NICKEL AS A POTENTIAL NUTRIENT

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NICKEL AS A POTENTIAL NUTRIENT

By

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ABSTRACT

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By

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Ten experiments were conducted to investigate the possible involvement of nickel in the nutrition of the Japanese quail (Coturnix coturnix japonica). The experiments consisted of a generation study conducted in an attempt to deplete successive generations of quail of any possible body stores of nickel and, in turn, produce symptoms of a nickel deficiency. A second series of experiments, conducted simultaneously with the generation study, dealt with a postulated involvement of nickel in the activation of the enzyme, arginase, in which attempts were made to induce at least a partial in vivo activation of the enzyme by nickel. This was accomplished by altering the dietary levels of arginine and manganese in an effort to increase the demand for an active arginase while at the same time removing its normal in vivo activator, manganese. Appropriate nickel-supplemented control quail were also carried through each of the 10 experiments.

To prevent exposure of the quail to airborne nickel contaminants, they were housed in rigid plexiglass and/or balloon type plastic isolators, originally designed for gnotobiotic research, equipped with glass media air filters.

Feeders and waterers were made of plexiglass or plastic which contained no detectable nickel.

A highly purified diet was developed which supported normal growth, egg production, fertility, hatchability and chick weight. Casein was used as the protein source in the diet, and in the course of development of the diet, it was shown that arginine is the first limiting amino acid in casein for the Japanese quail as it is for the chick. Supplementation of a 35 percent casein diet with 1.13 percent of L-arginine hydrochloride alleviated the growth limiting effects of the casein diet and increased plasma arginine, indicating that the arginine requirement had been met. Dietary vitamin levels recommended for the chick proved to be inadequate for the quail. The high vitamin requirement was not due to the stress of being housed in isolators since quail housed under conventional conditions exhibited the same high vitamin requirements when fed the purified diet. Of course, this does not exclude the purified diet, itself, as a contributing factor.

In three of the experiments, the quail were raised to adults and used to produce eggs from which a succeeding generation was hatched. Two other experiments, involving fourth generation birds, were terminated before egg production began. Supplemental arginine was added to all of the diets used in these experiments, so the only dietary variable was the nickel concentration. The basal diet was

assayed to contain 74 ppb of nickel, and the nickel supplemented diet was assayed to contain a final concentration of 1780 ppb of nickel. The dietary nickel concentration had no significant effect on weight gain in four of the five experiments and no apparent effect on reproductive performance in any of the three trials in which egg production occurred. Weight gain was significantly increased by supplemental nickel in one experiment involving fourth generation quail. An attempt to repeat this performance in another group of fourth generation birds was unsuccessful.

At the conclusion of each experiment, the birds were killed by decapitation and blood collected for plasma amino acid analysis. The lungs, liver and pancreas were collected for tissue nickel assays, and the kidneys were collected for kidney arginase assays. Due to the very small size of the organs, an accurate quantitation of the tissue nickel concentration was not possible by the methods used. Increased arginase activity was not consistently associated with the higher dietary nickel concentration and no conclusions can be drawn as to the *in vivo* activation of arginase by nickel. Hematocrit, hemoglobin and total plasma protein determinations revealed no consistent correlation between the values obtained and the nickel concentration of the diet.

The other five trials were devoted to a study of the

possible role of nickel as an activator of arginase. Two levels of nickel and two levels of arginine were included in the diets in a 2 x 2 factorial design. The first level of supplemental arginine used, 0.88 percent of the diet, proved to be somewhat inadequate and the supplemental level was increased to 1.13 percent. Significant increases in weight gain were produced by supplemental arginine in all five experiments. There was some indication that the addition of nickel to an arginine deficient diet stimulated growth. Plasma amino acid analyses revealed a lower essential to non-essential amino acid ratio in the nickel supplemented, arginine deficient quail. Thus, nickel may increase the efficiency of utilization of arginine when arginine is relatively deficient. Dietary nickel concentration had no significant effect on growth of quail given diets adequate in their arginine content. The dietary concentration of nickel had no effect on arginase activity, but the addition of arginine to the diet stimulated arginase activity in diets otherwise adequate in all the other known nutrients.

With respect to the last statement, two experiments were conducted in which the protein content of the diet was decreased by 8 percent and further imbalanced by the addition of one percent of tyrosine. The addition of tyrosine had been reported to increase arginase activity, but no increase was

observed, possibly due to the low level added. Decreased weight gain occurred on all treatments, as compared with previous trials, and the decrease was attributed principally to an inadequate protein level. An attempt was made to lower the nickel concentration of the basal diet used in these two trials by washing of the calcium, magnesium and phosphorus sources with dimethylglyoxime. The nickel content of each of the minerals was considerably lowered but a net decrease of only 3 ppb, from 74 to 71 ppb, occurred in the mixed basal diet. A decreased dietary manganese concentration was also employed in an attempt to deprive arginase of its normal activating ion and, in turn, induce nickel to act as an *in vivo* activator. The nickel concentration of the supplemented diets was increased to 4660 ppb for these two trials. The results obtained from the arginase assays were variable and inconclusive. The dietary concentration of nickel employed had no apparent effect on hematocrit, hemoglobin or total plasma protein values.

The results obtained suggest that the nickel requirement of the Japanese quail fed an otherwise adequate diet is less than 74 ppb, if indeed there is a bona fide need for nickel in the diet.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF APPENDIX TABLES

I. INTRODUCTION

The relatively recent discoveries of the importance of the trace elements selenium (Schwarz and Foltz, 1957) and chromium (Schwarz and Mertz, 1959) in nutrition has accentuated the probability that essential metabolic functions for other minerals remain to be elucidated.

Analyses of human tissues have revealed that there are at least 15 trace elements consistently present for which no metabolic function is yet known (Tipton, 1960). Although some of the 15 may be accumulated with age as inert contaminants with no biological function, others very possibly are essential for life with their function awaiting discovery by some enterprising scientist.

A quantity of indirect evidence indicating that nickel may exert a biological function is accumulating. For example, nickel is consistently present in animal tissue including the newborn human (Schroeder et al., 1962); it activates various enzyme systems in vitro, including arginase (Hellerman and Perkins, 1935); and nickel is present in ribonucleic acids from various sources (Wacker and Vallee, 1959a; 1959b). Nickel is also reported to be greatly elevated in the serum of patients suffering from myocardial

infarction (D'Alonzo and Pell, 1963). However, a nickel deficiency in animals or man has not yet been reported.

For an element to be considered essential, Underwood (1962) has suggested that the following criteria must be met:

(1) Lack of the element results in a state of deficiency.

(2) Supplementation of the element results in a reversal of the deficiency which may be accompanied by a growth response.

(3) A correlation between the deficiency symptoms and subnormal levels in blood or tissues in some instances.

At present several trace elements are classified as toxic and have not yet been demonstrated to be essential. However, as was the case with selenium, the toxic properties of an element are often recognized prior to proof of its essentiality.

Based on the indirect evidence of a physiological function for nickel, research concerned with elucidating such a function was considered worthy of pursuit. The Japanese quail was chosen as the experimental animal, and attempts were made to produce a state of deficiency and to demonstrate a role for nickel as an <u>in vivo</u> enzyme activator.

II. REVIEW OF LITERATURE

Nickel

Nickel as a toxic element

Long before selenium was found to be an essential element in nutrition, the adverse effects of ingestion of excess selenium had been reported (Potter and Elvehjem, 1937). Although fluorine has not been definitely established as an essential nutrient, fluorosis was recognized as a problem before observations were reported that fluorine, at least under certain conditions, also exhibits beneficial effects (Cass, 1961; Davis, 1961).

So it must be with nickel. At present no biological function has been demonstrated for nickel. However, the toxic effects of excess nickel have been recognized at least since the reports of Laborde and Riche (1888) and of Lehmann (1908). These workers reported that oral ingestion of inorganic nickel salts over long periods of time by guinea pigs, rabbits, dogs and cats did not produce toxic effects, while subcutaneous or intravenous injections produced toxic manifestations.

More recently, Arnold (1939) found no evidence of nickel toxicity when young rats orally ingested amounts of

nickel pectinate varying from 98.4 to 1256.0 mg per kg of body weight over an 8 week experimental period. No detectable differences in weight gain between the nickel pectinate treated group and the controls were observed, and no pathological lesions were found at necropsy.

Nickel, at the level of 5 ppm in the drinking water, was found to have no effect on the growth and survival of mice up to 18 months of age (Schroeder et al., 1963). In a follow-up article, the same authors (Schroeder et al., 1964) reported on the effects of the continued ingestion of 5 ppm of nickel in the drinking water of the same mice until all had died. The total elapsed time was 36 months. They reported that nickel was not carcinogenic at the level used and, in fact, reduced the number of tumors observed in 473 nickel-supplemented mice as compared with controls. However, they reported a decreased survival rate beyond 18 months of age for the group given nickel and concluded that nickel seems to have an innate toxicity of an undefined nature, expressed by lessened male survival. No accumulation of nickel was observed in the tissues of the mice over their life time.

Using weanling mice, fed much higher levels of nickel as the acetate salt for a period of 4 weeks, Weber and Reid (1969) found that 1600 ppm of supplemental nickel in the diet significantly reduced growth rate of male mice; but no

such reduction was observed at a level of 1100 ppm. A significant growth reduction at both levels was noted in female mice. The amount of nickel consumed by the female mice was slightly less at the 1100 ppm level and about equal at the 1600 ppm level to the amount consumed by the males. These results suggest that growing female mice have less tolerance to high levels of nickel than do males. Nickel supplementation resulted in no marked changes in apparent digestibility of either energy, fat or protein and no apparent changes in bone metabolism as measured by bone calcium, phosphorus and citrate levels. Enzyme activity studies indicated that nickel exerts its influence in the kidney and liver where the element is known to accumulate, and, while nickel did not appear to affect the activity of any given enzyme, it did decrease the overall activity of both the Kreb's cycle and the electron transport system. The same authors also reported that the high levels of nickel had no effect on mature body weights or on conception rate. The average number of pups born was not significantly decreased with increasing dietary levels of nickel. However, the average number of pups weaned was significantly decreased in animals receiving 1600 ppm of dietary nickel as compared to those receiving 1100 ppm of dietary nickel or a basal diet containing no additional nickel.

Nickel toxicity in the growing chick was demonstrated by Weber and Reid (1968) by including from 0 to 1300 ppm of supplemental nickel as the acetate or sulfate salt. No differences in growth rates were reported in chicks fed the two forms of nickel. As the birds ingested increasing amounts of nickel, weight gain decreased as dietary nickel increased, and this effect of nickel was thought to be in addition to its effect in reducing feed consumption. However, when feed intake was equalized in a subsequent study by paired feeding, no significant effect of dietary nickel on the growth rate of chicks to 4 weeks of age was observed. The two highest levels of nickel supplementation decreased nitrogen retention in both studies.

Nickel toxicity has also been reported in the bovine (O'Dell et al., 1970). Supplemental nickel as the carbonate, at levels of 0, 62.5, 250 and 1000 ppm was given in the ration of male calves for a period of 8 weeks - from 12 to 20 weeks of age. Growth rate was not affected by the 62.5 ppm level while slight growth retardation occurred at the 250 ppm level. At the 1000 ppm level, growth was arrested and feed consumption markedly decreased. However, the animals which received this higher level did not appear starved nor were they lethargic. The general appearance was that of a younger animal. Histopathological examination of selected tissues revealed no abnormality associated with the feeding

of nickel. When supplemental nickel was removed from the diet, growth rates were comparable for those formerly given 0 and 1000 ppm nickel indicating the transitory nature of the growth retardation seen with high levels of nickel supplementation.

The toxic effects of excess nickel and cobalt were observed by Griffith et al. (1942) to be less severe as the protein content of the diet was increased from 10 to 25 percent and were less severe on the low protein diet if supplemental sulfur amino acids were also added. Cysteine was much more effective than cystine or methionine. The authors postulated the formation of a detoxification complex between the metals and sulfur containing compounds such as cysteine, glutathione and homocystine in the body. They postulated that the main effect of cobalt poisoning is the result of binding of sulfhydryl compounds in the tissues and interference with cellular oxidation due to the formation of such complexes, with glutathione for instance, may be the stimulus to the hematopoietic system which causes cobalt polycythemia. Presumably the same type of mechanism could be responsible for the adverse effects seen when high levels of nickel are ingested.

Nickel as a carcinogen

Closely related to the toxic effects of nickel is its

apparent ability to cause carcinoma of the respiratory tract. This is especially prevalent in miners who are in constant contact with air containing nickel and nickel compounds. Among workers in the nickel refineries of South Wales prior to 1924, the incidence of lung cancer was reported to be five times, and cancer of the nose to be 150 times, the normal rate (Doll, 1958). The causative factor was thought to be nickel carbonyl ($Ni(CO)_4$). Carcinoma of the respiratory passages was observed after approximately 25 years of exposure to nickel in nickel miners in England (Kincaid, 1956).

Nickel carbonyl has been implicated as a pulmonary carcinogen in tobacco smoke. In 1957, Sunderman et al. demonstrated that rats inhaling small amounts of nickel carbonyl, equivalent to 1930 mg of nickel, developed extensive squamous metaplasia of the bronchial epithelium. Six brands of cigarettes analyzed by Sunderman and Sunderman (1961) were found to contain an average of 1.99 µg of nickel per cigarette. They analyzed the smoke given off by a burning cigarette for carbon monoxide and found the smoke to contain five to seven percent carbon monoxide. Carbon monoxide and nickel or nickel containing compounds will readily unite at the temperature of a burning cigarette to form nickel carbonyl. Based on the nickel content of cigarettes and the amount of carbon monoxide formed in the burning of the tobacco, these workers estimated that a person smoking two to three packs

of cigarettes per day would inhale approximately 5400 μg of nickel in one year, assuming that all of the smoke was inhaled. This amount of nickel would be about three times the observed carcinogenic level for rats. Cigarette smoke contains about 140 ppb of nickel carbonyl while the maximum amount considered safe in air has been estimated by the American Conference of Governmental Industrial Hygienists (1957) to be one ppb.

In further studies on the carcinogenic effects of nickel carbonyl, Hackett and Sunderman (1967) parenterally administered nickel carbonyl. Quantitative doses could more easily be given by injection and this method was less hazardous to the researchers. They reported LD_{50} values for rats of 2.2 ± 0.1, 2.1 ± 0.4 and 1.3 ± 0.1 μg of nickel per 100 g of body weight for intravenous, subcutaneous and intraperitoneal injections, respectively, and that the pulmonary parenchyma appeared to be the target tissue of nickel carbonyl regardless of the route of administration. Increased mitotic activity of the alveolar lining cells was noted.

It has been suggested that the toxicity of nickel carbonyl does not indicate toxicity of nickel *per se*, as other metal carbonyls, including iron carbonyl, are toxic (Schroeder *et al.*, 1962).

In contrast to the increased mitotic activity observed by Hackett and Sunderman (1967), Swierenga and Basrur (1968)

have reported a marked decrease in the mitotic index of rat embryo muscle cells cultured on a medium with a nickel concentration of 1 µg of nickel per ml of culture medium. In addition to a decreased mitotic index, cultures of all ages when exposed to a nickel-containing medium exhibitied a large percentage of pyknotic nuclei, with a majority of such affected cells at some stage of mitosis. They concluded that the occurrence of a percentage of abnormal mitotic figures exhibiting multipolar spindles, altered polarity and unequal segregation of nuclear and cytoplasmic material suggests that the carcinogenic effect of nickel may be involved with the spindle mechanisms within the cell.

The exact mechanism by which nickel produces cancer has not been elucidated, but Weisburger et al. (1963) believes that the presence of large amounts of ions capable of being chelated leads to an imbalance in some crucial process.

Nickel in soil and plants

The presence of nickel in the earth's crust was discovered by Mokragnatz (1922). Since this discovery, numerous attempts have been made to determine the nickel concentration of the soil and of the plants grown thereon.

A nickel concentration of 3.9 ppm was found in soil from Kentucky by McHargue (1925) and a nickel concentration of 3.9 ppm was found in soybeans grown on the soil. A soil

concentration of 22 to 66 ppm of nickel has been reported by Hunter and Vergnano (1952) for soils in Scotland. An average concentration of 20 ppm of nickel was found by Painter et al. (1953) for soils of New Jersey. The same workers reported higher nickel concentrations in coastal soils indicating that the nickel apparently was carried downward by drainage waters, or inward from the sea by the winds. Thus, it is quite evident that the nickel content of the soil varies from point to point on the earth's surface.

The nickel content of plants is dependent upon the species of the plant, part of the plant, stage of maturity, nickel content of the soil and soil acidity (Mitchell, 1945). Plants normally have a higher nickel concentration than animals (Schroeder, 1956). Concentrations of nickel in various plant species as reported by Schroeder et al. (1962) include: oats, 2.6 ppm; corn, 0.70 ppm; rice, 0.70 ppm; raw potatoes, 0.56 ppm; cocoa, 5.00 ppm and tea, 7.60 ppm. The leaves of plants contain more nickel than the stems, the younger parts of the plant more than the older parts, and the grain contains more nickel than the straw (Hunter and Vergnano, 1952).

A decrease in the nickel content of grasses with advancing maturity has been reported by Kirchgessner (1965). He reported a decrease of the nickel concentration to only about 20 percent of the level present at the time the first

shoots appeared.

Numerous reports have appeared indicating that available, or exchangeable, nickel in the soil is favored by a low pH and that liming of the soil, with the resultant increase in pH, decreases the amount of exchangeable nickel (Hunter and Vergnano, 1952). There may be little correlation between soil levels of nickel and nickel levels of plants grown thereon if the pH is alkaline (Painter et al., 1953). The same authors indicate that a concentration of 40 to 60 ppm of nickel in certain plants is toxic to the plants. An inhibition of plant growth by high concentrations of available nickel in the soil was suggested by Mitchell (1945), and he indicated that the problem could be alleviated by liming of the soil.

Nickel in animal tissues

The relative lack of data available prior to 1960 on tissue concentration of nickel, prompted Herring et al. (1960) to conclude that not enough data were available to be able to state "normal" values with certainty. In fact, the failure of Herring et al. (1960) to find nickel in the plasma and erythrocytes from all subjects was regarded by them as evidence against the existence of a physiological function for this element.

If an element has a physiological function, it stands to reason that it should be present in the newborn. The first report of nickel in the newborn was made in 1938 by

Rusoff and Gaddum who detected the presence of nickel in the newborn rat. Since then, Leonov (1960) and Schroeder et al. (1962) have detected nickel in human fetuses and newborn.

The distribution of nickel in the various tissues of the animal body has received considerable attention. Using radioactive $^{63}NiCl_2$ dissolved in saline and injected intraperitoneally, Wase et al. (1954) studied the distribution of ^{63}Ni in the tissues of the mouse. They related that the maximum uptake of injected ^{63}Ni was achieved in two to twelve hours and that the kidney, lung and plasma contained high concentrations of ^{63}Ni, with brain and muscle containing the least. They also noted the rapid disappearance of ^{63}Ni from all tissues except the lungs and brain. No values were given as to the absolute concentration of ^{63}Ni in the tissues.

The relative absence of nickel in most animal products as compared to vegetable products was reported by Schroeder et al. (1962). Tissue values for nickel concentration in mice, after their entire life had been spent exposed to drinking water containing either 5 ppm nickel or no additional nickel, revealed that nickel has a predilection for spleen and heart, with little tendency to accumulate in other organs in amounts much greater than the controls (Schroeder et al., 1964). The tissue values obtained are shown in table 1.

TABLE I. ORGAN CONCENTRATIONS OF NICKEL IN MICE (μg/g Wet Wt.)

Organ	-Ni[a]	+Ni[b,c]
Kidney	0.50	0.99
Liver	0.49	0.77
Heart	0.30	0.90
Lung	0.41	0.86
Spleen	0.39	3.39

[a] Basal diet contained 0.40 ppm nickel.
[b,c] 5 ppm of nickel supplied in the water as nickelous acetate.

The predilection of nickel for the spleen and heart was also found in the rat where generally higher concentrations were found than in mice receiving identical concentrations of nickel in the diet and water supply (Schroeder et al., 1967).

