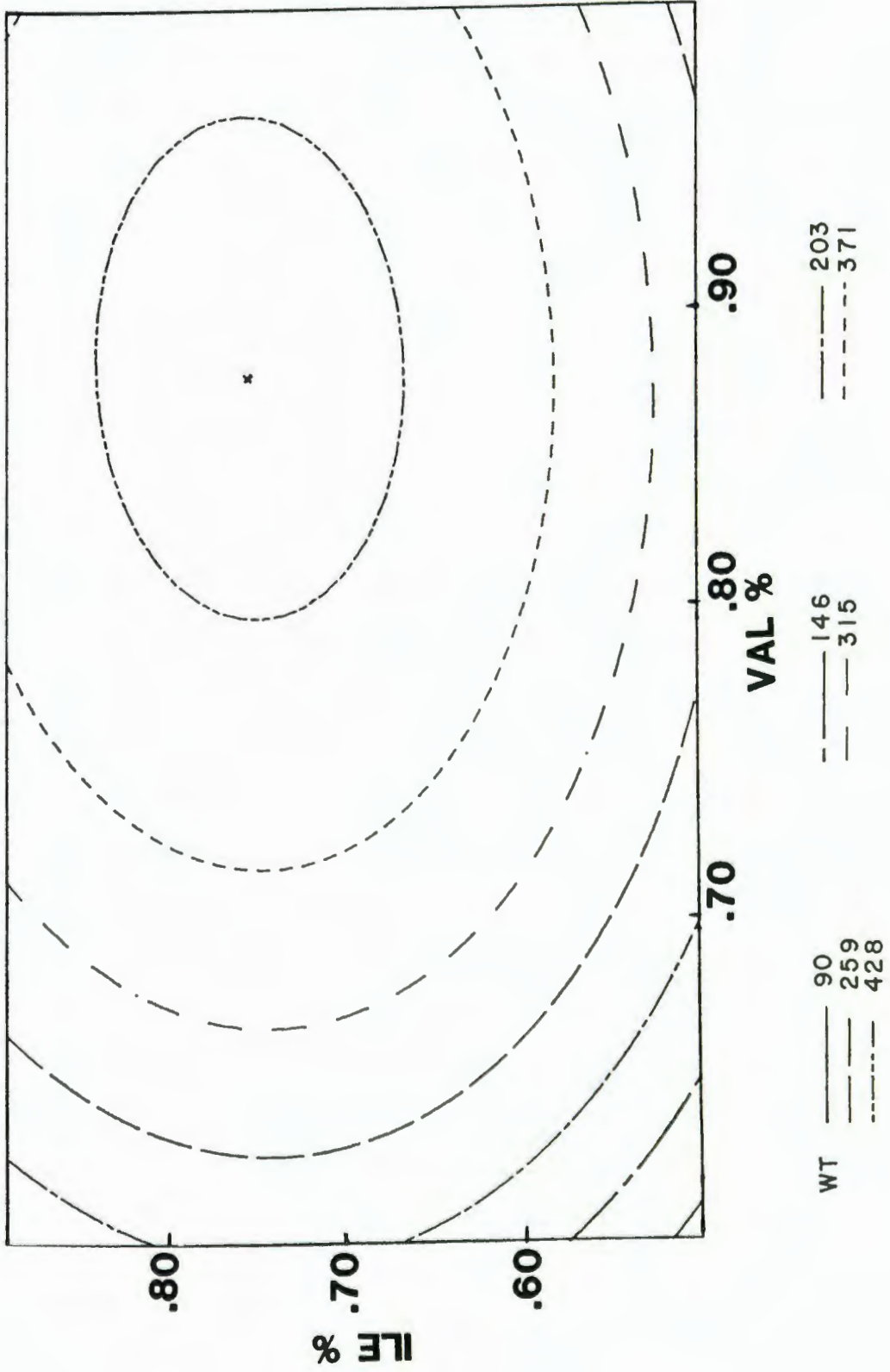
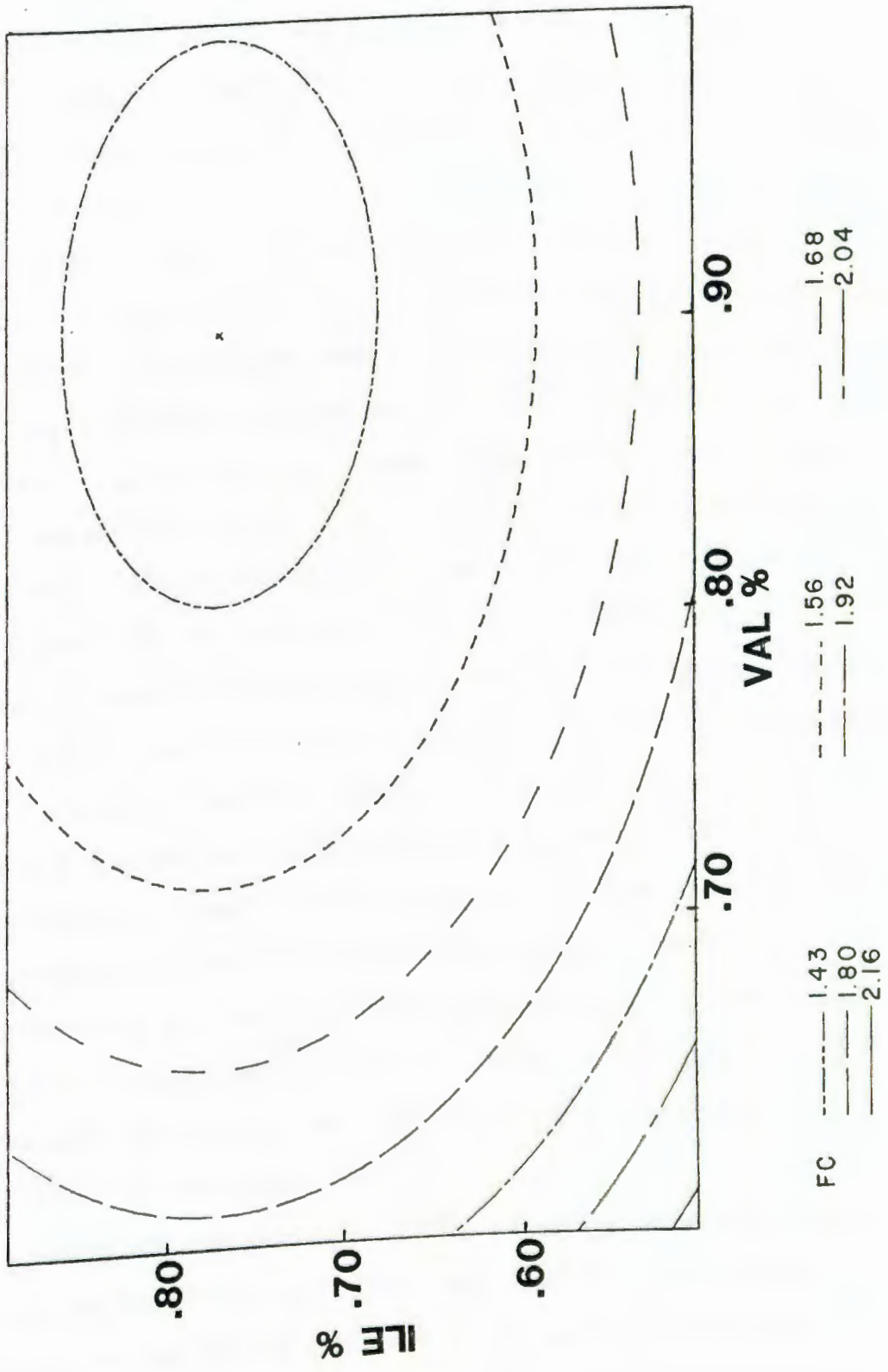


Figure 5. Study 5. Feed conversion of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.16%.

$$\sqrt{\text{MSE}} = 0.0485$$

$$R^2 = 0.871$$



isoleucine and valine respectively. The predicted values at these levels were 450 g and 1.39 for BWG and FC respectively. Feeding the positive control (23% protein) diet actually resulted in values of 451 g and 1.37 for the same criteria.

Figs. 6 and 7 and Appendices 8 and 9 summarize the results of study 6 which was conducted with no cerelese in the diets. An optimum BWG of 444 g would be obtained by feeding a dietary combination of 1.15, 0.81 and 0.88% of leucine, isoleucine and valine respectively. The optimum feed conversion value (1.38) would be obtained at 1.16, 0.80 and 0.90% following the same order. The results obtained with the positive control treatment (18.1% protein) were 436g and 1.41 for BWG and FC respectively.

Test diets in studies 4 and 5 contained approximately 18.5% protein, those of study 6 contained 20.6%. The protein content of a diet does not appear to affect the performance of the birds as long as the diet contains the required amount of the different essential amino acids expressed as a function of the energy level of the diet. This was illustrated in study 5 in which the test diets contained 18% protein and the positive control diet contained 23% protein.

