The effect of a dietary leucine excess on the immunoresponsiveness and growth of chickens

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The effect of a dietary leucine imbalance on chickens was investigated. The two criteria considered were growth and antibody production following immunization with sheep red blood cells. Three groups of chickens were fed diets containing increasing levels of leucine. Group 1 was fed a control diet containing 17,4 g leucine/kg, whereas Groups 2 and 3 were fed diets containing 25,6 and 37,8 g leucine/kg respectively. Five weeks after the dietary treatments had been introduced chickens were immunized, followed 4 weeks later by a further immunization. Antibody production following primary and secondary immunization was monitored at 3 – 4 day intervals. The average body mass of the chickens in each group was determined at weekly intervals. The dietary imbalance of leucine was shown to produce a marked depression in growth rate and a significant reduction in the level of serum IgM and IgG in birds in Group 1 and 2. The significance of these findings is discussed.

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Die invloed van 'n voedingsleusienoormaat op kuikens is ondersoek. Die twee maatstawwe wat oorweeg is na immunisering met skaaprooibloedselle (SRBS) was groei en teenliggaamproduksie. Drie groepe kuikens is met rantsoene gevoer wat verhoogde vlakke van leusien bevat het. Groep 1 is met 'n kontrolerantsoen met 17,4 g leusien/kg gevoer, terwyl Groepe 2 en 3 onderskeidelik rantsoene van 25,6 en 37,8 g leusien/kg ontvang het. Vyf weke na die aanvanklike rantsoentoediening is die kuikens geïmmuniseer, met 'n verdere immunisering na 4 weke. Die teenliggaamproduksie na die primêre en sekondêre immunisering is met gereelde tussenposes van 3 of 4 dae geëvalueer. Die gemiddelde liggaamsmassa van die kuikens in elke groep is weekliks bepaal. Van die resultate is dit duidelik dat die voedingswanbalans van leusien nie slegs 'n betekenisvolle afname in die vlak van serum-IgM en -IgG tot gevolg gehad het nie, maar ook 'n duidelike afname in groeitempo gehad het. Die belangrikheid van hierdie bevindinge word bespreek.

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Introduction

Previous research on rats has shown that excess dietary leucine induces a secondary depression in the plasma levels of free isoleucine and valine (Swendseid, Villalobos, Figueroa & Drenick, 1965; Tannous, Rogers & Harper, 1966; Clark, Yamada & Swendseid, 1968). It has also been reported that a lack of dietary isoleucine and particularly valine significantly reduces the production of lymphocytes (Aschkenasy, 1964; 1966; 1975). Because lymphoctyes play a crucial role in immunological responses, it is reasonable to expect that a deficiency of isoleucine and valine, whether due to a dietary deficiency or as a consequence of secondary induced depression by feeding excess dietary leucine, will have an adverse effect on the host's immune competence. This hypothesis was confirmed by Chevalier and Aschkenasy (1977) using rats as experimental animals.

Excess dietary leucine has been shown to depress the growth of rats fed a diet low in protein (Harper, Benton, Winje & Elvehjam, 1954; Harper, Benton & Elvehjam, 1955; Benton, Harper, Spivey & Elvehjam, 1956; Spolter & Harper, 1961; Rogers, Tannous & Harper, 1967). Similarly chickens have been shown to grow more slowly when a diet containing excessive dietary leucine is fed (D'Mello & Lewis, 1970). Because cereals such as maize contain nutritionally imbalanced concentrations of leucine relative to isoleucine and valine, the practice of feeding diets containing large proportions of such cereals could adversely affect the immune response and growth of chickens fed such diets.

The objective of this research was to examine the effect of a dietary imbalance of leucine, with respect to isoleucine and valine, on the immunoresponsiveness and growth of young chickens.

Materials and Methods

Chickens

Non-inbred, male White Plymouth Rock chickens aged 3 weeks were used. They were housed in groups of six or seven, in elevated wire cages (100 cm x 75 cm x 50 cm) with asbestos roofing. Drinking water and the experimental diets were provided *ad libitum*.