The nickel concentration of the various tissues has been highly correlated with the blood volume perfusing the tissues at 0.25, 2, 6 and 16 hours following a single injection of ^{63}Ni (Smith and Hackley, 1968). The kidney was the only organ with significant amounts of ^{63}Ni 72 hours following an intravenous injection of either 2.5 or 5.0 μC of ^{63}Ni. All of the ^{63}Ni activity in the blood could be accounted for by the activity in the plasma. A direct count of the washed erythrocytes showed no detectable ^{63}Ni. This is in contrast to the data of Wase et al. (1954) who found an increasing percentage of the ^{63}Ni activity of the blood of mice in the erythrocytes with increasing post-injection time. However,

^{63}Ni did not accumulate in the blood cells as has been reported for zinc (Robertson and Burns, 1963), selenium (Burke et al., 1967) and chromium (Gray and Sterling, 1950).

Nickel activity was found in the erythrocytes of rat blood following an LD_{50} dose injection of radioactive nickel carbonyl into the saphenous vein or administration by inhalation of a LD_{50} dose (Sunderman and Selin, 1968). The LD_{50} dose amounted to 2.2 mg of nickel per 100 g of body weight for the injected dose and 0.20 mg nickel per liter of air when inhaled for a period of 15 minutes. No absolute values for nickel concentration of tissues were given but muscle plus fat contained 7.3, bone plus connective tissue 5.5, viscera plus blood 4.7 and brain plus spinal cord 0.2 percent of the injected dose after 24 hours. The remainder of the dose was excreted. The ^{63}Ni activity present in the erythrocytes one hour post-injection accounted for 48.1 percent of the activity of the whole blood. After six hours the activity of the erythrocytes accounted for only 8.4 percent, with the translocation of ^{63}Ni from the erythrocytes to the plasma correlated with the disappearance of nickel carbonyl from the blood. Measurements were made of the distribution of the injected dose in the serum six hours after injection, and 80.5 percent of the total serum ^{63}Ni was bound to serum protein. Of that bound to the serum proteins, 88.1 percent

was bound to albumin and the remainder (11.9 percent) was presumed to be bound to the globulins. The latter figure was believed to be in error due to the tailing of the albumin fraction in the electrophoretic separation.

In an earlier report, Sunderman (1964) reported that 72 percent of the nickel remaining in the lungs following inhalation of nickel carbonyl was bound to macromolecules and could not be separated by dialysis.

Other reports concerned principally with the concentration of nickel in the blood have appeared. A range of 5.3 to 6.2 µg of nickel per 100 ml of human blood was reported by Butt et al. (1962), and an average value of 3.0 µg of nickel per 100 ml of human blood was given by Imbus et al. (1963). A positive correlation between increased serum nickel concentration and the occurrence of myocardial infarction has been observed (D'Alonzo and Pell, 1963). They suggest that high serum nickel levels are associated with infarction but not with other manifestations of coronary heart disease, but they could not determine if the high levels of serum nickel were a consequence of infarction or whether they were involved in the processes that led to infarction. If the latter is found to be true, they suggest that increased serum nickel can be used as a diagnostic indicator of acute myocardial infarction, and if nickel is

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the cause, treatment with a chelating agent to reduce serum levels micht be feasible.

The fulfillment of any potential requirement for nickel by the newborn mammal would depend upon a body reserve already present at birth or upon the nickel content of milk. Cow's milk was found to be free of nickel, even when 145 mg of elemental nickel were fed per day for two months (Archibald, 1949), and Archibald concluded that any nickel found in milk would be due to contamination from equipment used in handling the milk. Recent workers have shown that nickel is present in detectable amounts in milk from humans and cows (Leonov, 1958; Marano and Rainone, 1959; Tokovoi and Lapshina, 1962; Stoobun et al., 1962). The amount of nickel found was highest in the colostrum and gradually decreased as lactation progressed; however, no concentrations were given.

Excretion of nickel

In one of the earliest reports on nickel excretion, Flinn and Imouge (1928) determined that 98.5 to 99 percent of the nickel consumed in foods was excreted in the feces. Caujolle (1937) found significant amounts of nickel in the bile of dogs following an injection of nickel chloride. Fecal nickel excretion was found to be maximal in the first eight hours and urinary excretion maximal during the first four hours after an intraperitoneal injection of 1 μC of $^{63}NiCl_2$,

dissolved in saline (Wase et al., 1954). Total fecal excretion greatly exceeded total urinary excretion. Excretion studies on growing pigs indicated that 94 to 96 percent of an oral dose of nickel was excreted in the feces and 4 to 6 percent in the urine (Kirchgessner, 1965). Following an intravenous injection of $^{63}NiCl_2$, over 60 percent of the injected dose was excreted in the urine within 72 hours and less than 6 percent excreted in the feces during the same period (Smith and Hackley, 1968). Urinary excretion peaked two hours and fecal excretion eight hours following the injection.

The excretion of ^{63}Ni following an intravenous injection of $^{63}Ni(CO)_4$ was studied by Sunderman and Selin (1968). Four days after the injection, urinary excretion amounted to 31.2 percent and fecal excretion 2.4 percent of the injected dose. Biliary excretion during the first six hours after injection averaged only 0.2 percent; no further measurements were made on the biliary route. The major excretory route for injected $^{63}Ni(CO)_4$ was found to be through the lungs. During the first six hours after the injection, 38.4 percent of the ^{63}Ni was exhaled. No ^{63}Ni was detected in the breath after six hours. Nickel carbonyl was determined to be the exhaled form. The same workers recovered 81.4 percent of an injected dose of $^{63}NiCl_2$ in the urine and 3 percent in the feces within four days following the injection.

From the results reported, it is apparent that the route of nickel excretion is dependent upon the route of administration and, to some extent, upon the form of nickel administered. The majority of orally administered nickel appears in the feces as it is mostly unabsorbed; the major portion of an injected dose appears in the urine. When nickel is administered as the carbonyl, the lungs can be expected to play a major role in the excretion of nickel. The large percentage of an oral dose that is excreted in the feces and the much higher oral intakes required to produce a nickel toxicity imply that there is a mechanism limiting intestinal absorption as postulated by Schroeder (1962).

Evidence of a physiological role for nickel

Nickel has been implicated in a number of physiological roles throughout the years. Some of the postulated roles have been definitely disproven and some have not been studied adequately enough for any definite conclusions.

One of the first reports of a possible role for nickel in the animal body was published by Bertrand (1926). In his studies he found appreciable amounts of nickel in insulin and suggested that a proinsulin may be converted to insulin by nickel.

In the 1930's, several reports appeared which attempted to implicate nickel as an element necessary for the prevention of anemia. Nickel was reported to have a marked

erythropoietic action when fed in daily doses of 0.05 mg to rats receiving diets adequate in iron content (Myers and Beard, 1931). Copper was found to be more effective, but Myers and Beard concluded that copper was not specific as a supplement to iron for the prevention and cure of nutritional anemia. Reports by Keil and Nelson (1931) and by Underhill et al. (1931) established that there was a specific need for copper in the prevention of anemia and that this need could not be met by nickel.

Nickel has been reported to preserve the activity of the accelerator-globulin involved in the blood clotting mechanism (Leikin and Bessman, 1956), and Herring et al. (1960) reported that nickel may affect the clotting mechanism by stabilizing the labile factor.

One of the most potentially significant roles for nickel arises from the work of Wacker and Vallee (1959a, 1959b) who determined that nickel, along with several other elements, was found in all samples of ribonucleic acid (RNA) analyzed. A constant ratio of the sum of all the metals which were found in RNA to the number of phosphate groups of the RNA's from the various sources was found to be 2.2×10^{-2} gram atoms of total metals per mole of RNA phosphorus. Therefore, not all phosphate groups are saturated. The ratio was found to be somewhat higher in rabbit reticulocyte RNA and somewhat

lower in calf thymus RNA. Deoxyribonucleic acid (DNA) from the same sources contained lesser amounts of nickel.

The nickel content of RNA isolated from several sources is given in table 2.

TABLE 2. OCCURRENCE OF NICKEL IN RNA ISOLATED FROM VARIOUS SOURCES

Source	Nickel (μg/g RNA)
Calf pancreas	130
(sRNA) calf pancreas[a]	18
Calf thymus	74
Horse kidney	44
Rabbit reticulocyte	51
Euglena gracilis	60
Rat liver	64

[a]Soluble RNA isolated from supernatant of pancreas.

The metals found in RNA were firmly bound, as dialysis against metal-free water did not remove the metals. Some of the metals were partially or completely removed by dialysis against various chelating agents. The authors concluded that metals may play a role in the maintenance of configuration of the RNA molecule, perhaps linking purine or pyrimidine bases, or both, through covalent bonds possibly involving nitrogen atoms or π electrons of the bases. As they play a role in RNA, metals may bear a functional relationship to protein

synthesis and the transmission of genetic information. In an attempt to determine the effect of nickel on the incorporation of amino acids into protein in the lens of the eye, Devi and Banerjie (1965) found protein synthesis to be completely inhibited by nickel. Concentrations of 48 μg of nickel per gram of rat lung RNA and 29 μg of nickel per gram of rat liver RNA have been reported by Sunderman (1965). Both of these values are well within the range of values reported by Wacker and Vallee (1959b). The binding of ^{63}Ni to RNA in rat liver and lung following an injection of $^{63}Ni(CO)_4$ has been observed (Sunderman and Selin, 1968).

A more recent study conducted by Smith and Hackley (1968) revealed no significant correlation between the RNA content of selected tissues and the distribution of ^{63}Ni in these tissues. This does not conflict with the work of Wacker and Vallee (1959a, 1959b) as they gave no definite ratio between nickel and RNA, but rather a definite and constant ratio between the sum of all the metals present and RNA.

Nickel has been shown to strongly inhibit ribonuclease (Liu and Wang, 1964) and Kaindl and Altmann (1964) have suggested that at least one of the possible functions of trace elements in RNA is to stabilize it against the action of ribonuclease. Nickel carbonyl has been found to be an inhibitor of DNA dependent RNA polymerase activity in hepatic nuclei following an intravenous injection of 2.2 mg

of nickel (as the carbonyl) per 100 g of body weight (Sunderman and Esfahani, 1968). The inhibition of the incorporation of ^{14}C-orotic acid into RNA of rat liver following an injection of nickel carbonyl was reported by Beach and Sunderman (1969). A possible role for nickel in maintaining the conformation of ribosomes has been reported by Tal (1969). The implication of such a finding with respect to protein synthesis is quite evident.

One of the first reports of what was thought to be a response to nickel supplementation in an intact animal appeared in 1937 (Dixon, 1937). He found that dietary nickel (0.16 mg per week) plus dietary cobalt supplementation to sheep gave a greater growth response than cobalt alone (0.8 mg cobalt per week). The nickel source was stated to be cobalt-free so that the response supposedly was not due to additional cobalt supplied by the nickel supplement. Cobalt-free nickel is difficult to prepare, and quite possibly the nickel source was contaminated with cobalt.

Lo (1945) concluded that nickel has a physiological role in promoting both carotene and citrin formation in plant metabolism.

The first report indicating that nickel might be of importance in increasing crop yield was published by Roach and Barclay (1946) who found an increase in yield of wheat, potatoes and broad beans which had been sprayed with a

nickel solution. The nickel concentration of the solution was not given.

One of the most interesting roles postulated for nickel is its possible involvement in the pigmentation process. The *in vitro* ability of nickel to replace copper in the enzyme polyphenol oxidase (tyrosinase) has been demonstrated (Kertesz, 1951). On the basis of the strong affinities of melanin and its precursors for metals, Kikkawa *et al.* (1955) proposed that color depends upon specific genes which control the metals found in melanin. They reported that black rabbit hair contained cobalt, copper and nickel; yellow hair contained titanium, molybdenum and nickel; and white hair contained only nickel. Furthermore, the addition of titanium to melanin precursors produced yellow pigment; the addition of molybdenum produced red; and the addition of nickel produced white pigment. Similar relationships were also observed in analyses of fish skin, bird feathers, moth wings, guinea pig hair and human hair.

This possible relationship between nickel and hair color has been carried a step further by Abe (1956) who reported that the absorption of metals in various organs was found to depend on the hair colors of the mice used. He reported that more cobalt was taken into the bodies of black mice than of white mice, and that more nickel was absorbed by the white mouse. Future work with this possible role for nickel

should prove quite interesting.

The possibility that nickel, in addition to iodine, is necessary for the prevention of endemic goiter has been reported by Lyaonau (1960). Nickel, together with copper, cobalt, iron and manganese, is believed to contribute to the assimilation of iodine (Savchenko, 1964).

A possible involvement of nickel as a causative factor of bloat in ruminants has been reported by Harris and Sebba (1965). In their studies on the in vitro foam-forming tendencies of lucerne, they observed a greater stability of the foam when nickel was added. The foam-forming tendencies of aged lucerne solutions decreased, and Harris and Sebba concluded that the nickel in fresh lucerne appeared to be available for attachment to the protein while in aged lucerne, although nickel was still present, it was no longer in an available form. The significance of nickel, or other metals, in the cause of bloat in vivo has not been determined.

Nickel has been postulated to interfere with the active transport of specific sugars into yeast cells (Rothstein and Steveninck, 1966). They believe the transport to occur by the interaction of the sugar with the phosphoryl sites of the membrane carrier. This complex (carrier-phosphoryl sugar) is then translocated to the site of the glycolytic system where the phosphoryl group is transferred to the sugar

phosphate, which proceeds through the glycolytic scheme ultimately to carbon dioxide and alcohol. The carrier is released to the outer surface and the phosphoryl sites are regenerated presumably by the adenosine triphosphate (ATP) produced in the glycolytic chain. The nickelous ion is believed to interfere with the active transport by combining with the phosphoryl sites and thereby reducing or virtually eliminating the transfer of phosphate to the carrier. No phosphoryl sites can be regenerated, and the phosphoryl sites already present are used to transport glucose and to phosphorylate glucose. Once all of the phosphoryl sites have been used in the transport process, the active transport must stop. Hence, the presence of nickel ultimately results in the stoppage of active transport of glucose by the yeast cell.

Nickel, at the level of 5 ppm in the drinking water for rats, has been reported to affect the serum cholesterol level (Schroeder, 1968). Nickel nonsignificantly decreased serum cholesterol levels in males and significantly decreased serum cholesterol levels in female rats. Chromium and niobium had similar effects to those of nickel while increased serum cholesterol levels were associated with the 10 other metals tested.

Nickel has been shown to activate a number of enzymes, to have no effect on some and to inhibit others when added to <u>in vitro</u> enzyme systems. Since most of the biologically

active metals usually exert their effects through enzyme systems (Herring et al., 1960), any study concerned with establishing a physiological role for a metal should at least consider the role of that element as a potential enzyme activator. In this respect, Hellerman and Perkins (1935) determined that nickel, cobalt, manganese and iron (ferrous) could act as in vitro activators for the urea cycle enzyme, arginase. They observed that an arginase preparation would deteriorate with age if unactivated, but would remain active for months if activated with metal ions, and they suggested that a coordination complex between the metal and enzyme may be responsible for the metal ion activation of arginase.

Nickel has also been shown to activate trypsin (Sugai, 1944), oxalacetic carboxylase (Speck, 1949), enolase (Wold and Ballou, 1957), carboxypeptidase (Coleman and Vallee, 1960), wheat root phosphodiesterase (Ibuki et al., 1964) and acetyl coenzyme A synthetase (Webster, 1965). The latter enzyme appeared to have a double requirement for activating, divalent cations. A specific block in the alcohol dehydrogenase reaction leading to a partial inhibition of fermentation was observed in yeast cells exposed to a nickel containing medium (Fuhrmann and Rothstein, 1968). As pointed out earlier, nickel was shown by Liu and Wang (1964) to inhibit ribonuclease.

The possibility that nickel may be a biologically important metal has attracted the attention of Smith (1968) and of Nielsen (1970). The former worker fed a purified diet consisting of dried skim milk, sucrose, corn oil and supplemental minerals and vitamins to growing rats housed in isolators, for a period of 55 days. The nickel content of the diet was assayed to be 0.08 ppm. No significant difference in growth rates were observed before or after intraperitoneal injections of 70 µg of nickel per day. Since environmental contamination could be virtually ruled out, Smith concluded that, if nickel is required by the rat, the dietary requirement is less than 80 ppb.

The work reported by Nielson (1970) involved the feeding of purified diets to growing chickens. The chicks were maintained in an all-plastic, controlled environment and fed a diet containing 79 ppb nickel in 2 trials and a diet containing less than 40 ppb nickel in a third trial. Control chicks received the same diets supplemented with 3 to 5 ppm of nickel. In all trials, the chicks receiving the unsupplemented diets developed a slight thickening of the long leg bone and a slightly enlarged back, and seemed to walk with an abnormal gait. The length to width ratio of the tibias from the unsupplemented group was significantly decreased, and the leg color was bright orange-yellow instead

of pale yellow-brown. All of the observed changes were reported to be prevented by dietary nickel supplementation. Following oral administration of 25 μC of ^{63}Ni, an increased retention of the isotope after six hours was observed in the unsupplemented groups. The author concluded that nickel may have an important physiological role in the growing chicken.

A requirement of 1 mg of nickel per day has been reported for a 40 kg castrated Kirgiz fine-wooled ram (Odynets and Mambetov, 1962). No further explanation was given.

Analytical techniques for nickel

Atomic absorption spectrometry is currently the most sensitive method of determining nickel in biological materials (Sprague and Slavin, 1964; Sunderman, 1965; Takeucki et al., 1966; Mulford, 1966; Brooks et al., 1967; and Sachdev et al., 1967).

In cases where the nickel concentration of samples is too low to permit a direct measurement of the nickel content, a concentrating procedure, solvent extraction, is used to enable an accurate assay (Lockyer et al., 1961; Sprague and Slavin, 1964; Takeucki et al., 1966; and Mulford, 1966). In solvent extraction, two immiscible liquids are brought into contact to effect the transfer of one or more elements from one liquid phase to the other. In the usual case, one phase is an aqueous solution of the sample, and the other is an

immiscible organic solvent. Metal salts, being ionized, are usually much more soluble in aqueous media than in organic solvents, and to extract a metal into an organic solvent, the metal ion must be converted to an uncharged species. This is easy to accomplish as most metals form stable, neutral and extractable complexes with one or more of a group of organic compounds known as chelating agents. The solvent used must completely dissolve the metal chelate and be immiscible with the sample solution, and, in addition, should burn with a clear, steady flame. For this reason, esters and ketones are extensively used as the organic phase. Solvent extraction can be used to eliminate interferences which might ordinarily arise from the sample matrix, and can be used when the sample size is too small for direct analysis. With solvent extraction an increased signal can be obtained due to: (1) concentration of the metal from the aqueous phase into a much smaller volume of organic phase, and (2) the higher volatility of the organic solvent, as compared to water, which results in a higher concentration of neutral atoms in the flame (Mulford, 1966).

The Experimental Use of Japanese Quail and Isolators

If one hopes to establish an essential physiological role for a trace metal, such as nickel, in a living macro-

organism, the selection of that organism is very important.

The potential of the Japanese quail (Coturnix coturnix japonica) as a pilot animal for poultry research was first reported by Padgett and Ivey (1959). They discussed the advantages of using quail in place of chickens. Among the advantages reported were rapid growth rate, early maturity and short incubation period which allow for the production of the next generation in about 10 weeks. From their experience with the Japanese quail, Padgett and Ivey (1959) and Howes (1964) concluded that the coturnix must have a high rate of metabolism due to the rapid rate of growth and the high rate of egg laying.

Other advantages which the use of Japanese quail has over the use of chickens are of economic importance to the researcher with a limited budget, time and space (Wilson et al., 1959; Wilson et al., 1961). Quail are much cheaper to maintain than chickens and 20 to 30 birds can be kept for the same cost as one chicken (Howes and Ivey, 1961; Howes and Ivey, 1962). Because of the relatively small size of quail, space requirements are minimal which enables the quail to be easily adapted to germfree experimentation (Reyniers et al., 1960; Reyniers and Sacksteder, 1960).

A recent bulletin on the Japanese quail has been published by the National Academy of Sciences (1969). The publication includes information on breeding, care and management of

quail for research purposes. Most of the recommendations included in the publication were derived from the work of the various scientists cited earlier.