Based on the results of the response surface studies, it can be concluded that the requirements of leucine, isoleucine and valine are 1.16, 0.81 and 0.90% respectively. The advantage of these studies is that values of

Figure 6. Study 6. Weight gain of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.15%.

$$\sqrt{\text{MSE}} = 15.79$$

$$R^2 = 0.805$$

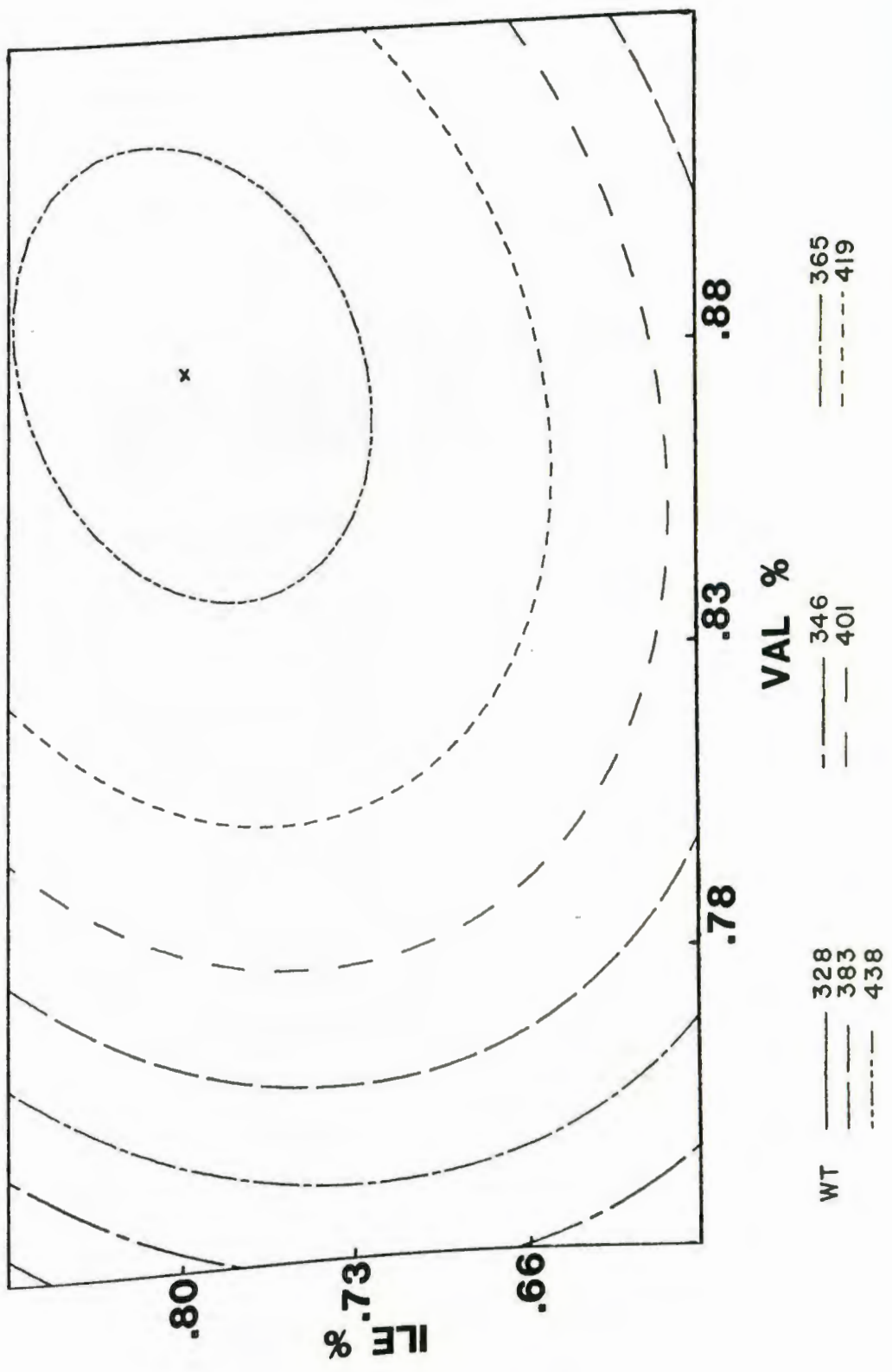
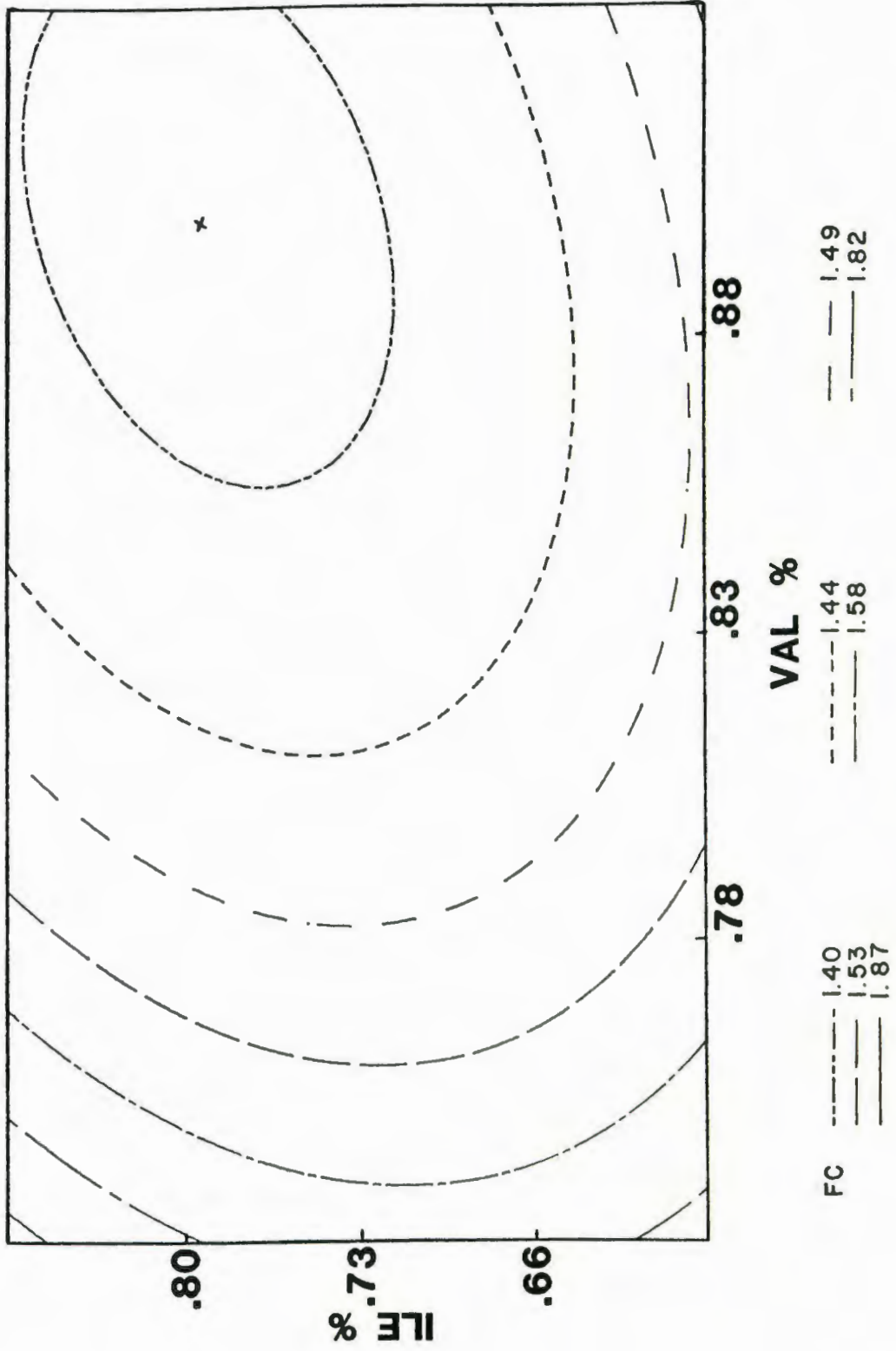


Figure 7. Study 7. Feed conversion of 3-week-old broiler males fed graded levels of isoleucine and valine. Dietary leucine was held constant at the level of 1.16%.

$$\sqrt{\text{MSE}} = 0.0248$$

$$R^2 = 0.875$$



leucine, isoleucine and valine were determined simultaneously. Compared to NRC values, the present results are different. In fact, leucine requirement is lower than that of the NRC value (1.35%), valine is higher. Isoleucine requirement remains, however, about the same. The values of Ile and Val being the actual values obtained when the diets were hydrolyzed for 72 hours (Thomas et al., 1981). For leucine, however, the hydrolysis time was 24 hours.

Antagonism at Suboptimal Levels

Study 7

Birds fed the valine deficient diets (0.63%) in study 7, with or without choline supplementation exhibited the same symptoms of leg and feather abnormalities described in study 2. These abnormalities were absent in birds fed the high valine diets (0.83%). In study 7, the results revealed that choline*Val interaction along with the main effect of choline were not significant for BWG and FC values ($P > 0.05$). Values for the grand means were $340 \pm 5.7g$ and 1.57 ± 0.014 for BWG and FC respectively. The main effect of valine, however, was highly significant ($p < 0.01$) for both criteria measured (Table 10).

Table 10. Study 7. Effects of feeding graded levels of choline chloride (50%) and valine on the performance of 3-week-old male broiler chicks.

		Choline chloride %			\bar{X} Val
		1.00	1.25	1.50	
Val%	0.63	247	253	244	248 ^a
	0.83	435	432	429	432 ^b
	\bar{X}	341	343	337	
		FC			
Val%	0.63	1.68	1.72	1.76	1.72 ^a
	0.83	1.42	1.44	1.43	1.43 ^b
	\bar{X}	1.56	1.58	1.60	

a,b

Means with different superscripts are significantly different ($p < 0.01$).

- SEM treatment: for BWG = 14.1; FC = 0.036
- SEM \bar{X} Val: for BWG = 8.1; FC = 0.021

The results of this experiment eliminated the role of choline in causing the leg abnormality and suggest that the feather abnormality and leg weakness are caused by valine deficiency per se.

The results of bone calcium, feather protein and the growth parameters of birds fed the Val supplemented,

valine deficient and all BCAA deficient diets are presented in Table 11. Chickens fed the valine deficient diet had the lowest values for all the criteria measured. A simultaneous deficiency of the 3 BCAA's resulted in values less than those of the Val supplemented treatment but much higher than the values obtained with valine deficiency ($p < 0.05$). The low bone Ca levels (134 mg/g dry bone) might partially explain the high incidence of leg abnormality observed among the birds fed the low valine diet. For unknown reason(s), a diet deficient in all the BCAA's had little or no adverse effects on the physical appearance of the birds.

Table 11. Study 7. Effect of Val deficiency on BWG, feed conversion, bone Ca and feather protein of 3-week-old male broilers.

Diet	Gain (g)	Feed Conversion	Bone Ca mg/g Dry Bone	Feather Protein %
Val Supplemented	435 ^a	1.42 ^c	172 ^a	88.0 ^a
BCAA Deficient	344 ^b	1.59 ^b	156 ^b	85.0 ^b
Val Deficient	247 ^c	1.68 ^a	134 ^c	82.7 ^c
SEM	8.3	0.013	3.5	0.43

a, b, c

Means with different superscripts are significantly different ($p < 0.05$).