Diets

The chickens were divided into three groups of 13 birds. Group 1 was fed a control diet containing 17,4 g leucine/kg; Groups 2 and 3 were fed diets of 25,6 g and 37,8 g leucine/kg respectively. The composition of the diets is given in Table 1.

The concentration of metabolizable energy and of each

Table 1 Composition (g/kg) of the diets fed in the trial

Ingredients	Diet 1	Diet 2	Diet 3
Yellow maize meal	354,4	480,2	480,2
Sorghum	194,7	–	–
Sunflower oilcake meal	20,0	147,0	147,0
Maize gluten	10,5	140,3	140,3
Rice bran	–	39,4	39,4
Fish meal	121,2	–	–
Husks	47,5	12,1	–
Oil	47,5	47,9	47,9
Cottonseed oilcake meal	142,8	50,2	50,2
Brewers grain	–	42,7	42,7
Skimmed milk powder	47,5	–	–
L-Leucine	–	–	12,1
L-Lysine-HCl	2,3	–	–
D, L-Methionine	3,2	–	–
Salt	0,4	5,5	5,5
Limestone powder	7,4	17,3	17,3
Monocalcium phosphate	–	16,8	16,8
Vitamins and minerals	0,6	0,6	0,6
Calculated analysis			
Crude protein (gN x 6,25/kg)	220,9	220,7	221,9
Metabolizable energy (MJ/kg)	13,4	13,4	13,4
Leucine (g/kg)	17,4	25,6	37,8
Isoleucine (g/kg)	8,2	8,2	8,2
Valine (g/kg)	11,3	11,3	11,3
Lysine (g/kg)	13,7	13,7	13,7

essential nutrient other than leucine was uniform in the three diets. Only the leucine concentration varied between diets, this being achieved by formulating two diets differing in their ingredient composition (diets 1 and 2) and by replacing indigestible husks in Diet 2 with synthetic L-leucine in Diet 3.

Antigenic stimulation

Sheep red blood cells (SRBC) stored in Alsever's solution (provided by the Natal Institute of Immunology, Pinetown, Natal) were used as antigen. The SRBC were washed three times in 0,9% (w/v) NaCl and resuspended to a 10% suspension in saline. After the chickens had been fed their respective diets for 5 weeks, they were immunized by intravenous injection of 1 ml of 10% SRBC suspension (Day 0); 4 weeks later (Day 28) they were re-immunized in exactly the same way.

Bleeding

At intervals of 3 or 4 days following immunization, the chickens were bled from the wing vein. The blood was allowed to clot for 2 – 3h at room temperature, after which the clot was cut and the sample centrifuged (2500 rpm, 30 min, 4°C). Serum collected was assayed the following day.

Haemagglutinin assay

A microtiter procedure was employed for haemagglutinin titer determination. Serial dilutions of each heat-inactivated (56°C) serum sample were prepared in phosphate-buffered saline (0,01M total phosphate, pH 7,3, 0,85g NaCl/100 ml), and a 1% SRBC suspension was then added to give a final dilution of 0,5% SRBC. The microtiter plates were allowed to stand at room temperature for 2 h, and were then held at 4°C overnight. Titers were expressed as the $\log_2$ of the reciprocal of the highest dilution giving detectable agglutination at 24 h.

Purification of chicken immunoglobulins for radial immunodiffusion assay

Chicken IgM and IgG were purified from serum using a combination of successive Na_2SO_4 precipitations and recycling on 2,5 cm x 100 cm Sephadex G – 200 columns, as described by Benedict (1967).

Quantitation of immunoglobulins

The serum immunoglobulins IgM and IgG were quantitated by single radial immunodiffusion, according to the method of Fahey & McKelvey (1965) except that 0,05 M barbital buffer pH 8,6, containing 0,5% ($^{W}/_{V}$) sodium azide was used.

Antisera specific for chicken IgM and chicken IgG were obtained from Miles Laboratories, Cape Town. Maximum economy of the antisera was achieved by using a 1:50 dilution of rabbit anti-chicken IgG and a 1:60 dilution of goat anti-chicken IgM. Undiluted test serum samples were assayed for IgM whereas a 1:80 dilution of test serum was required for IgG assays to maintain the clarity of the precipitate rings.