Careful management of the Japanese quail is very important to insure success with the bird. Eggs to be hatched should not be stored for longer than 14 days as the hatchability decreases greatly if the eggs are held for a longer period of time (Padgett and Ivey, 1959; Howes, 1964). However, eggs can be stored for a somewhat longer time if stored in plastic bags (Becher et al., 1963; Proudfoot, 1964a; Proudfoot, 1964b). Flushing of the plastic bag with nitrogen has been reported to increase storage time (Proudfoot, 1964b; Proudfoot, 1965). Storage of eggs at a temperature of 13 to 16° C is recommended (Howes, 1964).

Incubation should be carried out at a temperature of 37 to 38° C, and the eggs should be turned every 3 to 4 hours (Howes, 1964). Fertility of the eggs collected at the start of egg production is low, but after 50 days of production may be as high as 85 to 90 percent (Padgett and Ivey, 1959; Woodard et al., 1969). The hatchability of fertile eggs is 60 to 70 percent (Padgett and Ivey, 1959; Woodard et al., 1969). The greatest single cause of poor hatchability of quail eggs has been reported to be dehydration as a result of microscopic cracks in the shells which could not be de-

tected by the naked eye (Howes, 1964).

At hatching, the brooder temperature should be kept at 38° C for the first week and gradually lowered so that by four weeks of age the birds can be kept at room temperature, 24 to 25° C (National Academy of Sciences, 1969). The chicks weigh an average of 5 to 6 g at hatching (Reyniers and Sacksteder, 1960; Howes and Ivey, 1961) and it is necessary to place a small piece of screenwire or several small pebbles or marbles in the waterers for the first few days to prevent drowning (Wilson et al., 1961). Average feed consumption by growing Japanese quail, 20 to 35 days old, is about 11 g per day (Reyniers and Sacksteder, 1960).

As sexual maturity approaches, a ratio of approximately one male to three females has been reported to give optimal fertility (Padgett and Ivey, 1959; Woodard and Abplanalp, 1967). The density of the mature birds has been shown to be a factor in egg production and subsequent hatchability of the eggs (Ernst and Coleman, 1966). They report an increased number of cracked eggs at the higher densities studied (172 and 215 quail per square meter of floor space). However, they report that no delay in sexual maturity occurred as a result of rearing of the chicks at high density levels, since no difference in egg production was observed in full sibs which were raised at different densities and subsequently placed in individual cages during the laying period. An

earlier report by Wilson et al., (1961) indicated that development of quail was delayed one week by crowding, while Howes (1964) reported a depression of growth, increased mortality and cannabalism as a result of crowding. The National Academy of Sciences (1969) has recommended a minimum of 84 cm^2 of floor space per bird (120 birds per m^2) from 0 to 4 weeks of age and approximately 125 cm^2 per adult quail (80 birds per m^2).

Male Japanese quail weigh approximately 90 to 100 g at 120 days and females 120 to 130 g (Reyniers and Sacksteder, 1960). Adult males average 100 to 140 g and adult females 110 to 160 g (Howes and Ivey, 1961). The heavier body weight of the female has been attributed to heavier gonads, liver and intestines (Wilson et al., 1961). The average egg weight has been reported to be about 9 g or approximately 7 percent of the body weight of the female, and Japanese quail require about 3 kg of feed to produce 1 kg of eggs (Wilson et al., 1961). The same authors report that under good environmental conditions, quail may produce double the egg mass per unit of body weight as compared to good laying strains of chickens. Hence, the metabolic demands for egg laying should be exaggerated such as to allow the development of a nutritional deficiency which might not arise during growth of the quail, or might not arise at all in other species which have lower metabolic demands at all stages of the life cycle.

The amount and wavelength of light to which the developing quail are exposed has an influence on the development of sexual maturity and on subsequent egg production. At least 12 to 14 hours of light per day are required to stimulate gonadal development (Padgett and Ivey, 1959; Wilson et al., 1962; Tanaka et al., 1965) with intermittent 12 hours of light more effective than continuous 12 hours of light (Wilson et al., 1962; Tanaka et al., 1965). Japanese quail will continue to lay under continuous light (Daniels, 1968). Growth of female quail is depressed by green or blue light as compared to red or white light and testicular weights were less for males kept under green or blue light (Woodard et al., 1969). The same authors report that females reached 50 percent lay two weeks earlier under red or white light than under green or blue light, and the fertility of eggs from quail kept under blue light was lower than for birds kept under green, red or white light. However, egg weight, feed conversion and hatchability of the fertile eggs were not affected by wavelength of light.

In most trace element research, the use of purified or semipurified diets is usually dictated by the unavailability of natural feedstuffs low in the element in question. The first reported use of a purified diet for Japanese quail appeared in 1964 (Fox and Harrison, 1964), and was used for a study on zinc deficiency in quail. However, at least a

semipurified diet was used in a preliminary study with Japanese quail reported by Adkins et al. (1961). In the study, an attempt was made to replace the casein in the diet (20 percent) with an equivalent amount of crystalline amino aicds. The authors reported that this concentration of casein and the equivalent concentration of amino acids supported normal growth in the chicken, but depressed egg production and caused a loss of body weight when fed to mature quail over a period of four weeks. Low dietary protein was cited as the probable cause of the poor performance of the quail. Purified diets were extensively used by Ketola (1967) in an effort to identify a growth factor in soybeans for the Japanese quail, and to establish requirements for protein, energy, minerals and vitamins for the birds.

Undesirable effects resulted from feeding a sucrose-base purified diet to day-old Japanese quail (Alford et al., 1967). Water containing small quantities of dissolved sucrose passed into the air passages of the baby quail, and upon dehydration sucrose crystals formed in the air passages causing asphyxia. This problem was not noted when the diet was offered to two to three week old quail, but poor feather development resulted which the authors attributed to a lack of fiber in the diet.

The performance of Japanese quail fed purified or

commercial diets was studied by Gough et al. (1968) who reported, in general, rather poor performance of birds fed the purified diet. They observed a depression of growth rate, feather development and feed consumption, the incidence of a higher mortality during the first three weeks of life, decreased egg production and egg size with many soft-shelled eggs, and lower fertility and hatchability for quail receiving the purified diet.

The successful use of a purified diet for any species is dependent upon a knowledge of the nutrient requirements of that species. Very little work has been done on the nutrient requirements of the Japanese quail, and the poor growth and reproductive performance of quail fed purified diets is undoubtedly a reflection on the lack of knowledge in this area.

The nutrient requirements of the Japanese quail as estimated by various workers are summarized in table 3.

A complete listing of minerals and vitamins at levels which have been shown (Ketola, 1967) to be adequate for growth of Japanese quail is presented in table 4.

The levels of minerals included were assumed to be adequate as no growth response was observed when the levels were doubled. A depression of growth rate was observed when vitamin levels equal to one-half of those listed on page 38 were fed.

TABLE 3. APPROXIMATE NUTRIENT REQUIREMENTS OF THE JAPANESE QUAIL

Nutrient	Requirement	Reported by
Protein	24%	Weber and Reid (1967)
Glycine	1.74% (1-3 wks of age)	Svacha et al. (1969)
	1.17% (3-5 wks of age)	Svacha et al. (1969)
Lysine	1.3%	Svacha et al. (1969)
Methionine	0.7%	Svacha et al. (1969)
Energy (net)	1760 KCal/kg	Weber and Reid (1967)
Energy: protein ratio	36-38 (NE:protein)	Weber and Reid (1967)
	46 (ME:protein)	Ketola (1967)
Linoleic acid[a]	+	Calvert (1965, 1969)
Calcium	2.5% (Laying diet)	Nelson et al. (1964)
	4.0% (Laying diet)	Krishna and Howes (1966)
	0.44% (Grower)	Miller (1966, 1967)
	0.8% (to 2 wks)	Consuegra and Anderson (1967)
	0.48% (2-4 wks)	Consuegra and Anderson (1967)
Phosphorus	0.8% (Layer)	Nelson et al. (1964)
	1.25% (Layer)	Krishna and Howes (1966)
	0.59% (Grower)	Miller (1966, 1967)
	0.30% (to 3 wks)	Consuegra and Anderson (1967)
Vitamin A	3300 IU/kg (Layer)	Shellenberger and Lee (1966)
	4400 IU/kg	Georgis (1966)
Vitamin D_3	0.012 mg/kg (480 IU/kg)	Shue (1967)
Vitamin E	not > 40 mg/kg (Layer)	Price (1968)
Pantothenic Acid	30 mg/kg	Fox et al. (1966)

[a]Linoleic acid as 3% of diet was only level tested.

TABLE 4. LEVELS OF MINERALS AND VITAMINS ADEQUATE FOR GROWTH OF JAPANESE QUAIL[1]

Mineral	PPM	Vitamin	PPM
Calcium	16000	A	80 (26,000 IU)
Phosphorus	9000	D_3 (Premix)	1320 (3960 IU)
Sodium	2600	E (Premix)	360 (40 IU)
Potassium	6000	Menadione Sodium Bisulfite	12
Chloride	5100	Thiamine HCl	20
Magnesium	1000	Riboflavin	20
Manganese	100	D-Calcium Pantothenate	44
Zinc	80	Niacin	176
Iron	40	Pyridoxine HCl	20
Copper	5	Folic acid	2
Iodine	4	Choline Chloride	7000
Molybdenum	2	Biotin	0.44
Selenium	0.2		
Cobalt	0.1		

[1] From Ketola, 1967.

The requirements for sodium chloride and iodine for both the pheasant and Bobwhite quail are similar and have been reported to be 0.085 percent sodium, 0.048 to 0.11 percent chloride and not greater than 0.3 ppm iodine (Scott et al., 1960). The sodium requirement for broiler-strain chicks maintained on purified diets was determined to be between 0.11 and 0.13 percent (McWard and Scott, 1961; Nott and Combs, 1969). These results indicate that the sodium, chloride and iodine requirements can most likely be met by the inclusion of 0.15 percent iodized salt (containing 0.007 percent iodine) in the diet.

In addition to just meeting the requirement for an element by adding a minimum amount of that element to the diet, one also must be concerned with the form of the element which is used. This is especially true for calcium and phosphorus where the hydrous form of dicalcium phosphate has been determined to be more available than the anhydrous form (Supplee, 1962; Gillis et al., 1962; Rucher et al., 1968). Rickets and high mortality were reported in poults receiving two percent dietary calcium as the anhydrous salt whereas poults receiving the hydrous salt showed excellent gain and no mortality (Supplee, 1962). Tricalcium phosphate has been reported by Gillis et al. (1962) to be less effective than mono- or dicalcium phosphate for promoting bone calcification. These same authors reported that the hydrous salt of dicalcium phosphate was much more available for the poult and about equally available for the chick as the anhydrous form. Radioactive dicalcium phosphates were used by Rucher et al. (1968) who reported the need for 50 percent more of the anhydrous salt to give equivalent bone ash values in turkey poults, but equal bone ash values were attained in chicks given equal levels of the two forms.

There is a need for more research to determine the nutrient requirements of Japanese quail, since the quail is being so widely used in research today.

Research concerned with the possible requirement of a micro-nutrient must necessarily be aware of the distinct possibility of contamination of the diet and water by the feeders and waterers. The cages, and even the air, can be major sources of the micro-nutrient in question.

To alleviate this problem, plastic and plexiglass has been used extensively for feeders and waterers and for construction of isolators to which air filters can be attached (Trexler, 1959; Smith and Schwarz, 1967; Mertz, 1968). Plexiglass and certain plastics have been assayed and demonstrated to be very low in their content of trace metals (Smith, 1968), and the small amounts of the trace metals these materials do contain are considered to be tightly bound and unavailable.

Thus, the conditions employed are modifications of those used in gnotobiotic studies. If a slight positive pressure is maintained within the isolator, it can be opened for cleaning and feeding and watering of the animals without undue hazard of contamination (Trexler, 1959). A comparison of the performance of rats kept in controlled and conventional environments revealed no harmful effects on performance due to the isolators per se (Smith, 1968).

Air entering the isolator through glass fiber media filters was determined to be free of dust particles larger than 0.35 micron in size as well as all demonstratable

organisms, including bacteria, yeasts and molds (Smith and Schwarz, 1967).

In addition to contamination which results from metal cages, feeders and waterers, dietary contamination can result from the wrong choice of ingredients for use in a purified diet. Casein is a major source of contaminants since it strongly binds small amounts of metals (Smith and Schwarz, 1967). Convenient methods for their removal do not exist. Sucrose is normally a material of great purity while starch and reagent grade chemicals are often heavily contaminated with the trace elements of biological interest and may need to be purified (Smith and Schwarz, 1967). The ingredients can be treated with ion exchange resins or chelating agents in the hope that these agents have a greater affinity for the element in question than has the natural ligand in the diet (Mertz, 1968). Alternatives discussed by Mertz include: the growing of plants to be used for feed in hydroponic cultures containing a very low concentration of the element in question, the addition of some substance to the diet which will react with the element under study to make it unavailable for absorption, and/or the addition of an excess of an element which competes with the one under study. In the latter case, one needs to be concerned with the possibility of adverse effects on the utilization of

other elements in addition to the one which is of primary concern.

By a proper choice of dietary ingredients, Smith (1968) produced a skim milk diet with a lower nickel content than a purified amino acid diet. The use of skim milk would also reduce the amount of mineral supplementation required and eliminate at least part of that source of contamination.

After consideration of the characteristics of the Japanese quail as described in the research reported above, the use of this animal in the study of a possible physiological role for nickel seemed highly desirable for the following reasons:

(1) The possibility of depleting tissues of a trace metal, by feeding purified diets, is generally increased by following this practice through successive generations. The Japanese quail has a very short generation time, producing the first eggs 35 to 40 days after hatching (Wilson, et al., 1961). Incubation requires 17 to 18 days (Howes and Ivey, 1962). A practical generation time is, thus, not longer than 10 weeks.

(2) This species has a very high metabolic rate which tends to exaggerate nutrient requirements.

(3) The cost of highly purified diets per animal unit is less than for many other species because of the comparatively low food intake.

(4) The Japanese quail is small enough to conveniently house in a filtered air environment.

Arginase and Arginine

According to Mertz (1968), every element has the potential

of exerting harmful or helpful effects or being without effect depending upon the dose and nutritional state of the animal or system on which this dose is imposed; and any biological function for that element can only be assessed after a thorough study of any deficiency symptoms produced by an inadequate dietary supply of the element. The development of a deficiency state is not necessarily the complete lack of an element but can reflect a state of suboptimal supply. A deficiency is recognizable by the depression of some biological function. The function may be life itself or growth and these two are widely used as criteria of the existence of a deficiency state. However, it is not always possible to establish deficiencies which are incompatible with life, or even normal growth. This is especially true for trace elements with as yet undefined biological action. Therefore, other functions must be studied which are more sensitive to a mild deficiency. A deficiency state can cause impairment of lipid or protein metabolism, of membrane transport or of any particular enzyme reaction without any visible effect on growth or life (Mertz, 1968).

One of the functions common to most all of the biologically active trace metals is the specific activation of at least one enzyme (Herring et al., 1960; O'Dell, 1968). Nickel has been shown by Hellerman and Perkins (1935) to

activate arginase *in vitro*.

Arginase was discovered by Kossel and Dankin (1904). Early work indicated that the enzyme was concentrated primarily in the liver of mammals, amphibians, fishes and the tutrle but absent from the liver of birds and the majority of reptiles; however, arginase was present in the kidney of birds (Clementi, 1916, 1918, 1922). Arginase was subsequently found in the kidneys of mammals and fishes but in amounts much less than those present in the liver (Hunter and Dauphinee, 1924). Arginase was subsequently found in the liver of birds but at levels much lower than in the kidneys (Edlbacher and Röthler, 1925). The observations of Clementi (1916, 1918, 1922), Hunter and Dauphinee (1924) and Edlbacher and Röthler (1925) were confirmed by Chaudhuri (1927) who also noted that arginase was more abundant in males than females, expressed both as arginase per gram of tissue and as total arginase per tissue. Similar reports of a sex difference for arginase have been reported by Baldwin (1936), Wiswell (1950) and Mandelstam and Yudkin (1952).

The function of arginase in the bird is not known. The liver of birds lacks four of the enzymes involved in the urea cycle of Krebs and Henseleit (1932) and one of the urea cycle enzymes is absent from the bird kidney. Carbamyl phosphate synthetase is absent from both the liver and kidney and, in addition, ornithine transcarbamylase, argininosuccinate

synthetase and argininosuccinase are absent from the liver (Tamir and Ratner, 1963a, 1963b). Ornithine transcarbamylase was not detected in any of the tissues examined by Siren (1963). The urea cycle is thus incomplete in both organs and cannot function in removing ammonia in birds as it can in mammals (Fahey, 1957; Gullino et al., 1961; Goldsworthy et al., 1968). The lack of any apparent essential function for arginase in the bird was reported by Smith and Lewis (1963) who concluded that high arginase activity could only increase the dietary requirement for arginine. The main function of arginase appears to be related to the production of ornithuric acid, a compound which is used by the bird in detoxification of aromatic acids much as glycine is used in mammals (Baldwin, 1936; Nesheim and Garlich, 1963). Ornithuric acid is reportedly formed in the chicken kidney but not in the liver (McGilvery and Cohen, 1950). Addition of arginine to the diets of chickens given 1.5 percent dietary benzoic acid considerably lessened the growth depression induced by the benzoic acid indicating that arginine was either being used to form ornithuric acid or was acting to spare the requirement of some other compound (Nesheim and Garlich, 1963). Feeding a dose of benzoic acid shortly after arginine-1-^{14}C had been injected into chickens led to the recovery of much of the activity in the conjugation product of ornithuric

acid and benzoic acid, dibenzoyl ornithine. Based on the amount of activity recovered, they calculated that 40 percent of the ingested radioactive arginine would have been required to provide the ornithine used in the detoxification of the benzoic acid. Essentially no radioactive dibenzoyl ornithine was isolated from the urine of a group of hens given radioactive glucose, indicating that the hen does not possess a metabolic pathway to synthesize ornithine from glucose. Based on these results, Nesheim and Garlich (1963) concluded that dietary arginine was probably the source of ornithine in the fowl for conjugation with aromatic acids such as benzoic acid, and, apparently, pathways for ornithine synthesis from glucose, proline (Stetten and Schoenheimer, 1944) and glutamic acid (Stetten and Schoenheimer, 1944; Tamir and Ratner, 1963c) do not exist in the fowl as they do in the rat and probably other mammals.

The complete lack of carbamyl phosphate synthetase in the bird (Tamir and Ratner, 1963a; 1963b) is evident as ornithine cannot replace arginine in the diet, whereas citrulline can fully replace the dietary arginine (Klose and Almquist, 1940; Tamir and Ratner, 1963a; 1963c; Rock, 1969). These results were demonstrated by the feeding of radioactive precursors, $Na_2{}^{14}CO_3$ and citrulline-ureido-^{14}C.

Radioactive carbon dioxide was not incorporated into citrulline indicating that ornithine cannot be converted to citrulline because of the lack of carbamyl phosphate synthetase or ornithine transcarbamylase or both. Labeled arginine was detected after radioactive citrulline was fed, indicating that the enzymes necessary for its conversion to arginine are present in some extra-hepatic tissue of the bird (Tamir and Ratner, 1963b). Thus, ornithine released by the action of arginase on arginine cannot be reutilized to synthesize arginine in the bird as it can in mammals, and presumably the ornithine is utilized in the formation of ornithuric acid which is used for detoxification purposes (Tamir and Ratner, 1963c), as discussed earlier. The authors concluded that arginine has become an indispensable amino acid for birds as a result of the change in pattern of nitrogen excretion brought about by replacing arginine synthesis and urea excretion by purine synthesis and uric acid excretion.

As a result of the purely degradative action which arginase and the urea cycle as a whole appear to have on arginine, the arginine requirement of the bird is thus altered by any of a number of factors which alter the activity or the absolute amount of arginase in the kidney.

The dietary protein level has been demonstrated to have

a marked effect on arginase activity. This relation was first recognized by Lightbody and Kleinman (1939) who reported an increase in arginase activity, in the liver of rats, with increasing dietary protein levels ranging from 6 to 75 percent. The increase was observed when expressed either as activity per unit of liver dry weight or per unit of body weight. No nitrogen determination was carried out on the liver so there was no way of ascertaining whether the increase in activity was due to an increase in the activity of arginase already present or due to increased synthesis of the enzyme.