The amino acid composition of feathers is shown in Table 12, expressed as a percent of the total protein. Compared to the Val supplemented and the BCAA deficient treatment, the valine deficiency changed the pattern of feather amino acids by increasing the levels of Asp, Glu, Met, Tyr, His and lysine ($p < 0.05$). The level of cystine was significantly decreased. The mechanism by which valine deficiency affects feather amino acid composition is still obscure. This alteration, however, might explain the ragged appearance of the feathers. Aspartic acid, Glu, Tyr, His and lysine are called α -helix breakers (Leningher, 1978). The α -helix is prevented from forming by the presence of two or more consecutive residues with like charges. Similar charges (positive in the case of His and Lys, negative in the case of Asp and Glu) tend to repel each other, causing the rupture of the α -helix. The presence of bulky amino acids such as Tyr (due to its phenol group) would not fit the structure of α -helix. The decrease in feather cystine associated with the valine deficiency (Table 13) would deprive the helix from the disulfide bond necessary for proper structure (Segel, 1976).

Anderson and Warnick (1967) and Robel (1977) observed ragged feathers for chicks fed valine deficient diets. These authors, however, did not analyze feathers for amino acids. The pattern of feather amino acids presented in

Table 12. Study 7. Effect of valine deficiency on feather amino acid¹ pattern of 3-week-old male broilers.

<u>Diet</u>	<u>Valine Deficient</u>	<u>BCAA Deficient</u>	<u>Val Supplemented</u>	<u>SEM</u>
Protein %	82.7 ^c	85.0 ^b	88.0 ^a	0.43
Cys	8.04 ^b	-	8.72 ^a	0.100
ASP	7.43 ^a	7.12 ^b	7.00 ^c	0.038
THR	4.82	4.77	4.76	0.025
SER	11.20	11.40	11.50	0.092
GLU	11.20 ^a	10.60 ^b	10.40 ^b	0.092
PRO	10.30	10.50	10.40	0.146
GLY	7.07	7.08	6.93	0.044
ALA	4.97	4.90	4.46	0.186
VAL	8.40	8.37	8.54	0.061
MET	0.62 ^a	0.48 ^b	0.41 ^c	0.014
ILE	4.94	4.86	4.96	0.040
LEU	7.90	7.77	7.65	0.065
TYR	2.82 ^a	2.60 ^b	2.56 ^b	0.028
PHE	4.81	4.77	4.74	0.019
HIS	0.80 ^a	0.59 ^b	0.48 ^c	0.024
LYS	2.01 ^a	1.59 ^b	1.28 ^c	0.084
NH ₃	2.84 ^a	2.75 ^{ab}	2.51 ^b	0.074
ARG	7.59	7.52	7.42	0.043

¹ Values are expressed as % of the total protein in the feather.

a, b, c Means with different superscripts are significantly different ($P < 0.05$).

study 7 appears similar to that found by Penz et al. (1984b) with the exception that the latter was obtained with toxic levels of dietary leucine. When valine and isoleucine were added to the high leucine diet the feather abnormality was corrected. The amino acid composition of feathers was also similar to that of the control birds. These authors were able to detect differences among the BCAA levels in feathers. In study 7 the differences were not significant. This may have been due to the low levels of dietary BCAA's used. The antagonism among the BCAA's at suboptimal levels, therefore appears similar, to a certain degree, to that demonstrated with superoptimal levels.

It is possible that feed intake could have influenced the results of study 7. Therefore, the same experiment was run under equal feed intake conditions using force feeding and pair feeding techniques.

Study 8. Force-Feeding Trial

Birds force fed the valine deficient and all BCAA deficient diets gained approximately 65 g less than those fed the same amount of Val supplemented diet ad libitum (Table 13). Compared to Val supplemented chicks, feed conversion values were higher for all BCAA and valine deficient birds. Although these birds had similar size, the latter were lethargic and showed the symptoms of feather and leg abnormalities previously described.

Table 13. Study 8. Growth parameters, bone measurements and serum calcium in birds fed a Val supplemented diet or force-fed diets containing suboptimal levels of the BCAA's.

	Val Supplemented	BCAA	Val Deficient	SEM
Gain (g)	235 ^a	175 ^b	164 ^b	5.2
Feed Conversion	1.53 ^a	2.06 ^b	2.11 ^b	0.023
Dry Bone (g)	0.686 ^a	0.527 ^b	0.491 ^b	0.018
Bone Ash % DB	50.7 ^a	48.2 ^b	46.6 ^b	0.72
Bone Ca (mg/g/DB)	182 ^a	175 ^{ab}	163 ^b	4.2
Serum Ca (mg/dl)	10.4 ^a	10.8 ^a	10.2 ^a	0.250
Mortality %	-	6.25	37.5	

a,b,c

Means with different superscripts are significantly different ($p < 0.05$).

The weight of dry bone (g), bone ash (%) and bone calcium (mg/g/dry bone) were the lowest for birds force fed the valine deficient diet followed by those fed the BCAA and the Val supplemented diets. Statistical analysis of the data failed to show significant results for these criteria, between all BCAA and valine deficient treatments, most probably because of the high mortality rate (37.5%) among valine deficient birds (Table 13). Six out of the 16 birds force fed the valine deficient diet died

during the experiment (4 birds died on day 10) forcing to end the experiment by day 11. Only one bird in the BCAA deficient treatment died during the course of the study.

The results of high mortality rate associated with valine deficiency appeared similar to those published by Kimura and Tahara (1971). These authors reported that rats force fed a valine-free diet showed symptoms of "acute weakness" leading to a higher mortality rate (50%). Force feeding a valine and leucine-free diet retarded the weakness and prevented mortality of the rats. Their results suggested that the relationship between valine and leucine is more intimate than those of isoleucine and leucine since no mortality was recorded in the case of force feeding a diet lacking one or both of the latter amino acids.

Study 9. Pair-feeding Trial

Restricting feed intake of BCAA birds to the level of valine deficient birds resulted in similar weight gain and feed conversion values (Table 14). The growth parameters of these 2 groups were significantly different from that of the Val supplemented group ($p < 0.05$). In this study also, only valine deficient birds exhibited both the leg and feather abnormalities previously described. Further investigations showed that values of dry bone, bone ash and bone calcium for valine deficient birds, were the lowest among the 3 groups studied ($p < 0.05$). Serum

calcium on the other hand was not affected by any of the 3 treatments.

Table 14. Study 9. Growth parameters, bone measurements and serum calcium in birds receiving equal amounts of Val supplemented diet or diets containing suboptimal levels of BCAA's.

	Valine Supplemented	BCAA	Valine Deficient	SEM
Gain (g)	252 ^a	217 ^b	222 ^b	4.670
Feed Conversion	1.49 ^a	1.71 ^b	1.68 ^b	0.012
Dry Bone (g)	0.988 ^a	0.882 ^b	0.743 ^c	0.029
Bone Ash % DB	45.0 ^a	42.1 ^b	37.2 ^c	0.34
Bone Ca (mg/g/DB)	164.2 ^a	149.8 ^b	136.9 ^c	1.12
Serum Ca (mg/dl)	10.3 ^a	10.2 ^a	10.5 ^a	0.140

a,b,c

Means with different superscripts are significantly different ($p < 0.05$).

The leg and feather abnormalities associated with valine deficiency observed in studies 8 and 9, compared to a simultaneous deficiency of all BCAA's would indicate that a metabolic disorder was the consequence(s) of the valine deficiency. The low level of bone calcium

associated with valine deficiency (Table 14) could be the result of: (1) enhanced bone resorption by increasing osteoclastic activity or (2) decreased bone deposition by depressing osteoblastic activity. Both activities occur simultaneously in bone with a higher rate for the deposition process in growing animals (Leeson et al., 1985).

The influence of vitamin D₃ or its metabolite, 1,25 (OH)₂D₃ might be suspected but the fact that serum Ca was the same for all the treatments would eliminate this assumption.

The low level of % ash in the bone of valine deficient birds (37.2%) compared to 42.1% for all BCAA birds suggests that the 2 groups of bones had at least the same amount of bone matrix (valine deficient bone might have more on a dry weight basis). The lack of mineralization therefore, seems to be related to the "quality" and not to the quantity of bone matrix deposited in the case of valine deficiency. Anderson (1985) listed the quality of collagen among the factors that promote crystal proliferation (phase 2 of calcification).

Bone matrix consists of collagenous fibers united by a cementing substance (glycosaminoglycans). The fibers are made up of collagen fibrils, which have a cross-striated appearance. Collagen fibrils consist of recurring polypeptide subunits called tropocollagen, arranged head to tail in parallel bundles. The rod-shaped tropocollagen molecule in turn is composed of three helical polypeptide

chains (α_1 , α_2 and α_3) twisted around each other to form a superhelix (Leeson et al., 1985).

It was found that each chain of the tropocollagen is made up of 6 subunits (Gallop et al., 1967). These authors proposed a unified 3:2:1 (A,B,C) model for the tropocollagen molecule. According to this model each α chain contains 3 subunits of one type, two of another and one of a third so that:

$$\alpha_1 = 3A + 2B + C$$

$$\alpha_2 = 3C + 2A + B$$

$$\alpha_3 = 3B + 2C + A$$

In chicken bone matrix, Francois and Glimcher (1967) reported that α_2 contained more Leu, Ile and Val than in either α_1 or α_3 chains. Based on those results, Gallop et al. (1967) concluded that almost all the BCAA's are concentrated in the C subunits. They also suggested that the C subunits are near or at terminal positions in tropocollagen. Calcification has been known to be initiated and proliferated near the tropocollagen terminals in the hole zone which is located between 2 tropocollagen molecules (Glimcher and Krane, 1967; Glimcher, 1985).

The lack of dietary valine might cause leg abnormality in the following way: The consumption of valine deficient diet would disrupt the proper ratio of the available BCAA's in the osteoblasts. It is possible that

some proteins may be synthesized in preference to others in the osteoblasts making the collagen molecules unable to promote bone calcification or proliferation.