The immunodifussion gel plates were stored at 4°C in a humid container, until the precipitate rings had reached their maximum size. The gel plates were then soaked in several changes of 0,9% (w/v) saline (for 48 h), rinsed with distilled water overnight, and stained with Amido Black. When the background blue had been removed by washing several times in 2% ($^{V}/_{V}$) acetic acid, the gels were dried at 37°C. The ring diameters were measured by means of an ocular micrometer.

A calibration curve for each immunoglobulin was obtained using purified chicken IgM and IgG and by reference to the appropriate calibration curve the concentrations (mg/ml) of IgM and IgG in each test sample were determined.

Results

Gain in body mass

The mean gain in body mass of chickens fed Diet 1 from 8 to 14 weeks of age was 47,6 g/bird/day compared with growth rates of 35,7 and 29,3 g/bird/day on Diets 2 and 3 respectively. Growth of birds fed Diets 1 and 2 slowed considerably during the 15th week of age (Figure 1). At the termination of the trial (15 weeks of age) the body mass of those birds fed Diets 2 and 3 were 19,0 and 26,2% lower than the body mass of birds fed Diet 1.

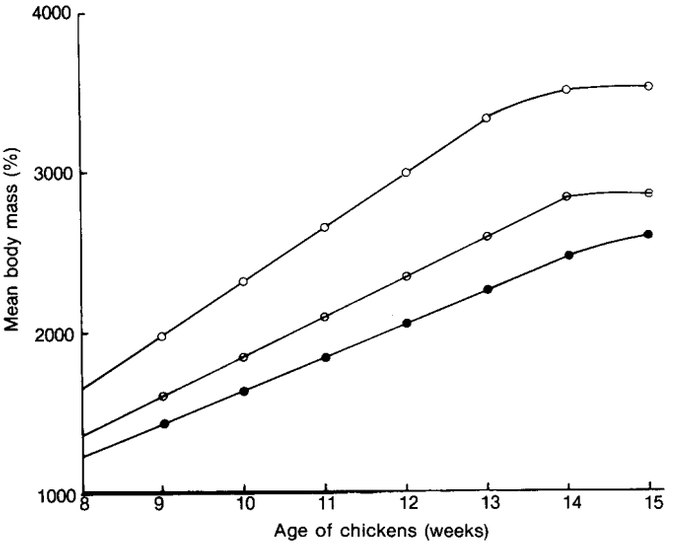

Figure 1 Mean bodymass of chickens from 8 to 15 weeks of age fed Diets 1 (O—O), 2 (⊖—⊖) and 3 (●—●)

Immunological studies

Radial immunodiffusion assay

The average concentrations of serum IgM for each of the three groups of chickens, during the periods of primary and secondary immune response are shown in Figure 2. Following primary and secondary antigenic stimulation each group showed a progressive rise in the serum IgM concentration reaching the peak primary response on Day 8 and the peak secondary response on Day 35 (i.e. 7 days after secondary immunization). A similar pattern of responses was evident in the case of serum IgG concentrations, the peak primary and secondary responses occurring 10 days after each antigenic stimulation (i.e. days 10 and 38 respectively).

The peak primary and secondary responses always occurred on the same day for each group (Figures 2 and 3); no lag phase for the onset of immune reactivity was observed for Groups 2 and 3. However, the magnitude of the antibody response was significantly depressed as the level of dietary leucine was increased, as is evident in Tables 2 and 3 in which the primary and secondary peak serum IgM and IgG responses are given. The values presented are means of eight readings per sample, there being 13 birds per dietary treatment from which blood samples were taken. Within-treatment mean squares were calculated from peak response data from which the statistical significance between treatment means was ascertained.