An increase in arginase activity, related to either the weight of the liver or to the hepatic nitrogen content, in response to increasing protein levels in the diet of the rat was reported by Mandelstam and Yudkin (1952). They reported the increase in activity to have achieved its maximum by three weeks, and that the arginase activity could be returned to the original level by decreasing the dietary protein level to its original level. They suggested that the adaptation observed was in response to the amount of protein being metabolized, and, hence, on the amount of urea being produced.

Similar results were reported by Ashida and Harper (1961) who observed increased arginase activity of rats as

the casein content of the diet was increased from 25 to 45 or 70 percent with the maximum increase observed after only four days at the 45 percent casein level and after seven days at the 70 percent level. When the casein levels were reduced back to 25 percent, the activity of liver arginase decreased with the rate of decrease being almost the inverse of the rate of increase observed when the casein content was increased. Total liver arginase was found to increase linearly with increasing urinary excretion when the diet was changed from 25 to 70 percent casein, indicating that arginase activity was proportional to the extent of protein catabolism in the body over the range of dietary protein levels studied. The adaptation to the high protein diet involved both an increase in liver size and an increase in enzyme activity. They also suggested that the high enzyme activity observed on the high protein was probably an adaptative response since the enzyme activity fell sharply within one day after the dietary protein content was lowered. An initial decrease in growth and feed consumption for one to three days when the protein content of the diet was increased was attributed to the inability of the animal to metabolize excess protein until it had undergone various adaptations, one of which is a substantial increase in arginase activity.

An increase in arginase activity as well as an increase in the activities of the other four urea cycle enzymes, was

noted by Schimke (1962a) when the dietary protein level was increased. He reported that the arginase concentration reached a maximum in eight days and that further changes in the liver content of the enzyme consisted in part of an increase in the size of the liver and total liver nitrogen. Maximum total arginase activity was reached after 18 days on a high protein diet, a value very similar to that reported by Mandelstam and Yudkin (1952). Urea excretion increased with increasing levels of protein.

A decrease in arginase activity in rats fed a protein-free diet was observed by Seifter *et al.* (1948) who reported that the decrease was probably due to a loss of enzyme protein *per se*. Arginase activity in rats fed a protein-free diet was observed by Rosenthal *et al.* (1950) to decrease at a rate faster than total liver protein. Regeneration of arginase, when protein is placed back in the diet, appears to lag somewhat behind the regeneration of total liver protein (Miller, 1950). A relatively constant concentration of arginase per gram of liver was noted by Millman (1951) when a protein-free diet was fed to rats. However, a decrease in total liver arginase activity, as a consequence of a decrease in liver weight, was observed.

Increased arginase activity has been reported in fasting dogs (Takehara, 1938) and rats (Lightbody and Kleinman, 1940). Urea excretion on a protein-free diet is decreased as body

proteins are being conserved and another energy supply is still available (Schimke, 1962b). However, urea excretion during fasting is increased as proteins must be catabolized for energy. An absolute increase of up to 300 percent in the activities of all the urea cycle enzymes has been noted in starved rats in which body protein is being catabolized. This increase in activity was noted even though the total protein content of the liver decreased, and Schimke (1962b) suggested that tissue protein may be broken down and re-utilized for protein (enzyme) synthesis in the presence of an overall negative nitrogen balance. An increase in the activity of enzymes associated with protein degradation is, according to Harper (1965), the only way by which carbon skeletons can be provided for gluconeogenesis. Gluconeogenesis is the only source of glucose for the starving animal and is so essential for the maintenance of the nervous system that conservation of amino acids for protein synthesis becomes secondary.

The level of arginase activity has been determined to follow an inverse relationship with the nutritive value of the dietary protein (Kean, 1967). More of the amino acids of a high quality protein can be used for protein synthesis with the result that less amino acids are deaminated and less ammonia enters the urea cycle. No correlation between urea

excretion and arginase activity was observed by Kean (1967) who suggested that the liver normally contains such an excess of arginase that its observed activity *in vitro* need not be correlated with urea excretion. No consistent correlation between increased urea excretion and arginase activity of rat liver was observed by Iwao and Ashida (1967). They fed a protein-free diet and 14 percent casein and 14 percent wheat gluten diets for a period of 11 days. Rats given the wheat gluten diet excreted more urea as was reported by Kean (1967) for rats receiving a poor quality protein. Increasing the level of casein increased the level of urea excretion and of arginase activity. Liver arginase activities of rats receiving wheat gluten were significantly lower than corresponding values for casein-fed rats. Rats fed the protein-free diet had values for arginase activity and urea excretion below those fed the gluten diet. A decrease in the dietary tryptophan level resulted in an increased urea excretion but decreased arginase activity. The authors concluded that urea excretion and arginase activity are positively correlated with regard to protein level, but negatively correlated with regard to dietary protein quality. A similar relationship between protein quality and arginase activity was reported by Muramatsu and Nakata (1967) who fed diets devoid of one or all of the essential amino acids to rats. A decrease was observed in urea cycle enzyme activity which was greater when

all of the essential amino acids were omitted as compared to the depression observed when only one was omitted.

Hormones which cause an increase in protein catabolism also cause an increase in arginase activity (Conrat and Evans, 1942; Freedland and Sodikoff, 1962; Schimke, 1963). Administration of large doses of cortisone, 25 mg per 100 g body weight, led to increased activities of the urea cycle enzymes which were proportional to the increased urea excretion; and adrenalectomy was associated with a significant decrease in the level of arginase activity (Freedland, 1964) with less striking effects on the other four urea cycle enzymes. Injection of cortisone into an adrenalectomized animal increased enzyme activity to the same extent as in the intact animal. Increasing the casein content of the diet, fed to adrenalectomized rats, from 15 to 60 percent resulted in increased levels of urea cycle enzymes in a pattern similar to that found in the intact rat (Schimke, 1963). The author concluded that corticosteroids act nonspecifically on the urea cycle enzymes, that the enzyme levels are changed only so much as the corticosteroids increase protein catabolism and urea excretion and that intact adrenal glands are not required for the increase in enzyme activities produced by increasing the protein content of the diet.

Dietary arginine level, as might be expected, has been demonstrated to effect arginase activity. Increasing

increments of dietary arginine resulted in an increase in arginase activity and an increase in urea excretion (Baldwin, 1936). No correlation between arginase activity and dietary arginine level in chicks was found by Smith and Lewis (1963). However, they used only one bird per level of arginine and reported a high variability in arginase activity, so their results can be considered inconclusive. Supplementation of a purified diet with 2.4 percent arginine caused an increase in arginase activity in the kidney of the chick (O'Dell et al., 1965). This amount of arginine was required for maximum growth rate and, presumably, maximum induction of arginase had occurred in all chicks but the excess of dietary arginine was sufficient to overcome any depressing effect on growth. The authors also reported an increase in arginase activity in chicks given a practical diet supplemented with arginine.

A dietary deficiency of arginine results in a characteristic enzyme response in those animals capable of synthesizing arginine, i.e., those with a complete urea cycle. Arginine-free diets are associated with decreased growth and slight decreases in urea excretion and arginase activity (Schimke, 1963). At the same time, however, the activities of all of the other urea cycle enzymes increase until enough arginine is synthesized to permit normal growth (Schimke, 1963; Muramatsu and Nakata, 1967).

Other amino acids have been demonstrated to have an

effect on arginase activity when they are added in excess. Arginase has been shown to be fully inhibited *in vitro* by excess ornithine (Bach *et al.*, 1944; Van Slyke and Archibald, 1946), and Hunter and Downs (1945) reported ornithine to be a potent competitive inhibitor of arginase and, in addition, that all amino acids of the L-configuration inhibited arginase. Intraperitioneal injection of ornithine, as well as arginine and citrulline were without effect on any of the urea cycle enzymes of rat liver (Schimke, 1963). Excess dietary levels of alanine, threonine, lysine, leucine, histidine, tryptophan, aspartic acid and methionine exerted no effect on urea excretion or levels of urea cycle enzymes (Schimke, 1963).

An intraperitioneal injection of 3.0 m mole of lysine into rats caused an inhibition of arginase which was 16.4 percent 30 minutes following the injection, reached a maximum of 56.0 percent at 60 minutes and disappeared at 240 minutes after the injection (Cittadini *et al.*, 1964). Excess dietary methionine caused an increase in liver arginase of rats, but the increase observed was probably due to the decreased feed consumption observed when methionine was added to the diet (Bergner *et al.*, 1968). An inhibition of arginase in chick kidneys has been demonstrated when 0.5 percent of α-amino-isobutyric acid is included in the diet and occurred even

when 3 percent arginine was also supplemented (Shao and Hill, 1969).

Added lysine (0.4%), histidine (1.6%), tryosine (3.0%) or isoleucine (2.0%) to the diet of chicks resulted in a 2- to 4-fold increase in arginase activities and rates of urea excretion (Austic and Nesheim, 1969a; 1969b; 1969c). They proposed that dietary excesses of amino acids may increase the arginine requirement of chicks by stimulating kidney arginase with a resultant acceleration of the rate of arginine degradation (Austic and Nesheim, 1969a). However, in another report, Austic and Nesheim (1969b) state that the increase in urea excretion resulting from an excess of lysine, histidine, tyrosine or isoleucine was equivalent to only about 6 percent of the ingested arginine, and they suggest that although the degradation of arginine to urea is increased, this pathway may not account for the total increase in arginine requirement resulting from excesses of dietary amino acids.

Thus, it would appear, at least in principle, that any dietary factor which alters the activity of arginase could affect the arginine requirement of the bird. Arginine was initially thought not to be a dietary essential for the growing rat (Scull and Rose, 1930). The rat has a complete urea cycle and some arginine synthesis should be possible.

However, Rose (1937) later determined that arginine was a dietary essential for the growing rat but not for maintenance of the adult.

Arginine was first demonstrated to be a dietary essential for the chick in 1935 (Arnold et al., 1935). These workers identified a factor in liver residue, which was very effective in promoting growth, as arginine and indicated that arginine from casein was not as effective for increasing growth as arginine from other sources. They also observed that the growth promoting effect of arginine was less after six weeks, indicating that the requirement is then less. In 1938, Klose et al. demonstrated that the chick is unable to synthesize arginine from ornithine, urea or a combination of the two and that arginine was therefore needed for maintenance as well as growth. They noted that arginine supplementation of a 20 percent casein diet increased growth but was without effect when added to a 30 percent casein diet, and this observation suggested to them that part of the arginine in casein was in such a form as to be unavailable to the chick.

An additional factor was presented by Bloch and Schoenheimer (1940a) which, they believed, could have an effect on the arginine requirement. They demonstrated that the amidine group of creatine originates from arginine. In the same year, Almquist et al. (1940) identified glycine as an essential amino acid for the chick, and Bloch and

Schoenheimer (1940b) determined that glycine was also involved in creatine synthesis. The source of the methyl group in creatine synthesis was determined to be methionine (Borsook and Dubnoff, 1940). Therefore, the entire scheme for creatine synthesis was basically outlined with arginine demonstrated to play a vital role.

The creatine content of muscle was demonstrated to increase following the addition of arginine to the diet of chicks which had previously been fed an arginine deficient diet (Hegsted et al., 1941). They also observed that rapid feathering birds showed more response to arginine and glycine supplementation than slowly feathering birds, and they concluded that feather formation is a primary factor in the high arginine and glycine requirements of chicks.

As might be expected from the role which arginine and glycine serve in creatine formation, the addition of creatine to the diet of the chick has a sparing effect on the arginine and glycine requirements (Almquist et al., 1941). However, Fisher et al. (1956) have reported that some arginine was still converted to creatine when creatine was included in the diet at a level which had previously been demonstrated to give "normal" muscle creatine values with an arginine-devoid diet. They also observed other signs of arginine deficiency, such as frizzled feathers and depressed growth rate, when only creatine was added to the diet, indicating that creatine

formation is not the only metabolic function for arginine. In addition, they observed that the muscle creatine content in the chick increases rapidly during the first four weeks of life and suggested this may, in part, account for the higher arginine requirement during early life. One percent of supplemental dietary creatine has been reported to be adequate for growth of the chick when no arginine was included in the diet (Fisher et al., 1956; Savage and O'Dell, 1960). A dietary arginine supplement of 1.9 percent was required for chicks on a 25 percent casein diet with no supplemental creatine (Fisher et al., 1956).

Numerous researchers have reported that the arginine requirement of chicks fed a diet with casein as the protein source is higher than when fed a natural diet. This was first indicated in 1936 by Arnold et al. It was suggested by Klose et al. (1938) that part of the arginine in casein was in such a form as to be unavailable to the chick, and thus, arginine was the first limiting amino acid in casein. It is interesting to note that arginine has been demonstrated to be the least limiting amino acid in casein for growth of the rat and that methionine and threonine are first and second limiting respectively (Harper, 1959a).

The amino acid composition of casein is presented in table 5.

TABLE 5. PERCENT AMINO ACID COMPOSITION OF CASEIN

Amino Acid	Hodson and Krueger (1946)[a]	Block and Weiss (1956)[b]	Ellinger and Boyne (1965)[b]
Methionine	2.6	3.3	3.3
Arginine	3.6	4.2	4.1
Tryptophan	1.2	1.5	
Threonine	4.2	4.5	4.4
Histidine	2.6	3.0	3.5
Isoleucine	6.4	6.6	6.0
Leucine	9.9	10.1	10.0
Lysine	8.3	8.2	8.8
Valine	6.2	7.4	7.4
Phenylalanine	5.2	5.8	6.0
Tyrosine	5.7	6.3	6.6
Cystine		0.4	0.4
Serine		6.3	
Glutamic acid		23.6	23.6
Aspartic acid		6.5	4.4
Glycine		2.1	2.2
Alanine		3.1	3.5
Proline		12.3	

[a]Microbiological assay.
[b]Column chromatography.

Because casein had been determined to be first limiting in arginine for the chick, it became a practice to add 10 percent gelatin, a rich arginine source, to the diet and reduce the casein content 25 percent. A 25 percent casein-10 percent gelatin was determined to be partially inadequate as a source of protein for chick growth and could be improved by the addition of 1.24 percent arginine (Wietlake et al., 1954). Similar results with a 25 percent casein-10 percent gelatin diet were reported by Hogan et al. (1956), and in addition, they stated that 1.8 to 2.5 percent was the optimum

amount of arginine to add to a 35 percent casein diet and that the rates of gain on casein-gelatin, casein or soybean meal diets were equal if suitable amino acids (arginine) were added to the casein-gelatin and casein diets.

A study was conducted by Griminger et al. in 1955 to determine if the browning reaction between carbohydrates and amino acids was a factor in causing the apparent increase in the arginine requirement of chicks fed a casein diet. Cerelose or dextrin was used as the carbohydrate source. Dextrin, having a smaller number of reactive end groups than an equal amount of cerelose, should have spared arginine if the browning reaction was occurring. In one instance, cerelose was added to the diet just prior to feeding to eliminate any browning reaction that might have occurred upon storage of the mixed diet. Neither dextrin nor cerelose addition at feeding time decreased the arginine requirement so the authors concluded that the browning reaction was not responsible for the increased arginine requirement observed on casein diets.

A corn-soy diet containing a total of 1.1 percent arginine has been reported to be adequate for chick growth, as no growth response was obtained when supplemental arginine was added (Snyder et al., 1956). They noted that 0.75 to 0.92 percent of supplemental arginine was required to promote adequate growth of chicks fed a 22 percent casein diet.

The casein was assayed to contain 3.7 percent arginine, so that the total arginine level in 22 percent casein diets was 1.56 to 1.73 percent.

No growth response was obtained by Krautmann et al. (1957) when they added arginine to either a corn-soy diet containing 0.94 percent arginine or a corn-soy-corn gluten meal diet containing 0.85 percent arginine. However, 1.67 percent total arginine was required in a 21 percent casein diet.

Data were presented by Anderson and Dobson (1959) which indicated that the amino acid composition of casein is responsible for the higher arginine requirement noted when casein provides most of the protein in chick diets. The same high arginine requirement was noted with diets based on other proteins (combinations of wheat gluten, blood fibrin and zein) to which amino acids had been added to provide the same essential amino acid content as a ration based on casein. The authors suggested that the essential amino acid levels and not the level of protein per se were responsible for the high arginine requirement of chicks fed casein diets. Earlier reports by Almquist (1947) and by Almquist and Merritt (1950) indicated that the arginine requirement of chicks fed a casein diet could be met if arginine were supplemented to supply 6 percent of the protein. The data presented by Snyder et al. (1956) and Krautman et al. (1957)

indicate a significantly higher arginine requirement as a percent of the protein.

A crystalline amino acid mixture, simulating the amino acid composition of casein, was formulated by Klain et al. (1959) and subsequently demonstrated to be inadequate for chick growth unless supplemented with arginine. The arginine requirement for chicks fed such a mixture of amino acid was 2.06 percent. Similar results were obtained by Fisher et al. (1960) using a crystalline amino acid mixture.

Thus, the high arginine requirement of chicks fed casein diets appears to be related in some manner to the amino acid composition of casein.

The addition of 2 percent lysine to a basal diet containing 50 percent milo gluten meal resulted in feather abnormalities which were attributed to an arginine deficiency, and the suggestion was made by the authors that the addition of lysine precipitated an arginine imbalance or deficiency which resulted in the abnormal feathering observed (Sanders et al., 1950). Supplementation of the lysine containing diet with 0.6 percent arginine resulted in normal feather development. Similar results were observed when excess lysine was added to a ration containing wheat gluten as the protein source (Anderson and Dobson, 1959).

The increased arginine requirement of chicks given excess lysine was postulated by Snetsinger and Scott (1961) to

be due to the increased involvement of arginine in a latent urea cycle which was stimulated by the excess lysine for the purpose of eliminating the excess nitrogen. The effect of excess lysine on the growth of chicks was attributed to a lysine toxicity (Jones, 1961). The inadequacy of casein was attributed by Patterson et al. (1961) to be due to an imbalance involving arginine rather than a simple deficiency or unavailability of arginine as neither the absolute amount of arginine in the diet nor the protein level were found to be solely responsible for the poor growth.

The existence of a specific antagonism between lysine and arginine was postulated by Lewis and Smith (1964) and Smith and Lewis (1966). In support of their claim, they noted that excess dietary histidine did not depress growth on an arginine low diet while excess lysine did not depress growth on a tryptophan-limiting diet.

The interaction between lysine and arginine was also termed an antagonism by Jones (1964) who reported that eight-day old chicks were less susceptible to excess lysine than the one-day old bird and that the growth depression was less. Lysine did not induce an arginine deficiency on a soy diet, and Jones concluded that lysine acts in some specific manner to decrease the availability of the arginine, i.e., a portion of the analytically determined amino acid is not used by the animal.

The conclusion reached by Boorman and Fisher (1966) was that the lysine-arginine interaction is a particular manifestation of the general phenomenon of amino acid toxicity and not due to a unique lysine-arginine interaction. This conclusion was reached after they had noted that the addition of 0.8 percent methionine to an arginine-deficient diet produced a growth depression, and the addition of excess arginine to a diet limiting in lysine did not result in a significant growth depression. Arginine did not alleviate the growth depression caused by an excess of all single amino acids tested, but its growth promoting effect was not solely confined to alleviating the growth depression caused by excess lysine. However, lysine was by far the most effective in producing a growth depression on an arginine-deficient diet. The data of Huston and Scott (1968) lend support to the report of Boorman and Fisher (1966) that the amino acid imbalance phenomenon in chicks is a general one. The data of Huston and Scott (1968) reveal that weight gain of chicks fed crystalline amino acid diets containing threonine at levels of 55 and 70 percent of the chick's requirement was not adversely affected by excess lysine; whereas, supplemental lysine markedly depressed growth when the concentration of threonine was adequate or approaching adequacy. Similar results were obtained when valine, isoleucine and leucine were first limiting. When arginine was first limiting, the

depression in weight gain due to excess lysine was greatest at the lowest level of dietary arginine and least on the arginine adequate diet. Thus, excess lysine did not depress weight gain when the crystalline amino acid diet was first limiting in either threonine, valine, leucine or isoleucine, whereas excess lysine depressed growth significantly when the concentration of the deficient amino acid was increased in an amount that would meet the chick's requirements. The authors suggested that this result could be due to an excess of arginine, not needed for protein synthesis, which could function to counteract the imbalancing effect of excess lysine in diets severely deficient in an essential amino acid. They further suggested that the arginine present in adequate diets was used for protein synthesis, and hence the arginine available for reversing the lysine-induced imbalance would tend to be minimal. A crystalline amino acid mixture was then formulated to be equally limiting in either threonine, leucine, valine or isoleucine and arginine, and the addition of excess lysine to such diets depressed weight gain, whereas no depression was noted when arginine was not also limiting. Growth was depressed on a threonine-deficient diet, that was also equally limiting in arginine, by the addition of excess histidine, tryptophan or methionine. Therefore, the authors concluded that the lysine-arginine interaction is not a specific one.