Plasma Amino Acids

Due to the similarity of results in studies 8 and 9, only chicken plasma from the pair feeding study was analyzed for amino acid content. Leucine and valine values reflected their levels in the diets (Table 15). Those of isoleucine, glutamic acid, glycine and tryptophan were not affected by the different treatments ($p > 0.05$). With the exception of isoleucine values, the current results agree with those published by D'Mello and Lewis (1970), D'Mello (1974), Smith and Austic (1978), and Penz et al. (1984a).

The hydroxyproline level was the lowest in the plasma of birds fed the valine deficient diet. This unusual amino acid is found in collagen and exists in plasma in at least three forms: free, peptide-bound and protein-bound (LeRoy et al., 1964). Its values (Table 15) represent the first 2 forms combined since the samples were deproteinized (Appendix 3).

The final breakdown products of collagen contain both free and peptide-bound hydroxyproline, found in the plasma, and neither are utilized for the resynthesis of new collagen (Varghese et al., 1981). The peptide-bound-hydroxyproline is excreted through the kidney. The free

hydroxyproline is catabolized in the liver by hepatic oxidase (Kopple, 1983).

Table 15. Study 9. Plasma concentrations ($\mu\text{M}/100\text{ ml}$) of selected amino acids in birds receiving equal amounts of Val supplemented diet or diets containing suboptimal levels of BCAA's.

Treatments	Val	Leu	Ile	Glu	Gly	Trp	Hydroxyproline
Val Supplemented	15.3 ^a	17.2 ^a	19.8 ^a	19.7 ^a	96.6 ^a	25.2 ^a	18.6 ^a
BCAA Deficient	11.3 ^{ab}	9.2 ^b	20.0 ^a	22.4 ^a	118.2 ^a	22.9 ^a	20.9 ^a
Valine Deficient	9.5 ^b	17.8 ^a	15.3 ^a	19.7 ^a	110.6 ^a	22.4 ^a	15.0 ^b
SEM	1.50	0.69	1.82	1.28	7.31	2.13	0.96

a, b, c Means with different superscripts in the same column are significantly different ($p < 0.05$).

Goidanich *et al.* (1965) and Weiss and Klein (1969) showed that, in mammals, total hydroxyproline in urine (mg/day) is correlated with bone metabolism. The free and peptide-bound hydroxyproline in the plasma are also

considered to be a reliable biochemical marker of bone turnover, skeletal tissue being the major reserve of collagen in the human body (Laitinen, 1974). Minisola *et al.* (1985) found that the level of free hydroxyproline in plasma and total urinary hydroxyproline increased in patients with bone disease including primary hyperparathyroidism, osteomalacia, Paget's disease, bone metastases and chronic renal failure. They also showed that the level of the free hydroxyproline decreased in the patients with Paget's disease following chronic administration of calcitonin. It also decreased in the plasma of patients following a successful parathyroidectomy. Neither calcitonin, nor parathyroid hormone were assessed, since the studies 8 and 9 were not designed to investigate the relationship between hydroxyproline on one hand and thyroid and/or parathyroid gland on the other hand.

A high level of hydroxyproline is excreted in the urine of young rats (Lindstedt and Prockop, 1961) and growing children (Goidanich *et al.*, 1965). It is an indication of high collagen degradation which occurs simultaneously with active bone formation (bone growth and remodelling) in growing mammals. Minisola *et al.* (1985) showed that hydroxyproline in plasma urine is quite high. Therefore, a hydroxyprolinemia is expected in young chicks. The decrease in hydroxyproline level in plasma of valine deficient birds might be the result of either increased catabolism of the free form in the liver or

enhanced excretion of this amino acid in urine. Hydroxyproline in the urine of both Val deficient and supplemented birds could not be detected with the amino acid analyses. The low level of plasma hydroxyproline in valine deficient birds may be due to less bone matrix being broken down.

Kidney Function

Urine flow (ml/min) and GFR values (ml/min/kg body wt) were similar for both control and valine deficient birds, indicating that kidney function was not altered by valine deficiency (Table 16). The values of these 2 criteria were similar to those obtained with young pullets (Wideman et al., 1985). They are, however, lower than those of Reed (1986), who used a high rate of inulin-mannitol solution (0.8 ml/min).

Fractional excretion of calcium in valine deficient birds was 3 times higher than that of control birds. It confirms that the kidney was efficient in ridding the body of the extra Ca coming either from bone resorption or intestinal absorption, thereby maintaining a normal blood calcium level (Table 16).

The results of bone calcium, serum calcium, plasma hydroxyproline and kidney function measurements would lead to the following hypothesis: The osteoblasts need available BCAA's in a proper ratio in order to synthesize a bone matrix able to promote bone calcification and proliferation. If this ratio is disrupted, Val may not be

available for the synthesis of the C subunits of the α chains in the tropocollagen molecules, leading to improper bone calcification. Osteoclastic activities would therefore prevail and the ricket-like condition would develop. It is illustrated by low bone ash and low plasma hydroxyproline, suggesting less degradation of bone matrix. The extra calcium depleted from bone should be excreted through the kidney. Urinary calcium increased by 3 times for Val deficient birds compared to Val supplemented chickens. If this hypothesis holds true, it suggests a vital role for valine in collagen formation and bone calcification.

Table 16. Urine flow, glomerular filtration rate (GFR), fractional excretion of Ca and blood calcium of birds fed valine supplemented and valine deficient diets.

Treat- ment	n	Urine flow ml/min	GFR ml/min/ kg wt	Fractional excretion of Ca (FE Ca)	Blood Ca mg%
Valine Supple- mented	6	0.076 ^a	1.09 ^a	0.04 ^b	9.87 ^a
Valine deficient	5	0.070 ^a	1.05 ^a	0.13 ^a	9.71 ^a
SEM		0.008	0.030	0.011	0.370

a,b

Means with different superscripts in the same column are significantly different ($p < 0.05$).

Antagonism at Superoptimal Levels

Study 10

In mammals and according to Lehninger (1978) leucine has been classified as a ketogenic amino acid since it yields free acetoacetate. Valine is a glycogenic amino acid since its degradation yields succinate which is an intermediate of the tricarboxylic acid cycle. Isoleucine is classified as both glycogenic and ketogenic since it is degraded to produce acetyl-CoA and succinate.

Based on the classification of the BCAA's and on the fact that antagonism among BCAA's does exist in both chicken (birds) and rat (mammals), one can hypothesize the following: Excess dietary leucine should bring about ketosis which could be corrected by adding dietary valine and isoleucine. No research has been done concerning the determination of ketone bodies in birds following an induced leucine toxicity. Therefore study 10 was designed to test the above hypothesis. The determination of β -OH butyrate and not the total ketone bodies was made. According to Nash et al. (1954) and Hultman (1974) acetone represents less than 5% of the total ketone bodies. In addition acetoacetate is not stable even if the samples are stored at -20°C (Gibbard and Watkins, 1968; Salway, 1969) and the ratio of $\frac{\beta\text{-OH butyrate}}{\text{acetoacetate}} = 2.4$ (Gibbard and Watkins, 1968).

The results of BWG, FC and β -OH butyrate concentration are presented in Tables 17, 18 and 19 respectively.

Table 17. Study 10. Effect of 3.06% dietary Leu on body weight gain (g) of broilers (1-3 weeks old); role of Ile and Val in alleviating the toxic effect of Leu.

Ile %	Val %	Leu %			
		1.36		3.06	
		0.86	1.20	0.86	1.20
0.83		434	420	353	384
1.16		412	410	344	424

- SEM = 7.4.
- Except for Ile, other main factors and interactions were significant $p < 0.05$.

Table 18. Study 10. Effect of 3.06% dietary Leu on feed conversion of broilers (1-3 weeks old); role of Ile and Val in alleviating the toxic effect of Leu.

Ile %	Val %	Leu %			
		1.36		3.06	
		0.86	1.20	0.86	1.20
0.83		1.30	1.32	1.40	1.43
1.16		1.35	1.31	1.42	1.33

- SEM = 0.024.
- Except for Leu and Ile*Val, other main factors and interactions were nonsignificant $p > 0.05$.

Table 19. Study 10. β -OH butyrate concentration in plasma (mg/l). Birds were fed high levels of Ile and Val to overcome the toxicity induced by 3.06% dietary leucine.

Ile %	Val %	Leu %			
		1.36		3.06	
		0.86	1.20	0.86	1.20
0.83		52.6	60.9	50.0	60.1
1.16		59.3	53.1	53.9	49.7

- SEM = 5.68.
- All main factors and interactions were nonsignificant $p > 0.05$.

Compared to the control diet which corresponds to the lowest combination of the BCAA's, the 3.06% dietary leucine used with the 0.83% isoleucine and 0.86% valine retarded growth rate by 80 g. A complete restoration of BWG occurred when dietary isoleucine and valine were simultaneously increased by 40% above the NRC required levels. A similar trend was observed concerning the feed conversion results (Table 18). The ANOVA applied to β -OH butyrate concentration revealed that neither the interactions nor the main effects were significant ($p > 0.05$). The mean values of the different treatments used in this study (Table 19) are not significantly different from each

other ($p > 0.05$) and the general grand mean value was 55.0 ± 2.01 .

The results of this study with respect to weight gain and FC were similar to those obtained by D'Mello and Lewis (1971), Boldizar et al. (1973), Smith and Austic (1978), and Penz et al. (1984b). More than 3% dietary leucine was also toxic to rats (Harper et al., 1955; Benton et al., 1956; Tannous et al., 1966).

The mechanism by which isoleucine and valine ameliorate the ill effects of dietary leucine is not understood and conflicting reports have appeared in the literature. Ueda et al. (1981) suggested that the depression of feed intake is the primary effect of leucine toxicity. They showed that chicks force fed an excess of added dietary leucine (1.32%) to the level of control chicks resulted in 100% restoration of the ill effect associated with the toxic level of leucine. D'Mello and Lewis (1971), however, showed that the decrease in feed intake of chicks which resulted from feeding an excess of added dietary leucine (1.5%), was not the primary cause of growth retardation. In their study, chicks pair fed the control diet and those fed the toxic leucine diet ad libitum had different weight gain values. Pair fed chicks gained less than the other birds. They concluded that the antagonism might then be caused by a metabolic disorder. The results of study 10 failed to show that ketosis occurs when high levels of leucine are fed.