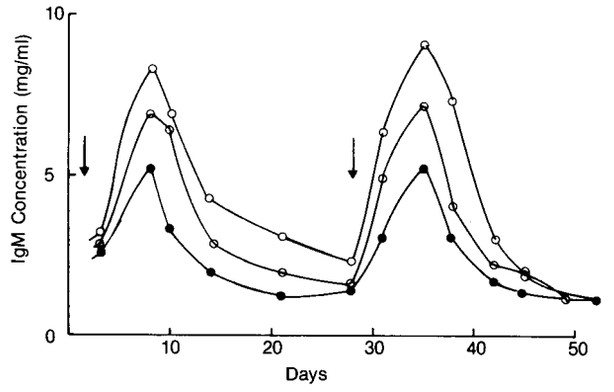

Figure 2 Primary and secondary serum IgM concentrations in chickens fed Diets 1 (O—O), 2 (O—O) and 3 (●—●). Chickens were injected with S R B C on days 0 and 28 as indicated (↓)

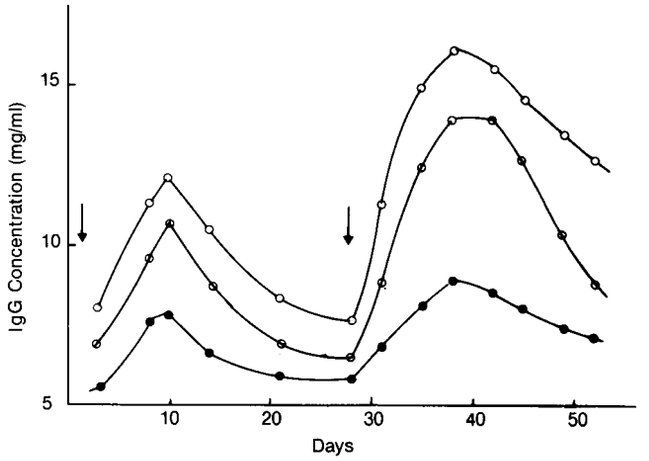

Figure 3 Primary and secondary serum IgG concentrations in chickens fed Diets 1 (O—O), 2 (⊙—⊙) and 3 (●—●). Chickens were injected with SRBC on days 0 and 28 as indicated (↓)

Table 2 Primary and secondary peak serum IgM responses

Dietary treatment	Primary response: Serum IgM mean (and SEM), mg/ml	Primary response: Per cent change	Secondary response: Serum IgG mean (and SEM), mg/ml	Secondary response: Per cent change
1	8,27[a] (1,05)		8,68[a] (1,08)	
2	7,52 (0,94)	−9,07	7,76 (0,96)	−10,60
3	6,34 (0,96)	−23,34	6,34 (0,95)	−26,96

[a]Mean values within response periods were each significantly different ($P < 0,01$) one from the other

Table 3 Primary and secondary peak serum IgG responses

Dietary treatment	Primary response: Serum IgM mean (and SEM), mg/ml	Primary response: Per cent change	Secondary response: Serum IgG mean (and SEM), mg/ml	Secondary response: Per cent change
1	16,35[a] (2,98)		19,80[b] (3,00)	
2	13,98 (2,98)	−14,50	17,72 (2,98)	−10,51
3	10,28 (3,00)	−37,13	11,98 (2,98)	−39,49

[a]Primary responses each differed significantly ($P < 0,01$) one from the other

[b]Secondary responses on Treatments 1 and 2 differed significantly ($P < 0,05$), the other differences being significant at $P < 0,01$

Haemagglutinin assay

The average serum haemagglutinin titers for the three groups during the periods of primary and secondary immune response are shown in Figure 4. The response was of a pattern similar to that obtained for the individual immunoglobulins, but the peak primary haemagglutinin response for each group occurred on day 8 and the peak secondary response on day 38 (i.e. 10 days after secondary immunization).

Primary and secondary peak serum haemagglutinin titers are given in Table 4. Statistical differences were calculated in the same manner as described for Tables 2 and 3.