A similar lysine-arginine relationship has been demonstrated in the rat fed either an 18.0 percent casein or a 15.6 percent soy protein diet (Jones et al., 1966). Arginine had little effect when added to the basal diets but completely eliminated the growth inhibition caused by 3 percent lysine and significantly reduced that caused by 6 percent lysine. The rat was more susceptible to excess lysine when the protein of the ration was furnished by casein. An in vitro inhibition of the proteolytic enzymes trypsin and carboxypeptidase B by lysine was reported by Wolff et al. (1962). Some in vivo effect of lysine on the pancreatic proenzyme content was observed by Jones et al. (1966; 1967) but the effect could not be correlated with the effect on growth rate as lysine depressed growth even when a diet containing enzymatically digested casein was fed.

The ratio of lysine to arginine in casein is approximately 2.0 and is about 1.0 in soy protein. Supplementation of a casein diet with arginine to give a ratio of 1.0 was reported by O'Dell and Savage (1966) to give maximum growth in the chick. Similar data on the lysine to arginine ratio were also presented by Jones et al. (1967) and Nesheim (1968a, 1969).

An intensive study into the mechanism of the lysine-arginine interaction was conducted by Jones et al. (1967). They reported that release of the amino acids from casein

was not impaired, as lysine caused a significant growth depression even when added to a crystalline amino acid diet. Neutralizing the hydrochloride from lysine, arginine and histidine with sodium bicarbonate, potassium bicarbonate or sodium chloride did not prevent the growth depressing effects of lysine. Absorption of arginine was not impaired as the plasma arginine concentrations of chicks, fasted for 13 hours and then given a test meal of either basal (10 percent gelatin plus 18 percent casein) or basal plus lysine, were approximately equal one-half and two hours after the test meal. The plasma arginine value then decreased to the fasting levels in chicks given the basal diet and to below fasting levels in birds given the basal diet plus lysine. Plasma lysine peaked at one-half hour in both groups, decreased to normal within four hours in the controls but remained elevated in the group receiving excess lysine. Based on these observations, they suggested three possible mechanisms by which lysine could increase the requirement for arginine:

(1) The excess lysine could cause an increase in protein synthesis, although this was discounted since growth was depressed when excess lysine was fed.

(2) Increased synthesis of creatine might be evidenced by an increase in arginine-glycine transamidinase activity.

(3) Excess lysine might increase the degradation of arginine, possibly by chick arginase.

The persistence of decreased plasma arginine concentration

when creatine was fed indicated that the mechanism by which lysine controls the concentration of arginine is unaffected by growth or transamidinase activity. Plasma arginine concentration decreased as early as four to six hours after excess lysine was consumed, but kidney arginase activity was not significantly increased until four days after excess lysine was given, and no increase in plasma or muscle ornithine concentration was observed at six hours. Therefore, it was considered very unlikely that a change in arginase activity was a significant factor in the initial decrease in plasma arginine concentration. An increase in tissue arginine was not observed and therefore could not account for the decreased plasma arginine level. Finally, the authors indicated that kidney tubular transport probably was not involved in the mechanism.

This latter observation was supported by Smith (1968) who reasoned that if lysine decreased arginine reabsorption, then excess arginine should decrease lysine reabsorption and decrease growth, but instead added lysine again decreased growth rate rather than stimulated it as should have occurred if arginine made lysine limiting by competition at the reabsorption site in the kidneys. The author also ruled out the possibility that lysine causes an activation of a latent urea cycle in the bird as no benefit was derived from the addition of ornithine to the diet, and he concluded that an

alternate route of disposal of excess lysine must exist which utilizes arginine or its by-products.

Observations reported by Boorman et al. (1968) lend support to the postulate that excess lysine inhibits arginine reabsorption in the kidneys. They observed that an increase in the plasma concentration of lysine apparently caused an overloading of the tubular reabsorption site resulting in increased excretion and decreased reabsorption of both lysine and arginine, and this effect was observed with only small increases in the plasma lysine concentration.

Data presented by Nesheim (1969) and by Austic and Nesheim (1969c) suggest that the growth depression produced by excess tyrosine or lysine can be partly accounted for by an acceleration of arginine degradation by arginase, over a period of time, as the feeding of aminoisobutyric acid plus lysine or tyrosine did not produce as great a depression in growth as the feeding of lysine or tyrosine alone. Aminoisobutyric acid was reported by Shao and Hill (1969) to be an in vivo inhibitor of arginase. The greatest effect of lysine excess on the arginine requirement appears to be the result of factors other than arginase. As the protein content of the diet used by Austic and Nesheim (1968c) was low (14.6 percent casein), they concluded that the excess amino acid may have produced an amino acid imbalance as described by Harper (1964), and that the role of arginase under such conditions

appeared to be secondary but greatly compounding the primary effect of the amino acid imbalance on the arginine requirement.

A difference in the arginine requirements of White Leghorns and Barred Plymouth Rocks was noted by Hegsted et al. (1941). A later report by Snyder et al. (1956) stated that extreme variability in growth of chicks resulted from feeding an arginine-deficient diet. About 20 percent of the birds appeared normal in every way while pen mates exhibited signs of arginine deficiency. These observations suggested to the authors that those chicks which appeared to grow normally on arginine deficient diets were endowed with the ability to synthesize arginine at an accelerated rate. The strain and breed of chicks used was implicated by Edwards et al. (1958) as having an effect on the arginine requirement. Studies by Griminger and Fisher (1962) revealed the existence of an inherited difference in growth potential on an amino acid deficient diet.

These reports definitely indicated that there could be a genetic difference in the arginine requirements of chickens. The existence of a difference in the arginine requirements between two strains of White Leghorn chickens has been reported by Nesheim and Hutt (1962). They observed that both strains exhibited a decreased growth rate on an arginine-deficient diet, but growth of the strain which they labeled

the high arginine requiring strain (HA) was depressed significantly greater than the strain with the low arginine requirement (LA). They reported that chicks of the HA strain required at lease 25 percent more arginine than chicks of the LA strain to at least four weeks of age. The rate of feathering was the same for both strains, so the difference in growth rate was attributed to the genes directly concerned with the utilization of arginine. A subsequent report by Nesheim et al. (1964) indicated that the difference in growth rate between the two strains was eliminated if they were pair-fed. Therefore, the HA strain was using arginine as efficiently as the LA strain. The growth difference noted between the two strains fed ad libitum a crystalline amino acid diet with an amino acid pattern different from casein was considerably less than when casein was fed ad libitum (Nesheim, 1968b).

The levels of kidney arginase in the two strains have been studied and determined not to be different at hatching or when fed commercial chick starters with adequate arginine. However, when an arginine deficient casein diet was fed, kidney arginase increased four to five times in the HA strain after four to five days but remained low in the LA strain. The difference in arginase activity was still observed when the strains were pair-fed.

Urea excretion was not increased in either strain at

six hours probably because the arginase activity or level had not yet adapted to the excess amino acid. At later intervals, urea excretion was increased as the dietary level of lysine increased, and as much as 12.7 percent of the dietary arginine was recovered in the excreta of chicks fed excess lysine. No significant losses of arginine in the urine of the HA strain were observed when they were fed the casein basal diet. The effects of excess lysine or arginine metabolism occurred within a short time, before kidney arginase responded to the excess, and Nesheim (1968b, 1968c) concluded that the HA strain appeared to metabolize lysine more slowly and that the increased arginase levels could be due to an accumulation of lysine in the body amino acid pools.

Based on all the information published on the relationship of lysine and arginine in a casein diet, one can only conclude that the mechanism of the interaction remains to be elucidated.

Other amino acid interactions have been reported, but the mechanism involved is one of competition for absorption site, e.g., the interaction between leucine, isoleucine and valine (Benton et al., 1956), or one of an in vivo conversion of one amino acid to another (Sugahara and Ariyoshi, 1967; Baker et al., 1968; Akrabawi and Kratzer, 1968).

A reference diet of crystalline amino acids has been

developed by Dean and Scott (1965) and has been demonstrated to approximately meet the need of the chicken for all of the amino acids. The pattern is unlike that of casein and includes only one percent of arginine.

Much mention has been made of the symptons of an arginine deficiency; e.g., Sanders et al. (1950) noted abnormal feather development with the appearance of spoon shaped feathers and an abnormal gait, but only one extensive report has been published on the symptoms of arginine deficiency in the chick (Newberne et al., 1960). In this report are described the symptoms of an arginine deficiency developed by feeding a 35 percent casein diet to chicks. An ataxia was observed which could not be related to the muscle creatine content, as the muscles of chicks with muscle paralysis contained as much creatine as the muscles of birds which exhibited no gross symptoms. Other gross symptoms observed included poor growth and feathering and leg weakness. Histopathologically, lesions were observed in the feathers, bone, striated muscle, nerve, liver and lymphoid tissues of all birds on the casein diet; and the degree of severity of the lesions roughly followed the pattern of muscular paralysis. Bone lesions included a flattening and lateral rotation of the distal third of the tibia which directed the condyle from its normal axis and caused the legs to splay out from the hock joint. Microscopic

examination of bone revealed a depressed rate of bone growth with a narrow epiphyseal disc, degerative and pyknotic osteocytes and complete lack of osteocytes in some areas.

Muscle atrophy was observed and was most pronounced in the muscle groups of the legs. Muscle fibers were smaller than normal and lacked the characteristic striations, and in some cases, the muscle fibers had atrophied, leaving only the connective tissue stroma which contained an abnormal number of small, dark nuclei.

Loss of nissl substance and shrinkage of the neurons with some degeneration and demyelination was observed in the sciatic nerve and the lateral motor column of the lumbar segment of the spinal cord.

Liver cells appeared shrunken and detached, especially near the center of the lobule. Overt necrosis was not observed, and the changes noted by Jungherr et al. (1958) in the liver cord cells were not observed.

The thymus and bursa of Fabricius were small and atrophic, resembling conditions which are seen in other types of stress.

The feathers were frizzled in appearance, especially on the dorsal surface of the wing. The feathers were characterized by a curved shaft, poorly developed secondary components, brittleness, loss of epithelium and sclerosis of the feather follicles.

To be able to determine the effect of a particular dietary regime on kidney arginase of the bird, a convenient, accurate method of measuring arginase activity is required. All of the current methods utilize the production of urea, after a period of incubation of the enzyme with arginine, as a measure of enzyme activity. The urea produced can be measured by reacting with a number of color producing reagents or by hydrolysis of the urea with urease followed by titration of the ammonia released. Perhaps the most used color reagent is α-isonitrosopropiophenone which was first used for the determination of urea by Archibald (1945). A year earlier he used the same color reagent for the determination of citrulline and allantoin (Archibald, 1944); so if these compounds are present in a sample to be analyzed for urea, a false, high reading will result.

The determination, in general, involves homogenizing the kidneys in a suitable buffer; preincubation of the homogenate with a buffer, usually glycine, and an activating ion, usually manganese; addition of the substrate, arginine; incubation for a set length of time; stopping the enzymatic reaction with an acid which will precipitate the enzyme; and, finally, measuring the amount of urea produced (Schimke, 1962a; Smith and Lewis 1963; Tamir and Ratner, 1963a; O'Dell et al., 1965; Kean, 1967; Carulli et al., 1968). Since arginase is strongly inhibited by ornithine (Hunter and

Downs, 1945; Van Slyke and Archibald, 1946), the amount of substrate should be in excess so that the retardation resulting from ornithine is insignificant. Arginine has been reported by Van Slyke and Archibald (1946) to decrease appreciably the color developed in the reaction between urea and α-isonitrosopropiophenone; hence, the same amount of arginine should be added to the blank and standards as to the samples. If the enzyme is not activated prior to introduction of the substrate, Van Slyke and Archibald (1946) note that two to four times as much of the enzyme preparation should be used. The use of urease to measure the amount of urea produced by arginase was discounted by Smith and Lewis (1963), as it was not found possible to halt the arginase reaction in such a way that conditions remained appropriate for urease action.

Arginase is activated by a number of divalent metal ions. Arginase activity was found to be enhanced by divalent cobalt, nickel, manganese and iron with divalent cobalt shown to be the most effective in vitro activator (Hellerman and Perkins, 1935). Manganese was the only metal ion which Richards and Hellerman (1940) found to be able to restore some activity to partially inactivated arginase. It is interesting to note that Smith and Lewis (1963) found that manganese at increasingly higher levels actually decreased arginase activity in an unknown manner, and that the use of

a 0.001 M manganese sulfate-maleate buffer solution produced the most desirable compromise between the need to avoid the undesirable effects and the necessity to activate the enzyme.

The pH of the incubation mixture is of critical importance in the arginase assay. A pH optimum of 9.8 for arginase was reported by Hunter and Morrell (1933), and they reported a rapid fall of activity on the alkaline side of the pH optimum. In their report on the activation of arginase by metal ions, Hellerman and Perkins (1935) suggested that the pH optimum of arginase was 7.5 when divalent nickel was the activating ion. A similar pH optimum for arginase activated by nickel was reported by Hellerman and Stock (1938). They also reported that the pH optimum for manganese activated arginase was approximately 10. The optimum pH for arginase activation by divalent manganese, nickel, cobalt and iron was reported by Kocholaty and Kocholaty (1941) to be 9.5. A decrease in arginase activity was noted by Mohamed and Greenberg (1945) when divalent nickel or cobalt were used as activators at a pH of 7 to 8. More recent reports by Bach and Killip (1960) and by Smith and Lewis (1963) confirm the observations of most earlier workers that the pH optimum of arginase is approximately 9.5.

The amount of urea produced in the <u>in vitro</u> arginase assay is usually expressed as the number of micromoles of

urea produced per gram of fresh tissue per hour. No values for the arginase activity of the Japanese quail are available in the literature. However, a few values have been reported for chicken kidney arginase. An average value of 6880 μ moles of urea per gram of fresh tissue per hour was reported by Tamir and Ratner (1963a), and an average value of 10,600 μ moles of urea per gram of fresh tissue per hour was reported by Nesheim (1968c) in a high arginine-requiring strain of White Leghorns on an arginine deficient diet. An average value of 7000 was given for the same birds and a low arginine-requiring strain when fed a diet containing adequate arginine. The latter value is in close agreement with the value reported by Tamir and Ratner (1963a).

Plasma Amino Acids

Plasma amino acid analyses have been used to study the effects of many aspects of protein and amino acid nutrition. Many factors are known to affect the plasma amino acid pattern, and a few of these will be briefly discussed.

According to Harper (1968), the plasma amino acid concentrations reflect the balance between the entry of amino acids into the blood from the food that is eaten and digested, from tissue protein breakdown and the exit of amino acids from the blood to the tissues where they are used for protein synthesis and removed by degradative reactions.

For a plasma amino acid pattern to have any meaning, the changes in the pattern must be observed and quantitated under rather standard conditions. It would be very desirable to be able to predict the effect of supplementing one amino acid in the diet on the blood amino acid pattern, and, once the effect is known, to be able to correlate the blood pattern and the dietary amino acid levels so that dietary deficiencies of amino acids can be diagnosed.

Supplementation of a single amino acid, lysine, in the diet has been demonstrated by Richardson et al. (1953) to always lead to an increased lysine concentration of that amino acid in the blood of chicks. Addition of lysine to a wheat gluten diet has a similar effect on the plasma lysine level in rats (Morrison et al., 1961). They observed an initial lag in plasma lysine level after which the level rose rapidly in response to the increased dietary lysine level. They reported that until lysine ceased to be the factor limiting growth, plasma free lysine levels were much less influenced by dietary lysine concentration than were weight gains. Because of this fact, measurement of free lysine levels alone may provide a misleading estimate of the adequacy of the dietary lysine content. A reciprocal relationship between plasma lysine and threonine was noted; with increasing lysine, plasma threonine decreased until it leveled out at a dietary lysine concentration which appeared to meet the rat's requirement for lysine.

Lysine has been reported to accumulate in the plasma of chicks only after the dietary concentration was high enough to permit maximum weight gain (Zimmerman and Scott, 1965; Scott, 1967). Similar results were demonstrated for arginine and valine. Thus, the authors demonstrated that the first limiting dietary amino acid remains at a very low level in the blood irrespective of the severity of the deficiency, and supplementation of the first limiting amino acid may fail to increase the plasma level of that amino acid if the requirement has not yet been met. A reciprocal relationship between lysine and threonine as earlier reported by Morrison et al. (1961) was also reported. A severe deficiency of either lysine or arginine markedly increased plasma threonine and each increment of the first limiting amino acid (lysine or arginine) resulted in a progressive decline of plasma threonine. Plasma threonine did not reach a minimum level until the dietary concentration of the first limiting amino acid was somewhat in excess of the level required for maximum weight gain.

The detection of a dietary amino acid deficiency is possible, as the first limiting amino acid has been demonstrated to decrease in the plasma within eight hours after ingestion of a diet deficient in an amino acid (Clark et al., 1966). When a lysine-deficient diet was fed to chicks, a decrease of plasma lysine occurred which was accompanied

by an increase in most of the other amino acids, with the essentials increasing more than the nonessentials (Dean and Scott, 1966). The increased level of most of the other amino acids, when one is limiting, was explained on the basis that an insufficient amount of the limiting amino acid at the sites of protein synthesis limited protein synthesis and resulted in a decreased uptake of all the amino acids except the limiting one. The authors concluded that plasma amino acid patterns could be used to detect amino acid excesses and deficiencies but not necessarily the order in which the amino acids are limiting or in excess.

All of the research discussed thus far has pertained to detection of a deficiency or excess of a single amino acid, usually in a crystalline amino acid mixture. Plasma amino acid patterns have also been determined after the consumption of an intact protein, and Charkey et al. (1953) reported that blood amino acid levels, in general, reflected the amino acid composition of the ingested protein, while Frame (1958) has demonstrated that the postprandial increase of individual amino acids in the plasma did not correspond to the relative amino acid composition of the protein ingested. The discrepancy appeared to be due not only to differences in the efficiency of digestion and absorption but also to the relative tissue requirement for the individual amino acids. The

most limiting amino acid in a protein has been shown to be the one which exhibited the lease increase in the plasma (Longenecker and Hause, 1959), and these authors also postulated that those amino acids which were most needed by the tissues would disappear most rapidly from the plasma. It is interesting to note that Longenecker and Hause (1959) found arginine to be the first limiting amino acid in casein for the dog. In addition, they concluded that supplementation of a protein with a free amino acid is a practical method to use to correct a deficiency, as the absorption rate of the free amino acid into the blood is similar to that of the amino acids liberated by the digestion of the protein. Therefore, all of the essential amino acids will be at optimal concentration in the blood at the same time for protein synthesis, i.e., no time lag.

The essential amino acid pattern of the plasma was reported by Kumta and Harper (1962) to reflect the dietary essential amino acid pattern, while the nonessential amino acids showed no characteristic pattern. In general, the essential amino acids, as a group, have been reported to be absorbed more rapidly than the nonessential amino acids which has resulted in greater increases in the plasma levels of the essential than the nonessential amino acids (Adibi et al., 1967). They also reported that lysine absorption was competitively inhibited by arginine when the two were per-

fused together in an equimolar mixture. The highest feed efficiency was obtained when the dietary nonessential to essential amino acid ratio ranged between 1.0 and 1.5, and the growth rate was reported to be greatest when the ratio was 1.5 (Sugahara and Ariyoshi, 1968). The authors also reported that the nonessential to essential amino acid ratio in the blood was strongly influenced by the ratio in the diet.

The feeding of a nonprotein diet to chicks and rats has been reported to result in a decrease in the plasma concentration of the essential amino acids below the level noted when the animals were fasted or fed a low protein diet (Hill and Olson, 1963; Zimmerman and Scott, 1967; Bergen and Purser, 1968).