Study 11

The ANOVA performed on weight gain data from study 11 revealed that Leu*Val and Ile*Val interactions were significant ($p < 0.05$). Only Ile*Val interaction was significant in feed conversion analysis. Weight gain values averaged across Ile are shown in Table 20. The lower level of Ile (1.0%) with the first level and the 2 highest levels of Val yielded weight gain values similar to those of the 1.4% Ile. The combination 1.0% Ile and 1.4% Val resulted in an average BWG of 434 g which was 25 g higher than that of 1.4% Ile and 1.4% valine. There is no explanation for these phenomena except that of random variation. The same comment can be made on the feed conversion values shown in Table 21. The results of the isonitrogenous isocaloric diet (positive control treatment) were 439 ± 16.2 g and 1.45 ± 0.022 for weight gain and feed conversion values, respectively. Therefore it can be concluded that in a high protein diet corn gluten meal may be used at high levels without any detrimental effect.

In study 11, 4% dietary leucine did not induce toxicity when protein content of the diet was 26.5%. The leucine induced toxicity in study 10 and in those reported by D'Mello (1971), Smith and Austic (1978) and others were obtained with low dietary protein (18 - 22%). Toxicity in rats induced by feeding excessive dietary leucine was also obtained with low dietary protein (Harper *et al.*, 1955; Dobson *et al.*, 1965; Tannous *et al.*, 1966). The

Table 20. Study 11. Weight gain of 1-3 week-old male broiler chicks fed different dietary levels of leucine, isoleucine and valine.

		ILE %					
		1.0		1.4			
Leu %		2.75	4.0	2.75	4.0		
Val %				\bar{X}			\bar{X}
1.2		427	413	420	426	418	421
1.4		442	426	434	410	409	409
1.6		425	431	428	421	442	432
1.8		430	412	421	443	414	429

\bar{X} : values averaged across leucine.

SEM treatment = 7.9.

SEM \bar{X} = 5.6.

Except for Ile*Val and Leu*Val, other main factors and interactions are nonsignificant ($p > 0.05$).

Table 21. Study 11. Feed conversion of 1-3 week-old male broiler chicks fed different dietary levels of leucine, isoleucine and valine.

		ILE%					
		1.0		1.4			
Leu %		2.75	4.0	2.75	4.0		
Val %				\bar{X}			\bar{X}
1.2		1.49	1.48	1.49	1.47	1.45	1.46
1.4		1.44	1.47	1.45	1.47	1.47	1.47
1.6		1.47	1.46	1.46	1.46	1.47	1.46
1.8		1.46	1.49	1.47	1.46	1.45	1.45

\bar{X} : values averaged across leucine.

SEM treatment = 0.011.

SEM \bar{X} = 0.008.

Except for Ile*Val, other main factors and interactions are nonsignificant ($p > 0.05$).

results of study 11 supported those of Daniel and Waisman (1968) who showed that excessive amounts of Leu 7-10% in a high protein diet did not affect the performance of the rats. The reason why excessive leucine is toxic only with a low protein diet is not understood. It is speculated that the consumption of high dietary protein level implies an abundance of all amino acid residues at the absorptive sites of the small intestines. Therefore, even in the presence of competition among amino acids, belonging to the same group for the absorptive sites (in this case BCAA's) a fairly adequate amount of the least available amino acid(s) would still be absorbed. However, with low protein diet though meeting the amino acid requirements, an excessive amount of one amino acid (Leu) would accentuate the problem of competition. The other amino acid(s) would become marginal or even limiting. More research is needed to clarify this phenomenon.

SUMMARY

At low to moderate dietary protein levels (up to 22%) for the broiler in the starter period, the BCAA's are interrelated. Any decrease or increase in one of these amino acids will influence the requirements of the two remaining amino acids. Therefore an adjustment of their levels should be considered accordingly.

The poor performance of the birds is the common feature of the antagonism among the BCAA's at suboptimal and superoptimal levels. At low dietary protein, leucine toxicity may be considered as a valine deficiency since both result in ragged appearance of feathers.

At suboptimal levels, the ratio of 3 BCAA's to one another becomes more important. Any variation of this ratio(s), illustrated by lowering the Val level, would have a detrimental effect on bone calcification, therefore resulting in a ricket-like condition. Research is needed to investigate the relationship between the Val deficiency and Ca, Vit D₃, parathyroid hormone and calcitonin.

High protein diet appears to mask the effect of leucine toxicity. This would imply that the absorption of the BCAA's plays an important role in explaining the existing antagonism among these amino acids. The relationship(s) between the excess dietary nitrogen (from essential, non essential amino acid or both), and this an-

tagonism is unknown. Therefore, more research concentrated on those areas is needed.

The domestic chicken provides an excellent model to investigate the interrelationship among the branched chain amino acids at both suboptimal and superoptimal levels. Moreover, studies involving the determination of end products excreted in urine are now available and relatively easy to conduct.

APPENDIX 1

Amino Acid Analysis

The sample should contain the AA to be separated in a free form which is the case of physiological samples such as urine and deproteinized plasma. Solid samples, however, such as feed and feather samples should be hydrolyzed (Moore and Stein, 1963).

A sample of AA mixture is placed on top of a column packed with negatively charged sulfonated polystyrene resin. Elution by buffers then occurs through the column. The acidic AA exit the column first, followed by the neutral and finally the basic AA. The use of a pressurizing system and the gradual increase in pH will speed elution from the column. After separation of AA on the ion exchange column, the AA is mixed with ninhydrin to form a blue-colored compound which is measured colorimetrically for quantification of the amino acids.

Reagents

- Physiological standard "A" containing acidic and neutral amino acids, and physiological standard B (basic amino acids) with 2.5 $\mu\text{M}/\text{ml}$ each (Pierce Chemical, Rockford, Ill.).
- Hydrolysate standard, 0.5 $\mu\text{M}/\text{ml}$ (Pierce Chemical).

- Norleucine standard (NLE), 0.5 μ M/ml (Pierce Chemical).
- Sulfosalicylic acid, 30 and 50% (SAA).
- HCl, 6N.
- Ninhydrin DMSO-NIN-SOL AF Reagent Solution Kit (Pierce Chemical).
- NaOH, 4%.
- Sodium citrate buffers:

	Buffer*	1	2	3	4
pH		2.20	3.28	5.	7.90
Na normality		0.20	0.172	0.38	1.10
Na citrate (g)		19.61	17.00	19.61	39.22
NaCl (g)		-	-	10.52	40.90
Thiodiglycol (ml)		20.00	5.00	5.00	-
Concentrated HCl (ml)		16.50	11.50	4.00	0.30
Liquid phenol (ml)		1.00	1.00	1.00	1.00
Final volume (L)		1.00	1.00	1.00	1.00

* Buffers 1, 2 and 3 were used for physiological samples, and 1, 2 and 4 were used for feather and feed samples.

Deproteinization of Physiological Samples

A 50 μ l aliquot of 50% SAA (30% solution in the case of urine sample) was added to 1 ml plasma or urine into a centrifuge tube. The mixture was vortexed and centrifuged at 3500 rpm for 15 minutes. The supernatant was removed

and 4% NaOH (0.2N) solution was added at the rate of 5 μ l/75 μ l supernatant. This mixture was then centrifuged at 11,500 rpm for 10 minutes. The supernatant was finally placed into a storage vial and refrigerated.

Hydrolysis of Feed and Feather Samples

Approximately 120 mg of feed or 25 mg of ground feathers were weighed into hydrolysis tubes. Glass beads, a 100 μ l of NL and 4 ml of 6 N HCl were added. Tubes were then flushed with N₂ gas, sealed with covers and placed into preheated heating blocks (110°C) for either 24 or 72 hours. After the digestion period tubes were flushed with N₂ gas and attached to distillation units. Distillation was over when samples were completely dry. Samples were then dissolved in 20 ml of sodium citrate buffer (pH = 2.2) and refrigerated until analyzed.

Analysis of Samples

Hydrolysate (20 μ l), deproteinized plasma and urine samples (40 μ l) were loaded into sample holding units and analyzed on a Durrum amino acid analyzer (Dionex Corp., Sunnyvale, CA). A similar amount (40 μ l) of the mixture standards A and B (20 μ l each) and 20 μ l of the hydrolysate standard and 20 μ l of NLE standard were also loaded and analyzed. The absorbance of proline and hydroxyproline was read at 440 nm, that of the other amino acids was read at 590 nm.

CalculationsPlasma and urine AA

$$\frac{2.5 \mu\text{M}}{\text{ml}} \times \frac{\text{ml}}{1000 \mu\text{l}} \times 20 \mu\text{l} = 0.050 \mu\text{M/standard}$$

$$\frac{\text{Sample area}}{\text{Standard area}} \times 0.05 = \mu\text{M in sample peak}$$

or in 40 μl sample

$$\mu\text{M sample peak} \times \frac{1000 \mu\text{l}}{40 \mu\text{l sample}} = \mu\text{M/ml} \times 100 = \mu\text{M}/100 \text{ ml}$$

Feed and feather AA

$$\frac{0.5 \mu\text{M}}{\text{ml}} \times \frac{\text{ml}}{1000 \mu\text{l}} \times 20 \mu\text{l} = 0.01 \mu\text{M per standard}$$

$$\frac{\text{Sample weight (mg)}}{20 \text{ ml}} \times \frac{1 \text{ ml}}{1000 \mu\text{l}} \times 20 \mu\text{l} = \text{mg sample injected}$$

$$\frac{\text{Sample area}}{\text{STD area}} \times 0.01 \mu\text{M per standard} = \mu\text{M in sample peak}$$

$$\frac{\mu\text{M in sample peak}}{\text{mg sample injected}} \times \frac{1 \text{ mM}}{1000 \mu\text{M}} = \text{mM per mg sample}$$

$$\frac{\text{mM}}{1 \text{ mg}} \times \text{AA molecular weight} \times 100 = \text{AA\%}$$

Corrected value for recovery

$$\text{mg\%} \times \frac{\text{NLE STD}}{\text{NLE Sample}} = \text{AA\% of the diet}$$

All the above might be summarized in one formula:

$$\frac{\text{Sample area}}{\text{STD area}} \times \frac{\text{NLE STD area}}{\text{NLE sample area}} \times \frac{\text{AA M.W.}}{\text{Sample weight (mg)}} =$$

AA% of the diet or the feather

APPENDIX 2

Inulin Determination

The concentration of inulin in plasma and urine samples was determined using the method of Waugh (1977). This method is based on measuring the absorbance of the pink color formed from the reaction of inulin in the plasma with cysteine/tryptophan solution in the presence of sulfuric acid.