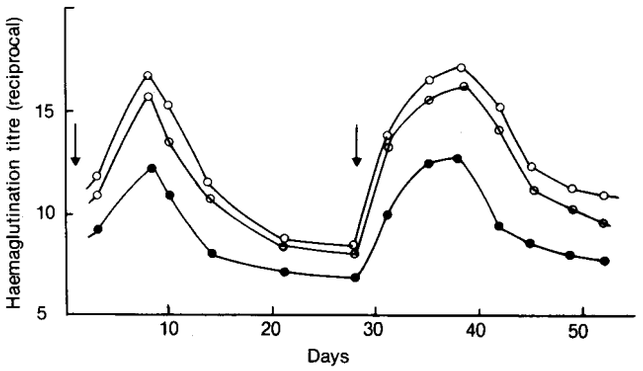

Figure 4 Primary and secondary haemagglutinin titers of chickens fed Diets 1 (O—O), 2 (⊙—⊙) and 3 (●—●). Chickens were injected with SRBC on days 0 and 28 as indicated (↓)

Table 4 Primary and secondary peak serum haemagglutinin titers

Dietary Treatment	Primary response: Haemagglutinin titer mean (and SEM)	Primary response: Per cent change	Secondary response: Haemagglutinin titer mean (and SEM)	Secondary response: Per cent change
1	10,692[a] (1,03)		10,857[a] (1,07)	
2	10,308[a] (0,48)	−3,59	10,500[a] (0,71)	−3,29
3	8,818[b] (0,60)	−17,53	9,111[b] (0,60)	−16,08

[a,b]Values with same superscript in each column do not differ significantly ($P < 0,01$)

Discussion

Growth depression induced by excess dietary leucine has previously been reported by D'Mello & Lewis (1970) using chickens, and by Harper, *et al.* (1954), Harper, *et al.* (1955), Benton, *et al.* (1956), Spolter & Harper (1961) and Rogers, *et al.* (1967) using rats. These authors also demonstrated that the growth depression could be partially overcome by the dietary inclusion of isoleucine or valine and completely overcome by dietary addition of isoleucine and valine given together. These observations imply that an excess of leucine inhibits the utilization of its two structurally related analogues, namely, isoleucine and valine.

Immunological studies conducted in the present research revealed that the kinetics of chicken IgM and IgG appearance in the serum, during the primary and secondary immune responses (Figures 2 and 3), followed a pattern similar to that characteristic of mammalian IgM and IgG (Hood, Weissman & Wood, 1978).

It is important to note that although the antibody peak responses occurred on the same day for each group of chickens, the magnitude of the response was significantly depressed by increasing the level of dietary leucine. This would indicate that the effect is not necessarily on T or B cell production but may reflect a reduction in the capacity to synthesize antibody from an inadequate amino acid pool. Aschkenasy (1979) reported that when rats were fed a low protein diet (4% casein) overloaded with L-leucine (70 g leucine per kg diet) there was not merely a depression in the serum IgG globulins (as observed in our experiment — Figure 3), but complete disappearance of these globulins. The discrepancy between these two results could be explained by the fact that higher leucine supplementation was used by Aschkenasy (7%) than was used in the present study.

Aschkenasy (1979) also emphasized that the immunosuppressive effect of excess dietary leucine was only observed when a low protein basal diet (4% casein) was used. No significant alterations in immune responsiveness (or growth) occurred with a leucine-overloaded, balanced diet (18% casein), or with a 4% casein diet supplemented with leucine.

Chevalier & Aschkenasy (1977) reported that rats need consume only a small amount of protein in order to maintain an almost normal immunological response, provided that the food consumed is balanced with respect to the essential amino acids (e.g. the branched chain amino acids — leucine, isoleucine and valine).

The above results clearly indicate that it is not so much a dietary excess of leucine, but the dietary imbalance of leucine with respect to isoleucine and valine that is deleterious.

The occurrence of interrelationships between leucine, isoleucine and valine have been established using rats (Harper, *et al.*, 1954; Benton, *et al.*, 1956; Bender, 1965; Rogers, *et al.*, 1967) and chickens (D'Mello & Lewis, 1970) as experimental animals. D'Mello & Lewis (1970) assessed the specificity of these interactions and reported that it is a combination of the specific interactions between leucine and isoleucine, and leucine and valine, that is responsible for the deleterious consequences of excess leucine. Of these interactions, that between leucine and valine was considered the most important because experiments revealed that valine was far more sensitive than isoleucine to a dietary excess of leucine, and also plasma amino acid showed that the level of valine in the plasma was controlled by the dietary balance of leucine and isoleucine.