A large increase in the plasma lysine concentration has been reported to occur during fasting (Charkey et al., 1953; Almquist, 1954; Hill and Olson, 1963; Zimmerman and Scott, 1967). The starved animal draws first upon the dispensable portion of soft tissues, i.e., blood and muscle. The lysine content of these soft tissues of the chicken is relatively high in comparison to the lysine content of the protein of the entire chick. This presents a condition in which, in effect, the starved chicken is nourished by a source of protein, particularly rich in lysine. The surplus of lysine

in such a protein, over the general requirement for all tissues of the chick, may be the reason for the exaggerated elevation of lysine in the blood (Almquist, 1954).

The plasma amino acid pattern has also been studied following the ingestion of an imbalanced protein. According to Harper (1959), an imbalance is any change in the proportion of amino acids in a diet that results in an adverse effect which can be prevented by supplementing the diet with a relatively small amount of the most limiting amino acid or acids. An imbalanced protein is usually created by adding a balanced mixture of essential amino acids, complete except for the first limiting amino acid of the protein, to a low level of the protein (Harper et al., 1964). The mixture of amino acids added to create the imbalance cannot be used for protein synthesis because it lacks one of the essential amino acids. The feeding of such a mixture in some way depresses growth and feed consumption to a level below that of animals receiving the low protein diet without the addition of the amino acid mixture, even though the amount of the limiting amino acid was the same in both cases (Harper et al., 1964). The author reported that a decrease in the plasma level of the limiting amino acid resulted which led to an increase in the levels of the other amino acids and caused a blood pattern similar to a much more severe deficiency. Such a blood pattern apparently triggers an appetite depressing mechanism

which depresses food intake which, in turn, acts as a protective response. In fact, a protein-free diet has been shown to be consumed in preference to a diet containing an imbalanced mixture of amino acids. The problem cannot be attributed to poor utilization of the limiting amino acid, as Harper et al. (1964) demonstrated that the limiting amino acid was apparently absorbed as well as, and not catabolized any faster than, the amino acid in the control diet. In that same year, Fisher and Shapiro (1964) confirmed by a paired-feeding technique, an earlier report (Fisher et al., 1960) that there apparently was no loss of efficiency with which the protein and limiting amino acid of an unbalanced ration were utilized. Thus, the growth depression observed when an imbalanced protein was fed could only be related to the reduced food intake.

A later report by Yoshida et al. (1966) suggested that incorporation of the limiting amino acid of an imbalanced protein into liver protein was actually enhanced by the imbalance, and that total retention of the limiting amino acid was slightly greater as a result of the imbalance.

The changes evident in the amino acid pattern of the blood occur within a short time after ingestion of a single meal, and their duration and magnitude depend upon the amount of amino acids added to create the imbalance and the size of the meal consumed. Such changes persist for several hours

after a meal and seem to be reinforced by consecutive meals (Harper, 1968).

From their recent research, Nethe et al. (1969) concluded that excess amino acids in a variety of patterns and concentrations do not impair the utilization of the first limiting amino acid even though their presence in the diet decreased feed intake and hence weight gain. In all instances, weight gain was reported to be a function of the absolute intake of the first limiting amino acid.

Based on the observations of all of the workers which have been discussed, it appears that the plasma amino acid pattern following the ingestion of an imbalanced protein is one which is very low in the most limiting amino acid and relatively high in the amino acids which were added to imbalance the protein, with the rest of the amino acids, following no predictable pattern.

An amino acid interaction which has created much interest is that between arginine and lysine, as discussed earlier. Several reports have appeared which discuss the plasma arginine and lysine levels that result from a relative arginine deficiency caused by an excess of lysine, as occurs in casein. Lysine supplementation of a peanut meal diet was reported to result in an increase in the plasma lysine and arginine levels (Richardson et al., 1953). In this case,

lysine was the limiting amino acid and was merely supplemented to requirement levels, i.e., lysine was not in excess. The addition of lysine to a diet containing 18 percent casein and 10 percent gelatin resulted in increased plasma and muscle lysine concentrations and decreased plasma and muscle arginine concentrations (Jones, 1964). An increase in plasma lysine on an arginine deficient diet has been observed by Zimmerman and Scott (1965). The addition of one percent excess lysine to a crystalline amino acid reference diet resulted in a decrease in plasma arginine (Dean and Scott, 1966). Similar results were reported by Jones et al. (1967) and by Squibb (1968). The addition of arginine to correct an excess of lysine does not cause an increase in the plasma arginine level until the arginine requirement for maximal growth is slightly exceeded (Zimmerman and Scott, 1965; Scott, 1967).

Thus, the effect of various dietary protein intakes on the plasma amino acid patterns can be summarized as follows:

(1) Plasma amino acid levels generally reflect, although not precisely, the amino acid composition of the ingested protein.

(2) A dietary deficiency of a single amino acid usually results in severely decreased levels of that amino acid in the plasma, but the dietary adequacy of an amino acid cannot always be diagnosed by relying on the plasma amino acid pattern, as the amino acid will be at a low level in the plasma until the requirement for for growth is slightly exceeded.

(3) In fasting, protein stores are catabolized to help meet the basal energy needs and amino acids accumulate in the blood, and these levels can be reduced, although not to normal, by the feeding of a nonprotein diet which supplies energy.

(4) A decrease in plasma arginine concentration can be expected when an excess of lysine relative to arginine exists in the diet. Such is the case for casein.

III. EXPERIMENTAL PROCEDURE

Introduction

Ten experiments were conducted to study the role of nickel in the nutrition of the Japanese Quail (Coturnix coturnix japonica). The experiments consisted of a continuing generation study in an attempt to deplete successive generations of quail of any possible body stores of nickel and, in turn, produce symptoms of a nickel deficiency. A second series of experiments, conducted simultaneously with the generation study, dealt with a postulated involvement of nickel in the activation of the enzyme, arginase, in which attempts were made to induce at least a partial in vivo activation of the enzyme by nickel. This was accomplished by altering the dietary levels of arginine and manganese in an effort to increase the demand for an active arginase while at the same time removing its normal in vivo activator, manganese. Appropriate nickel-supplemented control quail were also carried through each of the 10 experiments. These experiments were as follows:

Experiments I and II	Development of purified basal diet and establishment of first generation of low-nickel and nickel-supplemented quail.

Experiment III	L-arginine hydrochloride added to diet of one-half of birds at each level of dietary nickel.
Experiment IV	Development of second generation of low-nickel and nickel-supplemented quail.
Experiment V	Basal diet modified to contain 10 percent less casein and one percent of supplemental tyrosine; otherwise, the same as experiment III.
Experiment VI	Same as experiment V except manganese level of basal diet lowered.
Experiment VII	Development of third generation of low-nickel and nickel-supplemented quail; basal diet (presented in table 6) used in this and all subsequent experiments.
Experiment VIII	Repeat of experiment III.
Experiments IX and X	Development of fourth generation of low-nickel and nickel-supplemented quail.

Alterations were made in the purified basal diet as results of previous experiments deemed it necessary. Alterations in the dietary protein level, the tyrosine and arginine levels and the manganese level were made in attempts to alter arginase activity, and to establish the effects of supplemental nickel under each of these dietary conditions.

General Conduct of Experiments

The quail used in experiments I, II, III, V, VI and VIII were all hatched from eggs obtained from the Japanese quail

maintained by the Poultry Science Department at Michigan State University. The quail used in experiment IV (second generation) were hatched from eggs collected from the experiment I birds. The quail used in experiments VII (third generation) and VIII were offspring of the experiment IV birds, and the birds used in experiments IX and X (fourth generation) were offspring of the experiment VII birds. Eggs were collected from the first three generations of quail when the birds were between 10 and 12 weeks of age, or approximately between the fourth and sixth weeks of egg production. The quail used in experiment VIII were also hatched from eggs collected from the second generation birds (experiment IV), but when the birds were between 17 and 19 weeks of age or approximately between the 11th and 13th weeks of egg production. The quail used in experiment X were progeny of the third generation birds (experiment VII) and were hatched from eggs collected at 27 to 29 weeks of age or between the 21st and 23rd weeks of egg production. All eggs were hatched in commercially available chicken egg incubators.

The quail were removed from the incubator within 24 hours of hatching, weighed, leg banded and placed in plastic isolators which had been originally designed for gnotobiotic research. Housing of the birds in isolators was necessary to prevent exposure of the quail to airborne nickel contaminants known to arise from factories, automobile exhausts and tobacco

smoke. Weights were recorded to the nearest one-tenth of a gram, and the quail were weighed at weekly intervals. Birds raised to adults for the production of a succeeding generation were weighed weekly for the first six weeks and usually not again until just prior to exsanguination.

The quail were identified, to two weeks of age, with leg bands which were applied at the initial weighing. The leg bands were fabricated from a small strip of white paper onto which the identifying number was written. The paper was protected from moisture, on both sides, by transparent tape. The number was affixed to a small strip of masking tape by an equal sized strip of transparent tape. The band was held around the bird's leg by pressing together the free adhesive ends of the masking tape. To prevent loss of the bands, another small strip of masking tape was wrapped around the ends of the leg band. The use of this type of band was necessary as no nickel-free bands, of the proper size for banding day-old Japanese quail, could be purchased. At two weeks of age, the birds were wing banded with size #896 aluminum bands[1].

The isolators used for rearing the quail to four weeks of age were of rigid plexiglass construction (figure 1)[2].

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[1]National Band and Tag Co., Newport, Kentucky.
[2]Germfree Laboratories, Inc., 5644 N. W. 7th Street, Miami, Florida 33126.

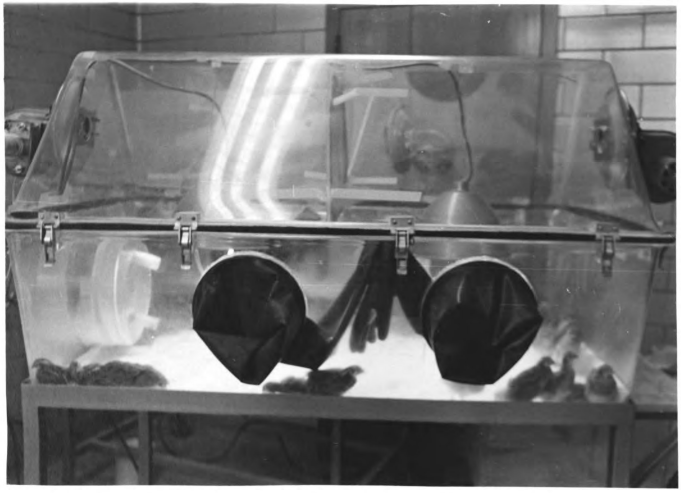

Figure 1. Isolator set up as used in the experiments.

The isolators were equipped with glass fiber filters[3] capable of removing particles of 0.3 micron diameter or larger. To eliminate other environmental contamination, plastics, which contained undetectable nickel levels, were used for feeders and waterers. The feeders were 2.2 x 5 x 50 cm for the growing birds and 4.5 x 7.5 x 50 cm for the adult birds. A small piece of all-plastic poultry netting[4] was placed on top of the feed to prevent feed wastage, and marbles were placed in the waterers during the first two weeks post-hatching to prevent drowning of the young chicks. All surfaces in contact with the birds were repeatedly washed with 6 N hydrochloric acid and rinsed with deionized, distilled, steam condensate to reduce the chance of nickel contamination from one experiment to the next. After thorough cleaning at the conclusion of an experiment, the isolators were disinfected with a 2% peracetic acid solution. The floors of the cages were covered with uninked newsprint to absorb the excreta. This paper was changed daily.

Heat and light were provided by two incandescent bulbs per isolator. Initially one bulb was a 150-watt bulb and the second a 100-watt bulb. The bulbs were placed in reflector

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[3]Fiberglass High-efficiency Filtration Media, Owens-Corning Fiberglass Corp., Toledo, Ohio.

[4]Thornber Bros., Ltd., Mytholmroyd, Halifax, Yorkshire, England.

holders suspended from the top of the isolator such that the light bulbs were approximately 43 cm from the floor. The temperature at floor level was approximately 38° C below the 150-watt bulb and 34° C below the 100-watt bulb. This light arrangement was used in experiments I, IV, VII, IX and X where only one treatment group was housed in each isolator. In experiments II, III, V, VI and VIII where two treatment groups were housed in each isolator, 150-watt bulbs were initially used in both ends of the divided isolator.

In all trials, bulb wattage was reduced from 150 to 100 at the end of one week to reduce the floor level temperature at both ends of the isolators to approximately 34° C. The temperature was further reduced at the end of two weeks to approximately 30° C and to room temperature, 24° C, by the end of the fourth week. Hence, lighting was continuous from the time the birds were placed in the incubator until they were four weeks of age.

At four weeks of age, the quail of experiments II, III, V, VI, VIII, IX and X were killed. Birds from experiments I, IV and VII, which were raised to adults and used for the production of the succeeding generation, were moved to balloon type isolators[5] and were housed on plastic flooring[6]

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[5]Product #4205-2, Standard Safety Equipment Co., Palatine, Illinois.

[6]Ashlar Louver, Eggcrate type, Item No. 449, Armstrong Cork Co., Lancaster, Pa.

which permitted the excreta to fall onto a plastic covered, metal pan below. The pan was covered with uninked newsprint which in turn, was covered with an inert absorbant[7] that served quite well to absorb the moisture and to keep the humidity and ammonia levels within the isolators at low levels. The pans were removed and cleaned at intervals of not longer than three days, depending upon the number of birds in the isolator.

The temperature in the cages for the adult quail was approximately 24° C (room temperature). The light regime for the birds from four weeks of age was 16 hours of light followed by 8 hours of darkness.

Feed and water were provided ad libitum throughout, and feed consumption was recorded as accurately as possible. Drinking water was glass-distilled, deionized distilled steam condensate and was provided fresh daily. The purified basal diet was similar to that used by Scott and Thompson (1967) and was modified to meet the requirements of the Japanese quail as far as they are known. The composition of the basal diet is shown in table 6. In the experiments where arginine and/or tyrosine were added to the basal diet, they were added at levels of 1.1 and 1.0 percent, respectively, at the expense of glucose. The casein content of the basal

[7] Eagle Picher Floor Dry, Eagle Picher Industries, Inc., Cincinnati, Ohio 45202.

TABLE 6. COMPOSITION OF BASAL DIET

	%
Casein[1]	35.0
Glucose[2]	41.8 (37.7)[5]
Corn Oil	5.0
Cellulose[3]	3.0
L-Glutamic Acid	5.7
L-Glycine	1.4
Mineral Mixture	6.7 (10.8)[5]
Vitamin Mixture	8.34 g/kg
D-α-tocopheryl acetate	300 mg/kg
Retinyl Palmitate	30 mg/kg
Choline Chloride	6000 mg/kg
Antioxidant[4]	0.0125

[1]High protein casein purchased from General Biochemicals, Chagrin Falls, Ohio.
[2]Cerelose purchased from Corn Products Company, Argo, Illinois.
[3]Solka-floc purchased from Brown Company, Chicago, Illinois.
[4]Santoquin provided through courtesy Monsanto Chemical Co., St. Louis, Missouri.
[5]Figures in parentheses indicate levels of glucose and mineral mixture in the basal diet for laying adults.

diet was reduced from 35 to 25 percent in experiments V and VI with an increase in glucose from 41.8 to 51.8 percent of the diet.

The final level of each of the minerals included in the diet is shown in table 7. The minerals were added to the rest of the diet as a premix, the formulation of which is given in table 1 of the appendix. The calcium level of the diet was increased to 2.8 percent at five weeks of age for those birds which were kept for the production of eggs. The inclusion of the increased calcium was made at the expense

TABLE 7. MINERAL COMPOSITION OF BASAL DIET

Element	PPM	Element	PPM
Calcium	11950 (28450)[1,2]	Zinc	88.8[2]
Phosphorus	10820[2]	Copper	12.4[2]
Potassium	4752	Iodine	5.9
Chloride	4207[3]	Molybdenum	3.2
Sodium	2494	Fluorine	1.0
Magnesium	1401	Cobalt	0.5
Sulphur	143	Selenium	0.1
Iron	90.9[2]	Chromium	0.1
Manganese	90	Nickel[4]	

[1]Calcium concentration of basal diet, used for laying adults was 28450 ppm.
[2]Includes amount furnished by casein.
[3]Includes chloride from hydrochloride forms of amino acids and vitamins used in basal diet.
[4]Nickel added at 2 ppm level to ½ of birds in all experiments except experiments V and VI where 5 ppm level of nickel was used.

of glucose. No change was made in the phosphorus level. All minerals used in the formulation of the mineral premix were of analytical reagent grade quality. The further purification of four of these will be discussed at a later point.

The final concentrations of vitamins in all diets used are given in table 8. The vitamins were also added to the diet as a premix, the formulation of which is given in table 2 of the appendix. Vitamins A and E and the antioxidant were mixed with the corn oil before incorporation into the diet. The choline chloride was added separately because of its hygroscopic nature.

TABLE 8. VITAMIN COMPOSITION OF BASAL DIET

Vitamin	PPM	Vitamin	PPM
Nicotinic Acid	210.0	D-α-tocopheryl acetate	300
Calcium Pantothenate	90.0	Retinyl Palmitate	30
Thiamine Mononitrate	30.0	Menadione	3.6
Riboflavin	30.0	Cholecalciferol	0.06
Pyridoxine HCl	30.0	Choline Chloride	6000
Folic Acid	12.0	Inositol	390
D-biotin	0.33	Ascorbic Acid	240
B_{12}	0.0408	Para-amino Benzoic Acid	39

All diets were mixed in a plastic shell blender[8] equipped with a rapidly revolving stainless-steel blending bar through which the corn oil, vitamins A and E and the antioxidant were added. Analysis of the mixed feed for nickel indicated that no nickel contamination of the feed occurred as a result of coming into contact with the blending bar. Eight kilograms of diet could be mixed at one time. To insure that the diets were identical except for their nickel content, two successive mixings (8 kg each) were performed on a nickel-low diet. The diets were mixed for a total of 35 minutes. Each of these mixes was then divided and one-half (4 kg) of each mix was blended together for five minutes for the final nickel-low diet. Nickel (as nickel carbonate except for experiment X

[8]The Patterson-Kelley Co., Inc., East Stroudsburg, Pennsylvania.

where nickel chloride was used)was then blended for 15 minutes into the remaining one-half of each mix.

Small amounts of the diets were scattered on the newsprint to encourage feed consumption in experiment I only. In subsequent trials, the birds readily consumed feed directly from the feeders.

Experiments II, III, V, VI, VIII, IX and X were conducted for a period of 28 days. Experiments I, IV and VII were continued until the succeeding generation was hatched. Records were kept of the number of eggs laid and of individual egg weights for experiments I, IV and VII. Individual laying cages were not used and consequently some cracking and breakage of eggs did occur. Prior to incubation, eggs were stored at 15° C until a sufficient number had been collected to insure an adequate number of chicks for the next generation. Consequently, the eggs from the first generation adults were stored 18 days, eggs from the second generation adults were stored 21 days and eggs from the third generation adults were stored 14 days before incubation. Chicks were removed from the incubator until the end of the 19th day of incubation at which time hatchability and fertility data were collected. Fertility was checked by visual appraisal after breaking of the unhatched eggs.

Approximately 150 quail chicks could be started in the isolators, which had a floor area of 7432 cm^2. If more than

100 birds survived to two weeks, the number was decreased to 100 by random selection to prevent overcrowding. The 100 surviving birds then had 74 cm^2 of floor area per bird. A further reduction in numbers occurred at four weeks in those experiments where birds were kept for egg production. The number of birds per isolator was reduced to approximately 30 with a ratio of one male to three females. Each bird then had 250 cm^2 of floor space.

At the conclusion of each trial, the birds were killed by decapitation. Pooled blood samples for plasma amino acid analysis were collected in test tubes to which two to three drops of heparin had been added. Care was taken to avoid contamination of the blood by crop contents. Each pooled sample represented an equal number of males and females. Two blood samples for plasma amino acid analysis were collected from each treatment of each experiment except experiments VII, IX and X where no blood was obtained. In addition to blood taken for amino acid analysis, heparinized blood was also collected from birds on these same experiments for determinations of plasma nickel, total plasma protein, hematocrit and hemoglobin. Tissues for nickel and arginase assays were removed immediately after killing each bird, frozen immediately in a dry ice-acetone mixture and stored at -80° C until assayed. Tissues collected included the

lungs, pancreas, liver and both kidneys. No tissues were collected from the birds of experiments II, VII and X. At the conclusion of each experiment, two quail from each treatment were necropsied and observations made for gross- and histopathology.