Reagents Required

Zinc sulfate solution (14.3 g/l).

Dilute H_2SO_4 (concentrated H_2SO_4/H_2O ; 70/30).

Cysteine/tryptophane solution (17 g, 400 mg/l).

Sodium hydroxide solutions (0.5 M and 1.6 M).

Benzoic acid solution (20 mM, pH = 4.2).

Plasma Deproteinization

Into a centrifuge tube containing one volume of plasma sample (1 ml) and one volume of distilled water, 7 volumes of zinc sulfate solution (14.3 g/l) were added and mixed. One volume of NaOH (0.5 M) solution was then added and the whole mixture was shaken thoroughly. The tubes were allowed to stand for 5 minutes and then centrifuged at 3500 rpm for 10 minutes where the supernatant was retained for analysis.

Standard Solutions of Inulin

A stock solution of reagent-grade inulin (Fisher Co.) 1 g/l, was prepared by dissolving 100 mg of dried inulin

in benzoic acid solution (20 mM) and volume was brought up to 100 ml. Working standards of 7.5, 15, 30, 60, 120 and 240 mg/l were prepared by diluting the stock standard with the buffered benzoic acid solution.

Analytical Procedure

An aliquot of 0.5 ml of standard solutions, deproteinized plasma sample (1/10), a sample of urine diluted 1/20 or distilled water was pipetted into a test tube. A volume of 0.5 ml of NaOH (1.6 M) was added, tubes were covered by glass marble and immersed in a boiling water bath for 10 minutes. Tubes were then cooled and placed in a room temperature water bath where 7 ml of the dilute sulfuric acid (70/30) and 0.5 ml of the cysteine/tryptophane reagent were added. The tubes were capped with parafilm, the content was vigorously mixed and tubes were placed in a water bath at 56°C for 25 minutes. Tubes were then cooled in a room temperature water bath for 5 minutes and the absorbance was measured at 515 nm. The absorbance values for the different standard solutions along with their corresponding concentrations served to develop a regression line as follows:

$$Y = a x + b \quad \text{where}$$

$$Y = \text{absorbance}$$

$$a = \text{slope}$$

$$x = \text{concentration}$$

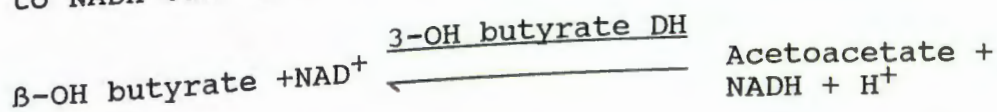
$$b = \text{intercept}$$

$$x = \frac{y - b}{a} \quad \text{x diluting factor was used to calculate the concentration of inulin in samples.}$$

APPENDIX 3

Determination of β -Hydroxybutyrate (Source, Hultman, 1974)

The enzyme 3-hydroxybutyrate dehydrogenase (3-OH butyrate DH) catalyzes the conversion of β -OH butyrate in the presence of NAD^+ to acetoacetate. The reduction of NAD^+ to NADH can be followed spectrophotometrically.

Reagents

Perchloric acid, 1 M.

Potassium carbonate, 3.6 M.

Glycine buffer, 0.4 M and pH = 9.5.

NAD, 37.5 mM (Sigma Chemical Co., St. Louis, MO).

3-OH butyrate DH (Sigma, Chemical Co., St. Louis, MO).

Preparation of Samples

One volume (1 ml) of plasma was added to an equal volume of ice cold perchloric acid (mixture was cooled and centrifuged at 3500 rpm for 5 minutes). An aliquot of 100 μl K_2CO_3 was added to 1 ml of the acid extract supernatant. This mixture was cooled in ice for 10 min and centrifuged at 3500 rpm for 2 minutes. Supernatant was retained and stored at $\leq -20^\circ\text{C}$ until analyzed.

Assay Procedure

A mixture of 4 volume glycine buffer and one volume NAD was prepared. An aliquot of 50 μ l of this mixture was added to 200 μ l of neutralized extract or distilled water (blank) into a 1 cm microcuvette. Cuvettes were placed in spectrophotometer and absorbance of samples (A_1) and blank (A_2) were read at 340 nm after 5 minutes. Then 5 μ l of the enzyme was added and absorbance of sample and blank was read after 20, 25 and 30 minutes. Reading at zero time was then extrapolated for both sample (A_3) and blank (A_4).

Calculation

Concentration in plasma (mg/ml):

$$\text{mg/ml} = \frac{(A_3 - A_1 - A_4 - A_2)}{\text{molar extinction coefficient}} \times \frac{\beta\text{-OH butyrate MW}}{\text{dilution factor}}$$

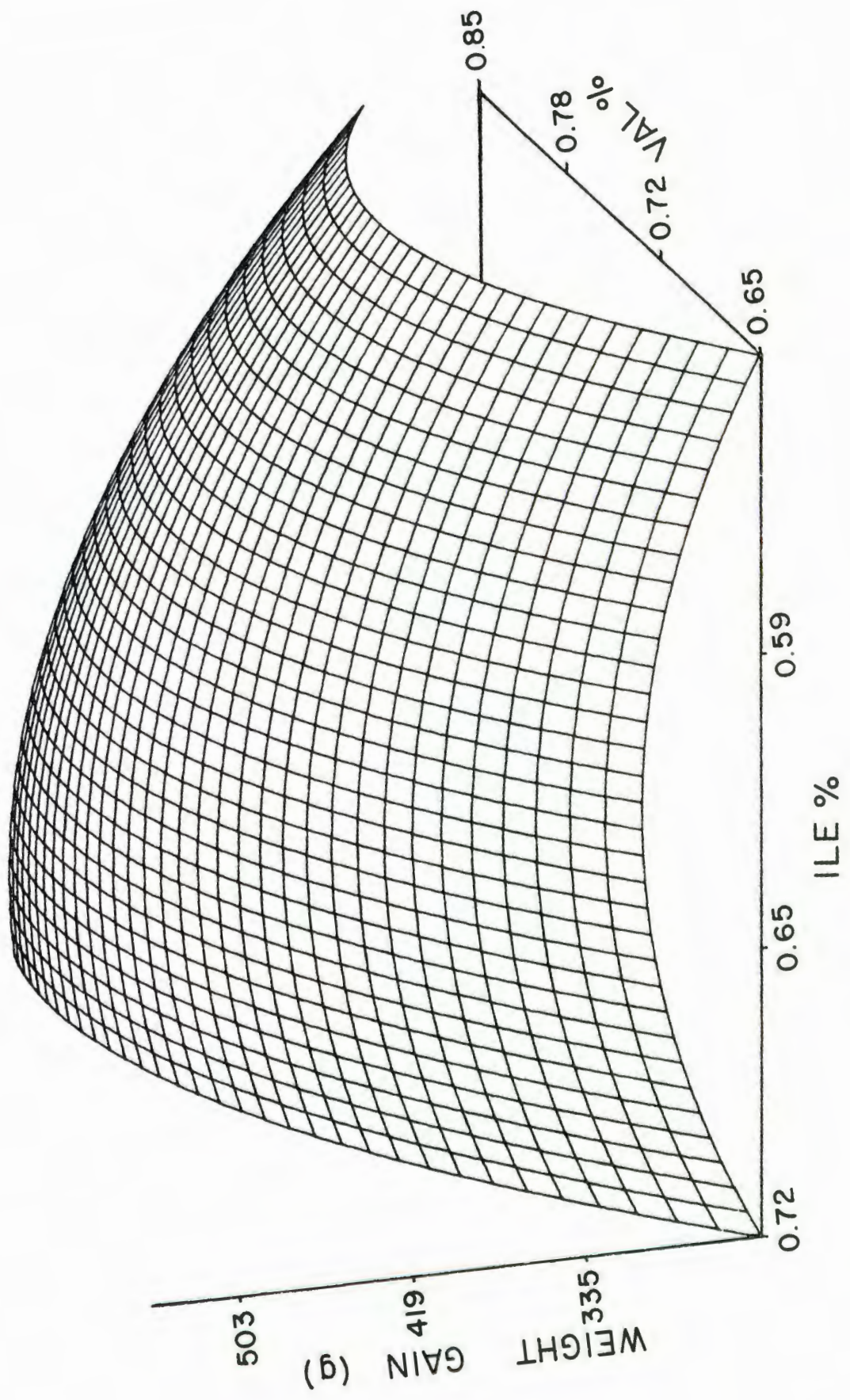
$$\frac{(A_3 - A_1 - A_4 - A_2)}{6.2 \times 10^3} \times 104 \times 2.805 \times \frac{1000 \text{ ml}}{1 \text{ l}} = \text{mg/l}$$

APPENDIX 4

Interaction of dietary isoleucine and valine and its effect on weight gain of chicks in the starter period. Leucine level was kept constant at 1.12%.