A deficiency of isoleucine and particularly valine has been shown to be extremely harmful to lymphopoiesis (Aschkenasy, 1964; 1966; 1975). However, at present little is known of the precise mechanism of involvement of isoleucine and valine in the production of lymphocytes. It has been reported (Aschkenasy, 1964; 1966; 1975) that a deficiency of valine is deleterious to thymus and peripheral thymus-dependent lymphocytes. These lymphocytes (T cells) are responsible for determining the amount, class, and affinity of the antibody produced by B cells (derived from Bursa or equivalent in mammals) in response to antigens (Miller, 1975). In the present investigation we made use of SRBC immunization to measure T cell-dependent antibody synthesis, and therefore an impairment of the T cells (owing to the leucine-induced deficiency of valine) could explain the depressed immunological reactivity observed (for Groups 2 and 3) in our experiment.

It has been established that a metabolic antagonism exists between leucine, isoleucine and valine (Harper, 1964) but the exact mechanism of interaction has not been fully resolved. It is possible that antagonism occurs during absorption at the intestinal level. *In vivo* experiments performed by Szmelcman & Guggenheim (1966), using rats, revealed that intestinal absorption of isoleucine and valine was inhibited by excess leucine and also that absorption of leucine was inhibited by excess isoleucine and valine. The criteria used to measure the inhibited absorption were increased retention of amino acids in the lumen, and a delayed rise of their level in the portal plasma.

Rogers, Spolter & Harper, (1962) also reported a delayed absorption of isoleucine and valine from the intestine of rats fed excess leucine. However, *in vivo* experiments performed by Kamin & Handler (1952) revealed inhibited absorption of isoleucine by leucine, but not that of leucine by isoleucine. *In vitro* experiments carried out by Hagihira, Ogala, Takedatsu & Suda, (1960) and Szmelcman & Guggenheim (1966) provided further evidence for reciprocal inhibition of absorption between leucine and valine, and leucine and isoleucine at the intestinal level.

Antagonism at the intestinal level may severely affect protein synthesis because of the unbalanced supply of amino acids that would be available to tissues e.g. excess leucine compared with isoleucine and valine under conditions of a dietary leucine overload (Szmelcman & Guggenheim, 1966). However, Harper (1964) reported that 'the very great absorptive capacity of the small intestine argues against this being a major effect *in vivo*. Even though the absorption of isoleucine or valine may be delayed by an excess of leucine, as more leucine is absorbed, the ratio of leucine to isoleucine and valine will fall and competition for absorptive sites would diminish'.

It is also possible that antagonism may operate at the level of protein synthesis rather than during intestinal absorption.

Harper, Benevenga & Wohlhueter (1970) suggested that incorporation of an amino acid into a critical protein may be inhibited by the presence of a structural analogue.

It has been reported (Ichihara & Koyama, 1966; Wohlhueter & Harper, 1970; Buse, Jursinic & Reid, 1975) that an excess of leucine activates the enzymes involved in the catabolism of branched-chain amino acids, causing increased degradation of itself as well as that of its antagonists. A transaminase enzyme degrades all three amino acids equally resulting in an almost complete loss of isoleucine and valine, whereas the level of leucine remains relatively high. This enhanced degradation of isoleucine and valine and/or a reduced intestinal absorption in the presence of excess leucine, could therefore account for their deficiency *in vivo* and the related deleterious consequences.

In conclusion, chickens consuming diets overloaded with leucine displayed not only a strong impairment of immunological reactions to SRBC, but also a marked retardartion of growth. These observations may have commercial significance because of the existence of a specific imbalance between leucine, isoleucine and valine in cereals which usually comprise the bulk of chicken feeds. Our results imply that chickens fed diets containing excess leucine would be more susceptible to infection (because of an impaired ability to produce antibodies) and would also exhibit a lower growth rate, than would chickens fed diets containing a more balanced ratio of leucine to isoleucine and valine.

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