Analytical Procedures

Hemoglobin

Hemoglobin was determined by the cyanmethemoglobin method of Crobsy, et al. (1954). A Coleman Junior II spectrophotometer was used for the optical density determinations.

Hematocrit

Hematocrit was determined by the micro method (McGovern et al., 1955). Blood samples were centrifuged for five minutes at 6000 x g in an International "Hemacrit" centrifuge.

Total plasma protein

Total plasma protein was determined by Miller's (1959) modification of the Lowry method. Ten µl of plasma were added to 5.0 ml of deionized, distilled water, and 1 ml of the resulting dilution was used in the determination.

Feed analyses

Analytical values for calcium, phosphorus, magnesium,

zinc, copper, iron and manganese were obtained as a check on the calculated content of the diet for each element and as a check on the uniformity of mixing of the feed. Assays, with the exception of phosphorus, were carried out by atomic absorption spectrometry following a wet-ashing procedure. Approximately 0.5 g of feed was digested using 30 ml of concentrated nitric acid and 3 ml of concentrated perchloric acid. The assays were run after appropriate dilutions. Phosphorus was determined in the same digest by the method of Gomori (1942) and read on a Coleman Junior II spectrophotometer.

Dietary protein (N x 6.25) determinations were carried out using the semi-micro Kjeldahl technique. A Kjeldahl determination was also made on the casein used as the protein source in all the purified diets.

Energy density determinations of the diets were carried out in a Parr adiabatic bomb calorimeter.

Plasma Amino Acid Analysis

Plasma amino acid analyses were performed on two pooled plasma samples from each treatment from all experiments except VII, IX and X. Determinations were carried out on a Technicon TSM Amino Acid Analyzer following removal of serum protein by sulfosalicylic acid precipitation. Samples were

prepared by adding 0.1 ml of norleucine and 0.1 ml of 50 percent sulfosalicylic acid to 1.0 ml of the pooled plasma samples. Either 50 to 100 µl of the resultant supernatant were then used for the analyses.

Nickel Analyses

Nickel analyses were performed on all the individual ingredients used in formulating the purified diets, with the exception of the crystalline vitamins and amino acids, as well as on the mixed diets. All samples were assayed at least in triplicate.

A solvent extraction procedure, as described by Mulford (1966), was utilized in all nickel analyses. Nickel analyses on the individual minerals used in the diet were performed after dissolving approximately 5 g of each mineral in 30 ml of 6 N hydrochloric acid, followed by the extraction to be discussed later. Approximately 10 g each of casein, cellulose, glucose and of the low-nickel diets were digested in 120 ml of concentrated nitric acid plus 7 ml of concentrated perchloric acid in 250 ml Phillips beakers. After digestion all samples were diluted to an equal weight (approximately 50 g). Approximately 0.25 g of the 5 ppm nickel diets and 0.40 g of the 2 ppm nickel diets were digested in the same volumes of acids and diluted to the same final volumes. Nickel

standards (see appendix table 3 for preparation of nickel standards) were carried through the same digestion procedure and diluted the same as the unknowns. Ten ml of nickel standard were added to at least one unknown sample of all materials analyzed to check for nickel recovery. As 10 ml of an aqueous solution of each nickel standard were carried through the digestion, 10 ml of redistilled, deionized distilled water were added to each of the unknowns to compensate for any possible nickel contamination of the water in which the nickel standards were diluted.

After digestion of samples and standards, all were quantitatively transferred from the Phillips beakers, by repeated rinsing with redistilled, deionized distilled water, into 120 ml Erlenmeyer flasks. The final net weight of all samples and standards was the same. The pH of all samples and standards was adjusted to 1.8 using 1 N distilled ammonium hydroxide and, where necessary, 1 N distilled hydrochloric acid. Two ml of a 5 percent aqueous solution of ammonium pyrrolidino dithiocarbamate (APDC) were added, and the flasks were shaken gently to insure complete chelation of all nickel present. The APDC was purified before use by shaking with an equal volume of methyl isobutyl ketone (MIBK). Five ml of MIBK were added, and the chelate dissolved in the MIBK by gentle swirling of the contents of each flask for

approximately one minute. Redistilled, deionized distilled water was then added to all flasks to bring the solvent layer into the neck of the flask. The solvent layer was then aspirated directly into the air-hydrogen flame of a Jarrell-Ash model 82-516 atomic absorption spectrophotometer equipped with a Hetco total consumption burner. An absorption wavelength of 2320 Å was used and the responses recorded on a Sargent Model SRL recorder. All glassware was cleaned by boiling in aqua regia.

Attempts were made to analyze the tissues of the quail for nickel both by neutron activation analysis and by atomic absorption spectrophotometry. Under the conditions employed for activation analysis, nickel was not detected even in the nickel standards, although neutron activation analysis has been successfully employed for nickel (Guinn, 1968). Nickel analysis was carried out on a group of liver samples from experiment VI. Livers from 8 to 10 birds (12 to 15 g of fresh liver tissue) were pooled into 250 ml Phillips beakers, 10 ml of a 0.075 ppm nickel standard added to all samples and the analysis completed as with the feed samples.

An attempt was made to digest the plexiglass and plastic used for feeders and waterers. A nickel analysis was carried out on the acid extract from each of the materials, as they were not digested by either nitric or perchloric acid. The analysis of the extract was completed as with

the digest from the feed samples. No nickel was detected, and the materials were assumed safe for use in the experiments.

Purification of Dietary Components

Purification of the cellulose, cobalt carbonate, calcium carbonate, magnesium carbonate and calcium phosphate was attempted after the nickel analysis of each indicated a relatively high concentration of nickel.

Cellulose

The cellulose was placed in an acid-washed, plastic bucket and made into a slurry by adding 4 N hydrochloric acid. After stirring for 5 to 10 minutes, the slurry was placed directly into a large Buchner funnel and vacuum applied until dry. The cellulose was then resuspended in triple distilled, deionized water and refiltered. The washing procedure with water was carried out three times to make certain that all the hydrochloric acid had been washed out. The cellulose was then oven dried for 12 hours at 90° C.

Calcium carbonate, magnesium carbonate and calcium phosphate

All three of these minerals were treated in a like manner. They were suspended in triple distilled, deionized water. One part of a saturated ethanolic solution of

dimethylglyoxime was added for every 10 parts of slurry. The mixture was stirred with a glass stirring rod for four hours and then filtered to dryness. The minerals were then resuspended in absolute ethanol to assure that all of the dimethylglyoxime was washed out, filtered, washed three times with triple distilled, deionized water and oven dried for 12 hours at 90° C.

Cobalt carbonate

Cobalt carbonate was essentially freed of its nickel contamination by using the ion-exchange method of Krause and Moore (1953). Approximately 50 g of cobalt carbonate were dissolved in 500 ml of 12 N hydrochloric acid, and 20 ml of the resultant deep blue solution were applied to a 1.5 x 30 cm column of Dowex 1 (50 to 100 mesh), pretreated with 12 N hydrochloric acid. The nickel was not absorbed and appeared immediately in the eluent. After washing the column with 20 ml of 12 N hydrochloric acid to insure that all the nickel had been washed through, the cobalt was washed off with 2 N hydrochloric acid. Cobalt carbonate was precipitated by adding sodium carbonate. Excess sodium carbonate was removed by washing with water, and the cobalt carbonate was dried at 90° C for 12 hours.

Subsequent analysis for cobalt and nickel showed the material to be almost pure cobalt carbonate with no detectable nickel present.

Arginase Assay

Arginase activity in the kidneys from the quail was measured according to the method of Tamir and Ratner (1963a), with slight modification, using the production of urea as a measure of arginase activity. Urea was determined by the method of Archibald (1945) using the color reagent α-isonitrosopropiophenone. The sulfuric-phosphoric acid mixture was modified to be 10^{-2}M with respect to ferric ions, as suggested by O'Dell, et al. (1965).

Kidneys were removed as soon as the quail were killed, immediately frozen in a dry ice-acetone mixture and held at -80° C until homogenized. After homogenization, the homogenate was immediately frozen on dry ice and stored at -80° C until the analyses were carried out.

A 4 percent homogenate (W/V) was made using 0.25 M sucrose in experiments I and III and 0.02 M KH_2PO_4 in experiments IV, V, VI, VIII and IX. No kidneys were obtained from the birds of experiments II, VII and X. In cases where there was insufficient kidney for the preparation of a satisfactory 4 percent homogenate, a 2 or one percent homogenate was made. Arginase assays were carried out with and without activation of the enzyme prior to introduction of the substrate. The incubation mixture contained 0.50 ml of 0.1 M glycine buffer, pH 9.5, 0.01 to 0.60 ml of 4 percent homogenate and 0.05 ml

of 0.1 M $MnCl_2$, if the samples were to be activated. The mixture was then incubated for 30 minutes at 37° C to allow for activation of the enzyme. The L-arginine hydrochloride substrate was also equilibrated at 37° C for 30 minutes. The amount of homogenate to use could only be accurately determined by a preliminary assay, with graded increments of homogenate, to see just how much homogenate was required to produce a quantity of urea which would fall within the range of the standards used.

After the 30 minute incubation period, 0.65 ml of 0.085 M L-arginine hydrochloride substrate, prepared fresh daily, was added as substrate, and incubation was continued for 15 minutes. The enzymatic reaction was stopped by adding 1.0 ml of a 15 percent solution of trichloroacetic acid (TCA). The contents of each tube were thoroughly mixed after the introduction of the substrate and after the addition of the TCA. After centrifugation, 0.1 ml of the supernatant was added to a test tube containing 4 ml of an acid mixture, consisting of one part water, one part concentrated sulfuric acid and three parts of concentrated phosphoric acid. The acid mixture was made to contain 10^{-2} M ferric iron. Also added to the acid mixture, just prior to the addition of the supernatant, was 0.4 ml of a four percent ethanolic solution of α-isonitrosopropiophenone. The solutions were then mixed

thoroughly, marbles were placed on the tops of the tubes and the tubes were then placed in a boiling water bath, in the absence of light, for one hour for the development of color. The water in the water bath was just above the level of the liquid in the tubes. After boiling, the tubes were cooled at room temperature in the absence of light. After 15 minutes, the optical density of the samples was determined with a Coleman Junior II spectrophotometer at a wavelength setting of 540 mμ with the reagent blank adjusted to zero. The color obtained was photolabile, especially during the color development phase. Hence, it was necessary to exclude light as much as possible. Urea standards and blanks were treated identically as the samples except that 0.01 ml of each of the urea standards was added to the appropriate tubes. One ml of 15 percent TCA was added to all standards and blanks before the arginine substrate was added. Urea standards were prepared fresh monthly and contained 150, 300, 600, 900, 1200 and 1500 μ moles of urea in the standard tubes during the assay. Duplicate activated and unactivated assays were carried out on all homogenates.

Statistical Analyses

The data from experiments II, III, V, VI and VIII were examined for statistical significance by least squares analysis. The data of experiments I, IV, VII, IX and X,

where only two treatment groups were involved, were tested for significance using the t-test. Data compiled from pooled samples were analyzed by the t-test for main treatment effects only, i.e., the effect of nickel and arginine levels. No statistical analysis was possible on the reproductive performance data as the laying birds were not kept in individual cages. No statistical analysis was carried out on the plasma amino acid data due to the pooling of blood, and the small number of samples per treatment which were analyzed.

IV. RESULTS AND DISCUSSION

Experiment I

The weights and analytical data of experiment I are presented in table 9. The purposes of this experiment were to develop a satisfactory purified diet to be used in subsequent experiments and to develop the first generation of nickel-low and nickel-supplemented quail.

Approximately 10 days into the trial, a number of birds on both levels of nickel exhibited symptoms similar to those seen in thiamine or vitamin E deficiency, but supplementation with either or both did not alleviate the problem. An ensuing high death loss resulted which considerably reduced the numbers by the 20th day of the experiment.

Since the purified diet contained 35 percent casein, the existence of an arginine deficiency seemed probable as such a deficiency had been clearly demonstrated for chickens receiving casein as the protein source. Supplementation of the diet with arginine did slightly improve the appearance and growth of the birds. Arginine was initially supplemented at 0.88 percent of the diet and later increased to 1.13 percent of the diet to bring the lysine to arginine ratio from 2.0 to 1.1. Improvement of growth and appearance of the

TABLE 9. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT I

Dietary Nickel, ppb	74	1780	±S.E.[1]
Weights, g			
Initial	5.8(128)[2]	5.7(126)	0.0
6 days	9.2(97)	9.1(91)	0.1
13 days	17.5(77)	17.7(60)	0.4
20 days	32.9(70)	35.6(41)	0.8
27 days	58.6(66)	64.2(38)*	1.1
34 days	77.5(66)	88.3(34)**	1.2
41 days	92.7(56)	98.9(32)*	1.3
48 days	103.5(54)	107.9(32)	1.6
Adult, 104 days	127.7(48)	132.7(30)	2.1
Males[3]	113.1(34)		
Females	142.4(44)***		
Arginase[4]			
Unactivated	5928(19)	4468(18)	689
Activated[5]	30463(21)	24221(19)	2670
Males[3]	23944(19)		3592
Females	31805(21)		3891
Liver Weight			
Fresh, g	3.86(44)	5.31(26)*	0.34
Males[3]	2.11(29)		0.19
Females	6.38(49)***		0.39
% Body weight	2.89(44)	3.92(26)*	0.23
Kidney Weight (both)			
Fresh, g	0.99(21)	0.97(19)	0.04
Hematocrit, %	53.1 (18)*	48.6 (10)	1.0
Hemoglobin, g/100 ml	13.5 (22)	13.1 (13)	0.3
Total Plasma Protein, g/100 ml	5.12(34)	5.33(34)	0.07

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]Statistical comparisons of males and females were conducted without regard to treatment.
[4]μ moles urea/g fresh tissue/hour.
[5]Activated with Mn^{+2} for 30 minutes.
*Significantly greater than lower value ($P < .05$).
**Significantly greater than lower value ($P < .01$).
***Significantly greater than lower value ($P < .005$).

birds continued with age so that by five weeks of age the quail appeared quite normal, and egg production began at 40 days of age. The improvement with age indicated that the requirement for a nutrient or nutrients had decreased with age such that the concentration of the nutrient in the diet, which was inadequate to three weeks of age, was then sufficient.

The weight data shown in table 9 indicate that the quail receiving supplemental nickel were significantly heavier at 27, 34 and 41 days of age. This difference was probably more a reflection of the less crowded conditions for the birds receiving supplemental nickel than to an effect of nickel per se, since there were only about one-half the number of quail in the supplemented group as in the low-nickel group. Females were significantly heavier than males, a fact which has also been reported by all of the earlier researchers who have used Japanese quail. Mature weights for males and females were well within the ranges of 100 to 140 g for adult males and 110 to 160 g for adult females as reported by Reyniers and Sachsteder (1960).

The pattern of death loss of the quail in this trial to three weeks of age suggested that the supplemental nickel was detrimental rather than beneficial, at least during the period of time before supplemental arginine was added to the

diets. Nickel has been shown to activate arginase *in vitro* (Hellerman and Perkins, 1935). If, in fact, nickel were activating arginase *in vivo*, one might expect that quail receiving supplemental nickel and an already insufficient level of arginine would become relatively more arginine deficient than birds receiving an arginine deficient diet but not receiving supplemental nickel. Therefore, arginase activity was determined in this and all subsequent trials where tissues were taken from the birds at the time of exsanguination. Assays were carried out using both an *in vitro* manganese activated enzyme preparation and unactivated preparation in an attempt to measure total kidney arginase potential and to approximate the arginase activity *in vivo*, where no excess of activating ion would be present as is the cause for an *in vitro* activation.

The results of the arginase assay for experiment I (table 9) indicate no significant difference in activity, activated or unactivated, due to the nickel treatments. Approximately five times as much 4 percent kidney homogenate was required for the unactivated assay as for the activated assay. This is very close to the two to four fold figure reported by Van Slyke and Archibald (1946).

Sex differences in arginase activity have been reported in the Bantam fowl (Chaudhuri, 1927), in the mouse (Wiswell, 1950) and in the rat (Mandelstam and Yudkin, 1952), with the activity in the male reported to be 15 to 50 percent greater

than in females. No significant sex difference in arginase activity was observed in the Japanese quail used in experiment I, although the arginase activity of the females tended to be higher than in the males.

The kidney homogenate used for the arginase assay in experiment I was prepared in 0.25 M sucrose according to the method of Kean (1967). This method proved to be quite unsatisfactory as the characteristic charring reaction between sulfuric acid and sucrose occurred during the color development phase of the procedure. The resultant brown color, rather than the normal pink, made an accurate colorimetric determination of urea difficult, even though an equal amount of sucrose homogenate was added to the standards and blanks. An unsuccessful attempt was made to use urease to determine the amount of urea produced by the sucrose homogenates. Apparently the enzymatic reaction could not be halted in such a manner that conditions remained appropriate for urease action, even after adjustment of the pH to 6.8. Similar findings were reported by Smith and Jones (1963).

Maximum urea production was obtained when a 0.085 M solution of L-arginine hydrochloride, pH 9.7, was used as the substrate. The use of a 0.85 M solution of substrate, as used by Tamir and Ratner (1963a) caused a 20 to 30 percent decrease in the amount of color produced between urea and

α-isonitrosopropiophenone. Such a color reduction has been previously reported by Van Slyke and Archibald (1946).

Optimal activation of the enzyme was achieved by adding 0.05 ml of a 0.1 M solution of manganese chloride to the incubation medium. The addition of a more concentrated solution of manganese activator either failed to further increase the activity of the enzyme or actually caused an apparent decrease in enzyme activity similar to that reported by Smith and Lewis (1963) for chick arginase. No attempt was made to activate the enzyme with nickel *in vitro*.

The amount of kidney homogenate used in the assay had to be adjusted such that a maximum of 0.1 ml of the urea containing supernatant was added to the acid mixture for the color development with α-isonitrosopropiophenone. The addition of 0.2 ml of the supernatant resulted in a cloudy solution which made a colorimetric reading impossible. Apparently 0.2 ml of the supernatant contained sufficient quantities of one or more of the reagents used in the incubation medium to interact with one or more of the reagents of the acid mixture. No attempt was made to isolate the cause of the interaction.

Nickel has been shown to occur in RNA (Wacker and Vallee, 1959a; 1959b) and as such may indirectly affect protein synthesis. Hematocrit, hemoglobin and total plasma protein

determinations were carried out in an effort to measure the possible effect of nickel on protein synthesis or on the incorporation of the protein into hemoglobin or red blood cells. No significant differences were observed in hemoglobin or total plasma protein concentrations. The quail receiving no supplemental nickel had a significantly greater hematocrit. These findings would suggest that, at least in this experiment and for the parameters checked, nickel had no effect on protein synthesis or on hemoglobin synthesis. kidney weights of the two groups of birds were almost identical and coupled with the observation that the activated arginase activities were not significantly different would indicate that the synthesis of arginase was not favored by the presence of nickel.

Of the other tissues taken--lung, liver and pancreas--only the livers were weighed due to the difficulty involved in obtaining a complete pancreas from all the birds and the difficulty involved in uniformly blotting the blood from the lungs. The livers from the quail receiving supplemental nickel were significantly heavier than those from the low-nickel group, whether expressed on a fresh basis or as a percent of body weight. The livers from the females in both groups were significantly heavier and more pale in color than livers from males. The group receiving supplemental nickel had 73 percent females while the low-nickel group contained

only 56 percent females. This difference in the number of females undoubtedly would account for most of the difference in liver weights observed between the two groups.

The quail of experiment I were raised to adults and eggs collected for the production of the second generation of low-nickel and nickel-supplemented quail. Reproductive data for these quail are presented in table 10. The birds were not housed in individual laying cages, so the egg production data are only a good approximation of the number produced by each group of quail. Due to the colony type of housing used, many eggs were broken or cracked, and since there was some tendency for the birds to break and eat the eggs, undoubtedly many were never recorded. None-the-less, the production figures of 54 and 65 percent for the low-nickel and nickel-supplemented groups, respectively, are much higher than those reported by Gough et al. (1968), who also fed a purified diet to Japanese quail. Average egg weight was very close to the 9 g average reported for the Japanese quail by Wilson et al. (1961) and was not decreased by feeding a purified diet as reported by Gough et al. (1968).