$$\sqrt{\text{MSE}} = 26.76$$

$$R^2 = 0.807$$

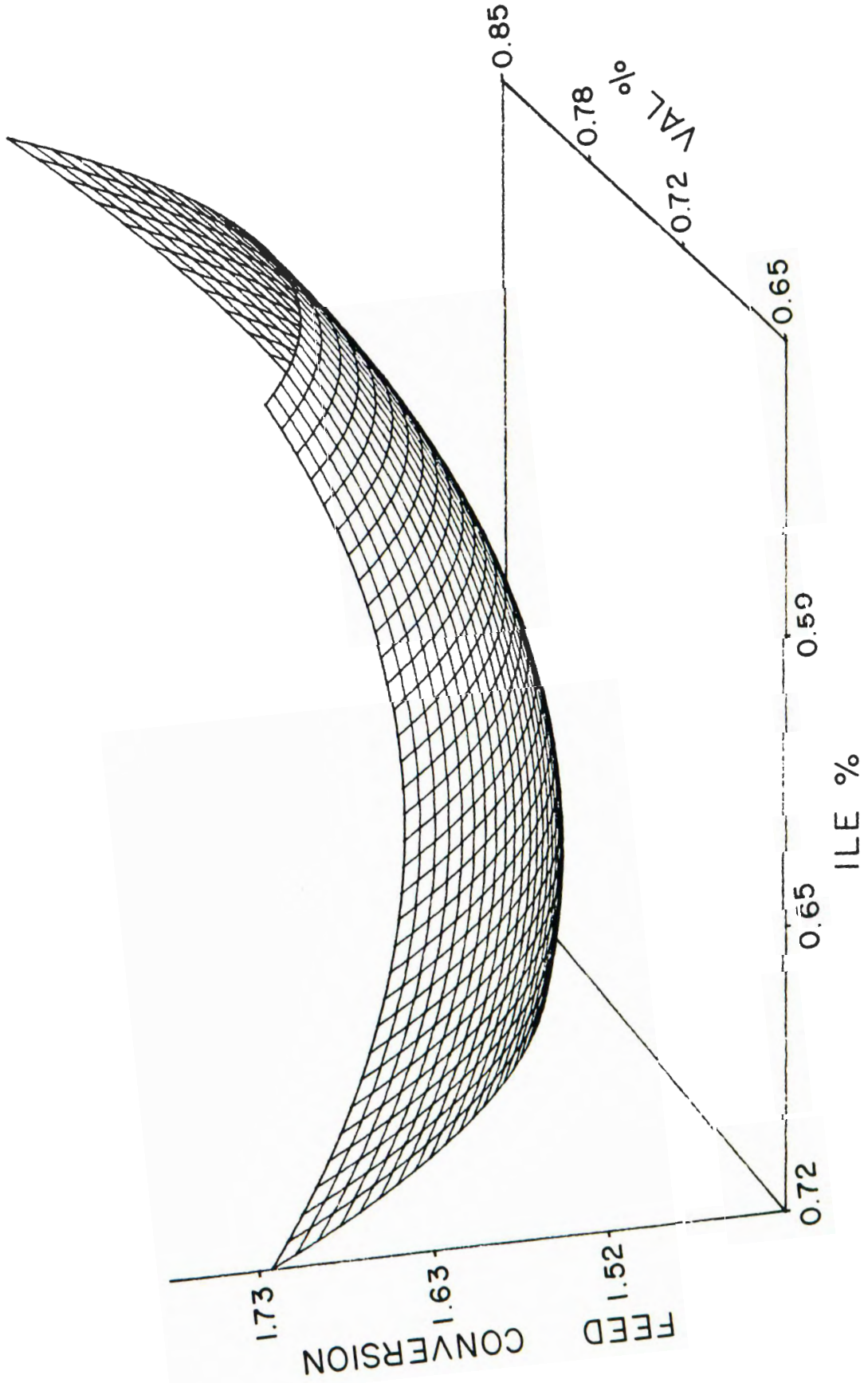


APPENDIX 5. Study 4.

Interaction of dietary isoleucine and valine and its effect on feed conversion of chicks in the starter period. Leucine level was kept constant at 1.08%.

$$\sqrt{\text{MSE}} = 0.0412$$

$$R^2 = 0.783$$

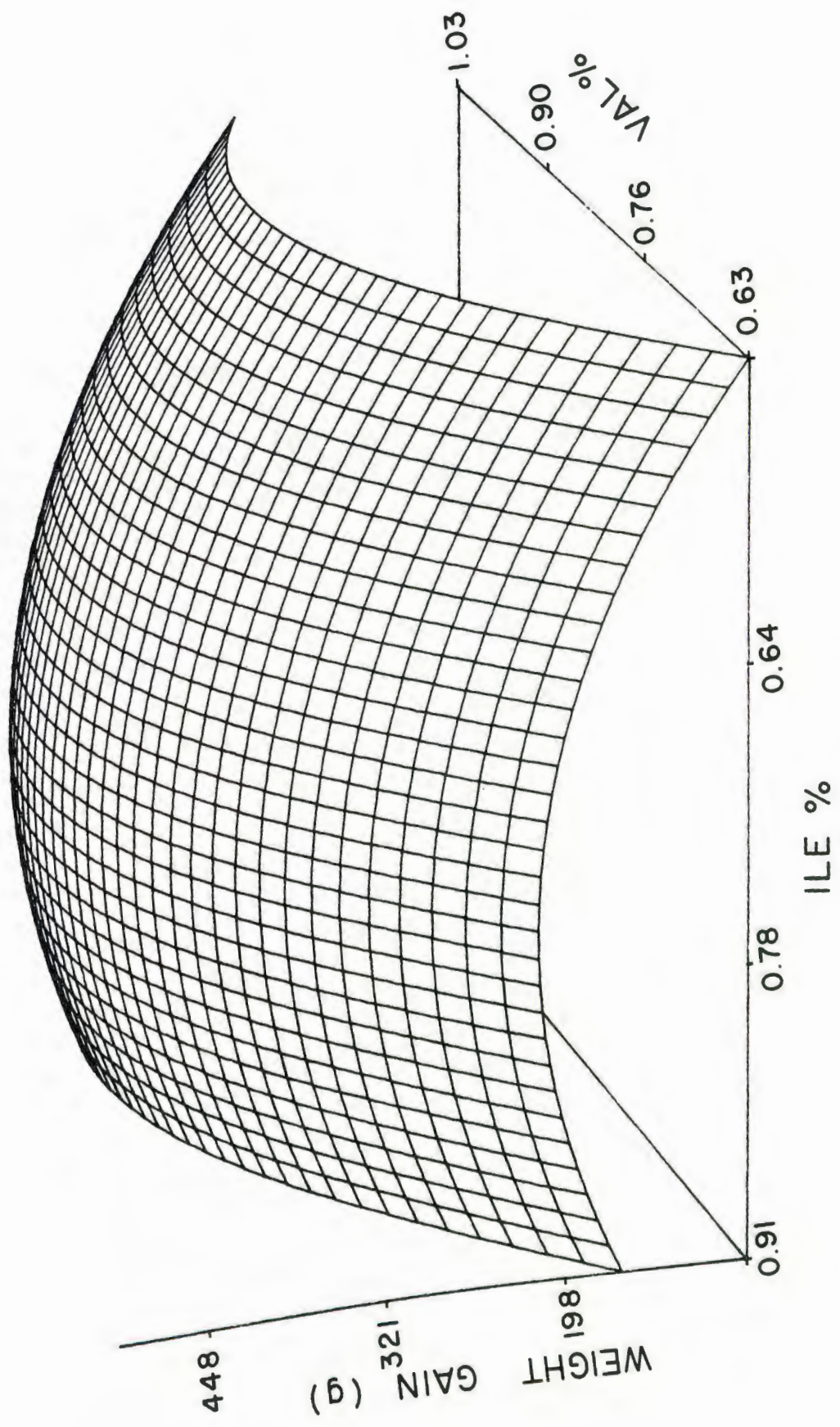


APPENDIX 6. Study 5.

Interaction of dietary isoleucine and valine and its effect on weight gain of chicks in the starter period. Leucine level was kept constant at 1.12%.

$$\sqrt{\text{MSE}} = 21.78$$

$$R^2 = 0.909$$

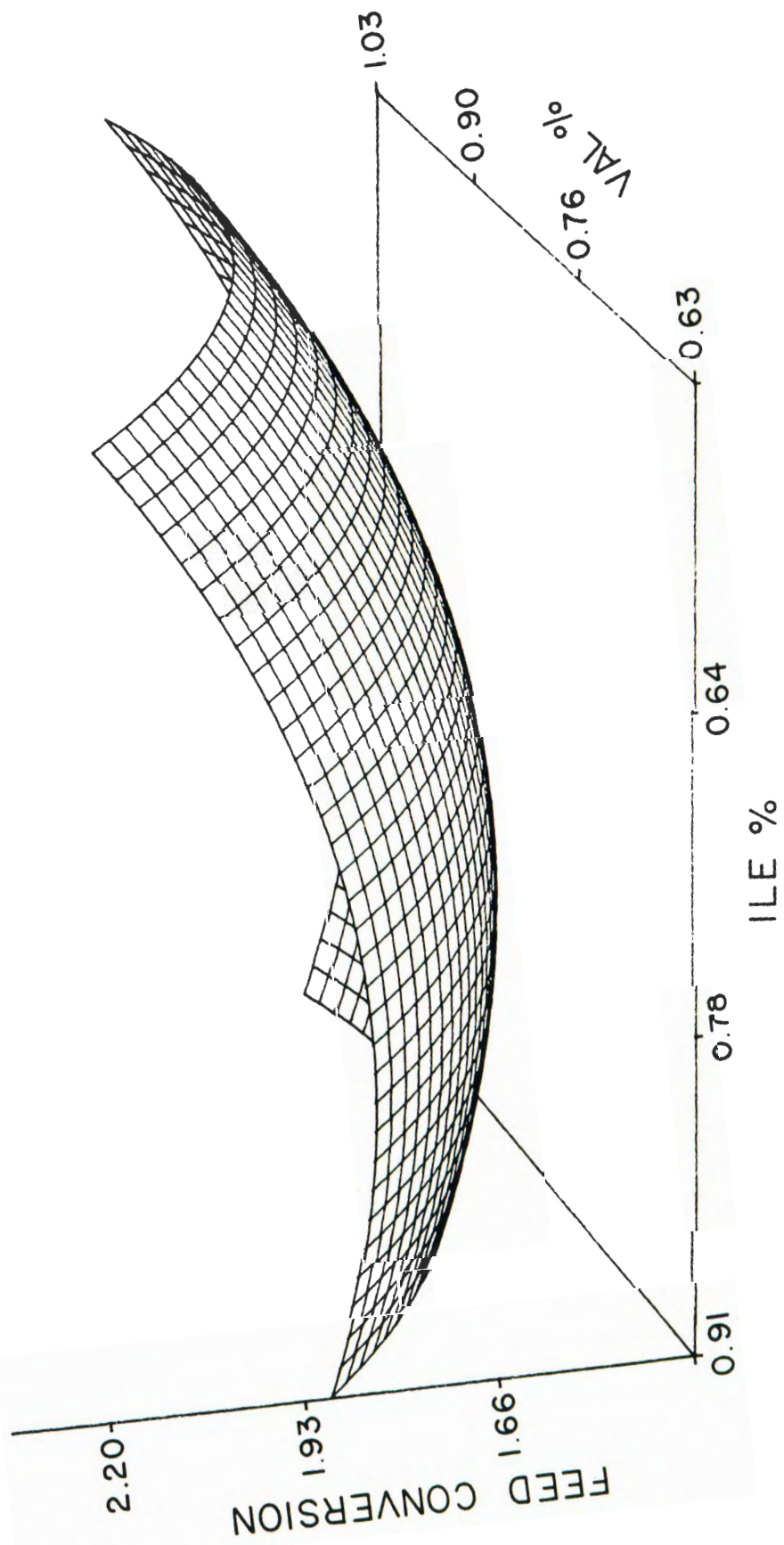


APPENDIX 7. Study 5.

Interaction of dietary isoleucine and valine and its effect on feed conversion of chicks in the starter period. Leucine level was kept constant at 1.16%.

$$\sqrt{\text{MSE}} = 0.0485$$

$$R^2 = 0.871$$

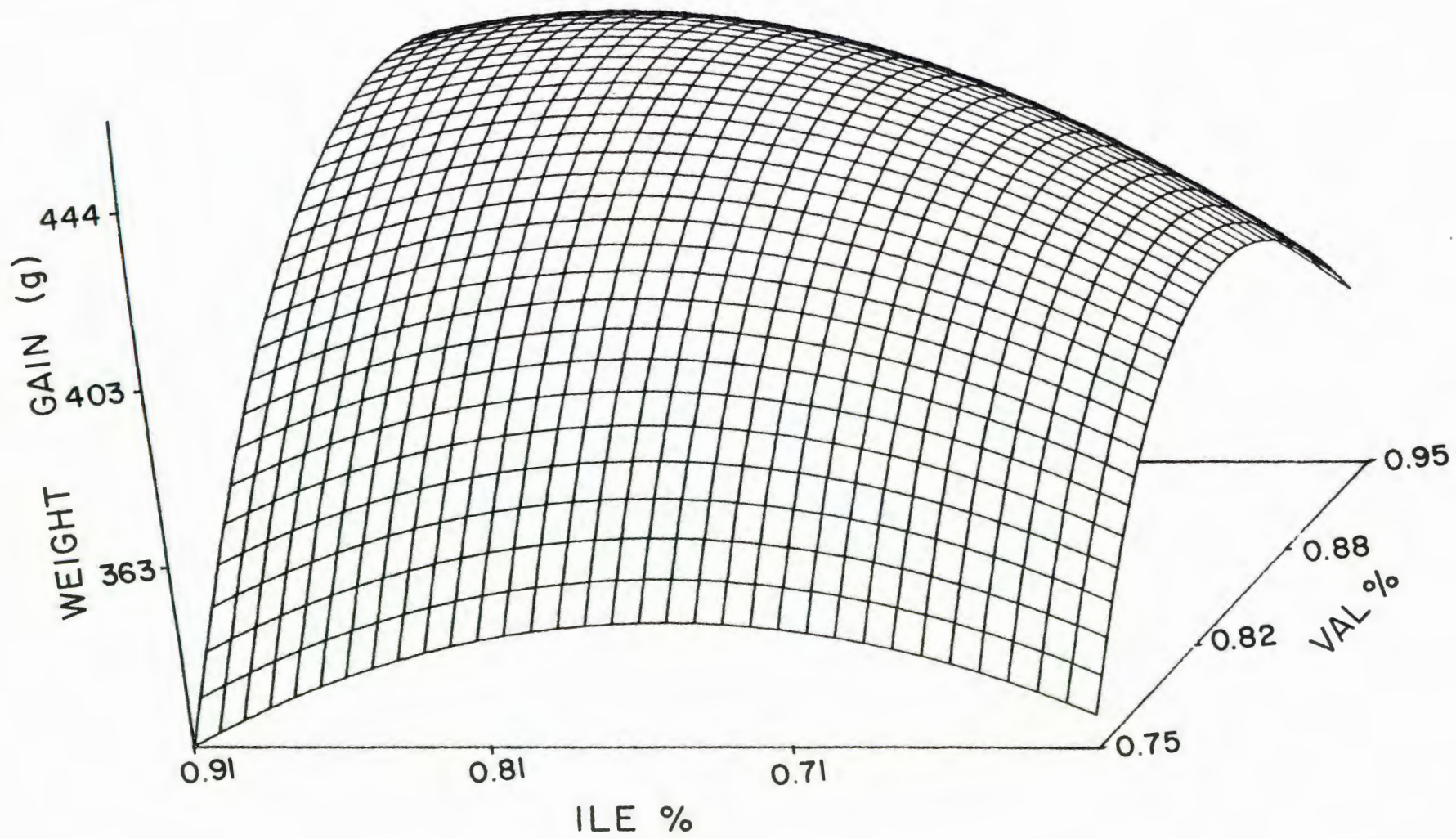


APPENDIX 8. Study 6.

Interaction of dietary isoleucine and valine and its effect on weight gain of chicks in the starter period. Leucine level was kept constant at 1.15%.

$$\sqrt{\text{MSE}} = 15.79$$

$$R^2 = 0.805$$

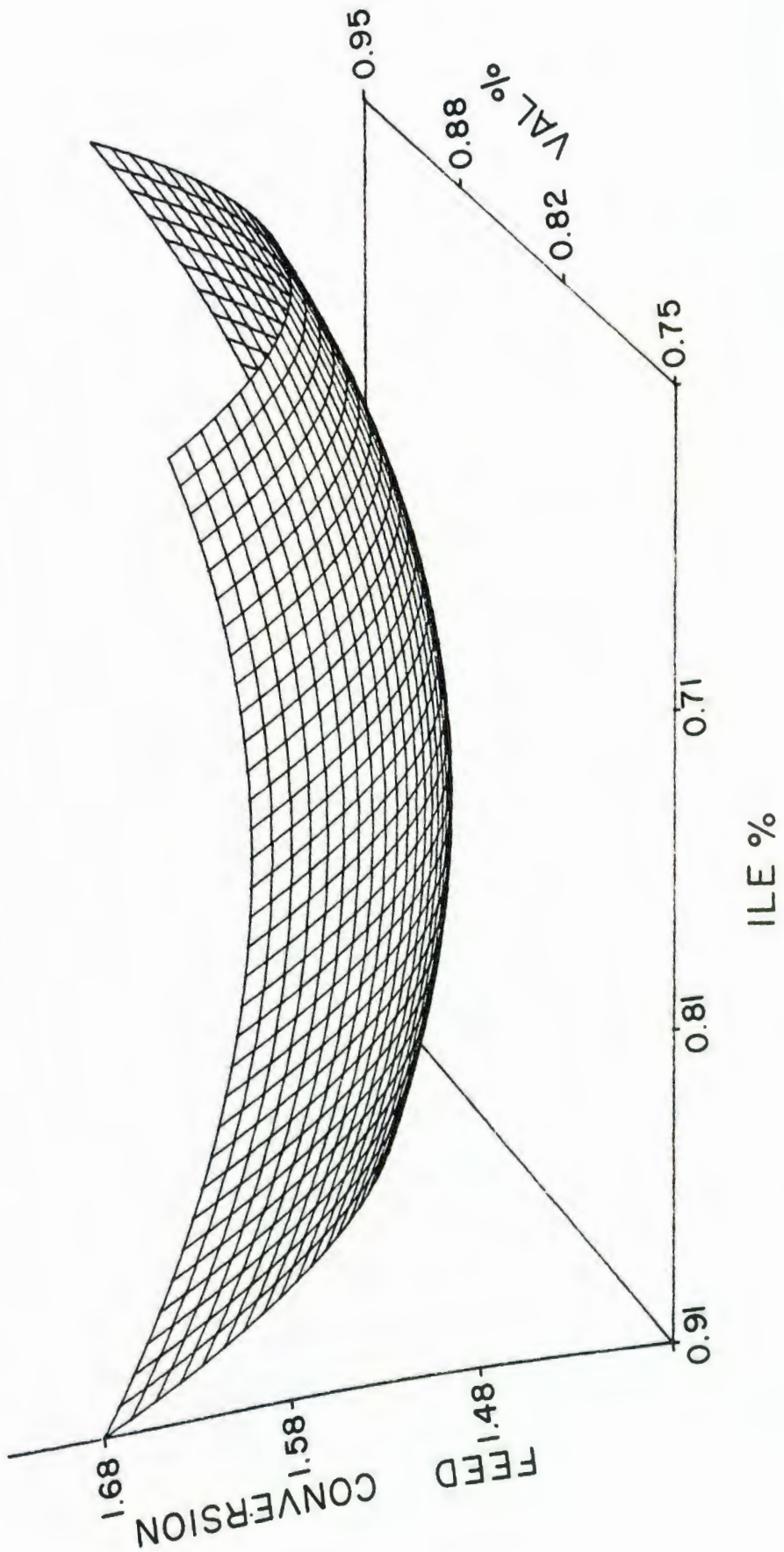


APPENDIX 9. Study 6.

Interaction of dietary isoleucine and valine and its effect on feed conversion of chicks in the starter period. Leucine level was kept constant at 1.16%.

$$\sqrt{\text{MSE}} = 0.0248$$

$$R^2 = 0.875$$



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