Fertility and hatchability data indicate no difference in performance between the two groups of birds. The figures for both fertility and hatchability are well within the range considered normal for Japanese quail of the same genetic

TABLE 10. REPRODUCTIVE DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT I.

Dietary Nickel, ppb	74	1780
First egg, days	40	43
Total eggs laid	876	866
Ave. wt., g	9.17	8.79
Eggs/female bird day	0.54	0.65
No. eggs set[1]	256	266
Net (uncracked)	199	229
No. fertile	164	183
% Hatch (of total set)	43.8	42.1
% Hatch (of net)	56.2	48.8
% Fertile (of total)	64.1	68.8
% Fertile (of net)	82.4	79.9
% Hatch (of fertile)	68.3	61.2

[1] Eggs collected when birds were 10 to 12 weeks of age for production of second generation, experiment IV.

background (maintained by the Poultry Science Department at Michigan State) but fed a commercial diet. According to Howes (1964), the greatest single cause of poor hatchability of quail eggs is dehydration during incubation as a result of microscopic cracks in the shells which cannot be detected by the naked eye. As indicated earlier, the type of housing used in the present study led to cracking of many eggs, and consequently many dry eggs were found after incubation. After eliminating the dry eggs, the fertility and hatchability figures were very close to those reported by Padgett and Ivey (1959) and Woodard *et al.* (1969). They reported a fertility of 85 to 90 percent and a hatchability of the fertile eggs of

of 60 to 70 percent.

Experiment II

This experiment began only four weeks after the start of experiment I, so experiment II began after the quail of experiment I had "outgrown" the problem of a dietary deficiency as previously discussed. The weight data for the second experiment are shown in table 11. In this experiment, a 2 x 2 factorial design involving two levels of nickel, 74 and 1780 ppb, and two levels of supplemental arginine, 0 and 0.88 percent of the diet, were used in an effort to definitely establish whether an arginine deficiency was the causative factor of the problems observed in experiment I. This experiment was terminated after 16 days as practically all of the birds had died. The deficiency symptoms, as described for experiment I, again appeared when the birds were about 10 days of age and a very high death loss ensued. A significant ($P < .005$) increase in weight gain was observed when 0.88 percent of supplemental arginine was added to the diet.

TABLE 11. WEIGHTS OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT II.

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg	
Concentration of Ni, ppb	74	74	1780	1780	
Supplemental Arginine, %	0	0.88	0	0.88	
Weights, g					±S.E.[1]
Initial	6.4(75)[2]	6.4(75)	6.4(75)	6.4(75)	0.0
5 day	9.1(70)	11.3(73)	9.5(71)	10.9(67)	0.2
12 day	14.2(43)	22.2(29)	15.2(32)	20.0(32)	0.6
16 day	17.2(25)	30.8(25)	19.8(20)	25.4(18)	1.1

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.

This effect was evident at all weigh periods subsequent to the initial weighing of the birds, so an effect of arginine on growth rate was observed as early as five days of age. Thus, the problems encountered were due to a yet insufficient level of arginine or to some other nutrient or nutrients or a combination of arginine and some other nutrient. A significant ($P < .05$) negative interaction was observed between nickel and arginine, i.e., arginine supplementation to a diet containing 1780 ppb nickel did not produce as much response in weight gain as the addition of the same amount of arginine to a diet containing 74 ppb nickel. One possible explanation for this interaction is that the higher level of nickel was activating arginase *in vivo* with the result that not all of the supplemental arginine was available for growth. No tissues were taken from the birds when they were killed so this possible explanation can neither be repudiated nor substantiated.

To enable a comparison to be made between growth rates of quail receiving the purified basal diet and quail receiving a commercial chick diet, the excess quail which were hatched but not needed for experiment II were housed conventionally and fed a commercial diet, the composition of which is given as a footnote to table 23. Average initial weight of the 51 birds fed the commercial diet was 6.5 g. One, two and three

week weights averaged 12.1, 25.4 and 34.0 g, respectively. This rate of growth was only as good as that observed on the arginine deficient purified diet and suggests that the commercial diet is deficient in one or more nutrients. The vitamin levels of the commercial diet could not be obtained, but based on the results of experiment III, to be discussed, it is possible that the levels of vitamins in the diet were inadequate.

Post-mortem examination of quail from the first two experiments by a pathologist resulted in a tentative diagnosis of a vitamin E deficiency. To determine if this in fact were the problem, four levels of vitamin E, in a purified diet, were fed to four groups of quail housed under conventional conditions. The levels of E included in the diet were 0, 50, 100 and 200 mg of d-α-tocopherol per kg of diet. Death losses were so high on all treatments that all of the surviving birds were killed at the end of two weeks.

Histopathological examination revealed an accumulation of a variable amount of fat in the livers which did not appear to bear any relationship to the treatment groups. The only other significant microscopic lesion noted was seen in the sections of skeletal and cardiac muscle of the birds which had received no added vitamin E or 50 mg of vitamin E per kg of diet. In both of these groups, the muscle fibers

appeared to be somewhat smaller in diameter, which resulted in a general appearance of a moderate increase in cellularity. No diagnosis was made as to the cause of the problem observed at all levels of supplemental E, but it was concluded that the level of vitamin E (100 mg per kg of diet) used in experiments I and II probably was not the cause of the high death loss observed in these two experiments.

Experiment III

Weights and analytical data for experiment III are presented in table 12. Once again a 2 x 2 factorial design involving two levels of nickel, 74 and 1780 ppb as in experiments I and II, and two levels of supplemental arginine, 0 and 1.13 percent of the diet was used. The arginine level was increased from the 0.88 percent level, used for the first five weeks of experiment I and all of experiment II, to 1.13 percent in order to bring the lysine to arginine ratio to 1.0. Because the problems encountered early in both experiments I and II were not thought to be entirely due to insufficient arginine, the levels of all of the vitamins in the diet were arbitrarily tripled in experiment III. The high death loss experienced in trials I and II did not occur and, in fact, 22 to 24 birds from each treatment had to be killed at the end of eight days to prevent overcrowding. After the numbers were reduced to approximately 50

TABLE 12. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT III.

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg	
Concentration of Ni, ppb	74	74	1780	1780	
Supplemental Arginine, %	0	1.13	0	1.13	
Weights, g					±S.E.[1]
Initial	6.7(76)[2]	6.8(77)	6.8(75)	6.5(75)	0.1
8 days	10.6(72)	18.2(74)	11.0(75)	17.8(72)	0.3
15 days	21.7(49)	41.9(51)	22.9(50)	41.9(50)	0.7
22 days	36.9(48)	65.7(50)	39.1(50)	65.5(50)	1.2
26 days	47.2(47)	76.4(49)	49.0(49)	77.4(49)	1.4
Feed Consumption					
Feed/bird day, g	3.1	5.5	3.3	5.3	
Gain/feed	0.43	0.45	0.44	0.46	
Arginase[3]					
Unactivated	3633(24)	4791(24)	4683(22)	5989(17)	511
Activated[4]	24760(22)	29206(20)	26766(20)	35488(14)	2009
Liver Weight					
Fresh, g	1.74(24)	2.38(24)	2.24(24)	2.76(19)	0.07
% Body Wt.	4.00(24)	3.10(24)	4.82(24)	3.56(19)	0.22
Hematocrit, %	40.4 (16)	37.4 (11)	40.6(8)	39.6 (9)	0.9
Hemoglobin, g/100 ml	11.6 (12)	10.6 (12)	11.8(9)	10.4 (12)	0.5
Total Plasma Protein g/100 ml	3.82(10)	3.72(14)	3.81(14)	4.20(18)	0.09

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]μ moles urea/g fresh tissue/hour.
[4]Activated with Mn^{+2} for 30 minutes.

quail per treatment, only two birds on each of the 74 ppb nickel treatments and only one bird from each of the 1780 ppb nickel treatments died. The quail not receiving supplemental arginine exhibited a depressed growth rate and frizzled and spoon-shaped feathers as described by Fisher et al. (1956) and Newberne et al. (1960). Several birds receiving the diets with no supplemental arginine developed splayed legs as described by Newberne et al. (1960). However, the birds did continue to live and grow slowly on the low-arginine diets indicating that one or more of the vitamins was at an insufficient dietary concentration in experiments I and II where high death losses occurred.

The only significant difference in weight gain in experiment III was due to the supplemental arginine, and once again, the effect was noticeable very early. The quail receiving supplemental arginine were significantly ($P < .005$) heavier at all weigh periods after the initial one. Thus, it appears that arginine is the first limiting amino acid in casein for the Japanese quail, and that this species is similar in this respect to the chicken (Arnold et al., 1936; Klose et al., 1938). Although only one level of supplemental arginine was used (1.13 percent), growth was as good as that reported by Jacobs et al. (1969), who fed a purified diet containing 55 percent of soy protein. They reported average

weights of 17.8, 36.0, 56 and 78 g for the first, second, third and fourth weeks, respectively. The amount of supplemental arginine required by Japanese quail fed a casein diet appears to be somewhat less than the 1.9 percent reported by Fisher et al. (1956) for chicks receiving a 25 percent casein diet and less than the 1.8 to 2.5 percent reported by Wietlake et al. (1954) for chicks receiving a 35 percent casein diet. The total arginine concentration of the diet, using the figure given by Block and Weiss (1956) (table 5) for the arginine content of casein, was 2.60 percent or about 9.3 percent of the protein, using an 80 percent protein casein. This is somewhat higher than the six to eight percent figure reported by Almquist and Merritt (1950), Snyder et al. (1956) and Krautmann et al. (1957) for the chick. Growth rates of all the quail receiving the arginine deficient diets were quite uniform indicating that, at least in the quail used in this study, there was no observable genetic difference in the requirement of the birds for arginine as has been reported to occur in chickens (Hegsted et al., 1941; Snyder et al., 1956; Edwards et al., 1958; Nesheim and Hutt, 1962; Nesheim et al., 1964; Nesheim, 1968b).

No difference in feed utilization was observed. The quail receiving supplemental arginine consumed more feed but made no better use of it than the arginine deficient birds.

Arginine deficiency delayed feather development, and in this experiment, nickel supplementation of the arginine deficient birds appeared to have a defininte favorable effect on feather development as compared to the arginine deficient birds receiving no supplemental nickel.

In this experiment, as in experiment II, an arginine-nickel interaction occurred such that arginine supplementation to a diet containing nickel did not produce the same response as arginine supplementation to the low-nickel diet. However, the differencesin the present experiment were not significant. Yet, it is interesting to note that the quail which had received supplemental nickel had significantly ($P < .05$) higher kidney arginase activities, both activated and unactivated, than those that had not received supplemental nickel. Arginine supplementation of the diets also caused a significant ($P < .01$) increase in the activated and unactivated kidney arginase activities. Such an effect of arginine on chick arginase activity has been reported by Baldwin (1936) and O'Dell *et al.* (1965).

Both nickel and arginine supplementation to the basal diet significantly ($P < .005$) increased liver weight and liver as a percent of body weight. As no record was made of the sex of the birds in this trial, no statement can be made concerning the relative size of the livers from males

and females before egg production began.

The quail receiving supplemental arginine had significantly ($P < .05$) lower hematocrit and hemoglobin values than the birds receiving the arginine deficient diets. No significant effect due to nickel was observed for hematocrit and hemoglobin values, and no significant effect of either arginine or nickel was observed for total plasma protein values.

Experiment IV

The quail used in experiment IV were second generation birds which had been produced from the adult quail of experiment I. Hatching weights (table 13) were somewhat greater than in experiment I, indicating that such weights were not depressed by the feeding of purified diets as reported by Gough et al. (1968). The only significant weight difference was in the initial weight. Arginine, at a level of 1.13 percent of the diet, was supplemented to both levels of nickel in this trial as the birds were to be used for the production of the third generation of nickel-low and nickel-supplemented quail.

Nineteen days into trial IV, the hose supplying air to the isolator in which the low-nickel group was housed came loose with the result that all but 10 of the birds suffocated. Of the 10 remaining birds seven were females, so enough eggs were eventually collected for the production of the third

generation. Adult females were again significantly heavier than adult males.

The feed consumption data reported in table 13 include only the first 37 days of the experiment as the gain to feed ratio is meaningless once growth has stopped, and the adult birds tended to waste much more feed so accurate feed data could not be kept.

Two sets of data are presented in table 13 for the unactivated arginase assay. The higher values were obtained from kidney homogenates which had been stored until the time of assay at -80° C. The lower values were obtained on the same homogenates after a storage time of 48 hours at 0° C. Such high, unactivated arginase activities had not been seen in previous experiments, nor, as will be discussed, were they to be observed in subsequent experiments. Thus, it appears that storage temperature can, under unknown conditions, be a factor in the preservation of arginase activity in Japanese quail kidney homogenates. The arginase activity of the nickel supplemented birds was significantly ($P < .05$) greater for the initial assay but was not significant, although still greater, after storage at 0° C. No significant differences were observed for the activated arginase assay or between sexes. The activated assay was carried out after the homogenate had been stored at 0° C for 48 hours, but as in experiments I and III, the values obtained were approximately six

TABLE 13. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT IV.

Dietary Nickel, ppb	74		1780	±S.E.[1]
Weights, g				
Initial	6.2(110)[2]**		5.8(113)	0.1
9 days	24.9(95)		24.2(97)	0.3
16 days	45.0(88)		44.6(94)	0.4
20 days	53.8(10)		58.4(92)	0.8
27 days	76.4(10)		79.3(51)	1.0
34 days	95.8(10)		92.8(51)	1.2
41 days	115.8(10)		110.2(50)	1.9
78 days	130.1(10)		124.2(44)	2.5
Adult, 110 days	128.3(10)		124.4(44)	2.2
Males[3]		109.0(18)		3.2
Females		133.9(36)***		1.7
Feed Consumption				
Feed/bird day, g(to 37 days)	5.1		6.2	
Gain/feed (to 37 days)	0.39		0.39	
Arginase[4]				
Unactivated	32844(10)		41599 (10)*	17154[6]
	2678(10)[5]		3851 (9)[5]	458[6]
Activated[7]	17021(10)		22813 (9)	1621
Males[3]		17089(6)		3675
Females		20999(13)		1661
Liver Weight				
Fresh, g	4.04(10)		4.61(10)	0.50
Males[3]		1.91		0.46
Females		5.63***		0.72
% Body weight	3.11(10)		3.50(10)	0.35
% Dry matter	36.4 (10)		41.0 (10)	1.6

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]Statistical comparisons of males and females were conducted without regard to treatment.

TABLE 13. (cont'd).

[4]μ moles urea/g fresh tissue/hour.
[5]Values for assay obtained on same tissue at two different times.
[6]Standard error for second set of values at each level of nickel.
[7]Activated with Mn^{+2} for 30 minutes.
*Significantly greater than lower value ($P < .05$).
**Significantly greater than lower value ($P < .01$).
***Significantly greater than lower value ($P < .005$).

times higher than those obtained for the unactivated assay. As in experiment I, the arginase values for females tended to be higher than those of males.

No treatment differences were observed in liver weights, but the livers from the females were significantly heavier ($P < .005$) than those from males. Some of the livers taken from the females were very oily. No hematocrit or hemoglobin determinations were made.

Reproductive data from the second generation quail are presented in table 14. The birds were housed under the same conditions as described for experiment I so many eggs undoubtedly were broken and therefore not recorded. Egg weights and eggs per female bird day were very similar to the figures presented in table 10 for experiment I.

TABLE 14. REPRODUCTIVE DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT IV.

Dietary Nickel, ppb	74		1780	
First egg, days	38		37	
Total eggs laid	281		1290	
Ave. wt, g	9.37		8.79	
Eggs/female bird day	.573		.648	
No. eggs set	94[1]	92[2]	364[1]	499[2]
Net (uncracked)	89	77	282	390
No. fertile	62	47	188	247
% Hatch (of total set)	38.4	19.6	31.0	19.6
% Hatch (of net)	40.5	23.4	40.1	25.1
% Fertile (of total)	66.0	51.1	51.6	49.5
% Fertile (of net)	69.7	61.0	66.7	63.3
% Hatch (of fertile)	58.1	38.3	60.1	39.7

[1]Eggs collected when birds were 10 to 12 weeks of age for production of third generation, experiment VII.

[2]Eggs collected when birds were 17 to 19 weeks of age for production of birds used in experiment VIII.

The experiment IV birds were used to provide eggs for hatching of birds used in two succeeding experiments, and, therefore, two sets of fertility and hatchability data are presented. The first set is similar to that observed in experiment I. In each case, eggs were collected for hatching when the birds were 10 to 12 weeks of age or in approximately the fourth to sixth weeks of egg production. The second set of data was compiled from eggs collected from the same birds but during the 17th to 19th weeks of egg production. Fertility and hatchability of fertile eggs exhibited a definite decrease as the birds grew older.

Experiment V

This experiment was designed to stimulate arginase activity to such an extent that the quail receiving the arginine deficient diets would not grow as well as in previous experiments, and to determine if nickel could activate arginase *in vivo*. If the latter were true, the group receiving supplemental nickel but no supplemental arginine should not grow as well, and have higher arginase activities, than the group receiving neither supplemental nickel nor arginine. Growth on the diet supplemented with 1.13 percent of arginine and 4660 ppb total nickel would be expected to decrease if nickel were capable of *in vivo* activation of arginase and if the dietary arginine level were marginal or

deficient. If excess dietary arginine were present, its degradation by arginase would not be detrimental to the bird, and such birds would be expected to gain as rapidly as quail not receiving supplemental nickel.

The attempt to stimulate arginase activity was carried out by reducing the casein content of all the diets from 35 to 25 percent and adding one percent of supplemental tyrosine to increase the tyrosine level from the original 1.36 percent in the 35 percent casein diet, to 1.98 percent in the 25 percent casein diet. Supplemental tyrosine has been reported to increase arginase activity two to four fold (Austic and Nesheim, 1969a; 1969b; 1969c). However, they added three percent tyrosine to chick diets.

In addition to the attempt to stimulate arginase activity, an attempt was made to lower the nickel content of the basal diet. Analysis of each of the reagent grade minerals used in the basal diet revealed that the magnesium carbonate, calcium carbonate and calcium phosphate were relatively highly contaminated with nickel, as shown in table 15. Nickel analysis of the same minerals, after washing with an alcoholic dimethylglyoxine solution revealed a considerably lowered nickel concentration in all three (table 15). The cellulose used in trials V and VI was acid washed and resulted in a reduction of the nickel concentration from 97 to 90 ppb. However,

TABLE 15. NICKEL CONTENT OF REAGENT GRADE MINERALS BEFORE AND AFTER WASHING WITH DIMETHYLGLYOXINE.

Mineral	Nickel Concentration	
	Before washing	After washing
$4MgCO_3Mg(OH)_2 \cdot n(H_2O)$	1300 ± 30[1]	388 ± 38
$CaCO_3$	138 ± 9	54 ± 10
$CaHPO_4$	395 ± 25	224 ± 26

[1]Standard error of the mean.

the nickel concentration of the basal diet only decreased from 74 to 71 ppb, an insignificant change. When unwashed calcium carbonate was added to the basal diet to increase the calcium content for the laying birds in experiments I, IV and VII, the nickel content was increased from 74 to 101 ppb indicating that the minerals, even though reagent grade, are contaminated with nickel and can make a significant contribution to the total nickel content of a purified diet.

The weights and analytical data of the quail used in experiment V are presented in table 16. Weight gain was not significantly influenced by the nickel concentration of the diet but was significantly ($P < .005$) increased at all weigh periods, except the initial period, by 1.13 percent of supplemental arginine. Growth rates of the birds on all treatments were considerably less than the growth rates observed in the earlier experiments. This was probably due to

TABLE 16. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT V.

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg	
Concentration of Ni, ppb	71	71	4660	4660	
Supplemental Arginine, %	0	1.13	0	1.13	
Concentration of Mn, ppm	90	90	90	90	
Weights, g					±S.E.[1]
Initial	6.0(61)[2]	6.0(61)	6.0(60)	6.0(60)	0.1
6 days	8.8(56)	11.8(54)	8.6(56)	12.0(56)	0.2
13 days	12.7(52)	23.9(54)	12.7(47)	22.3(55)	0.4
20 days	21.1(46)	34.8(53)	20.2(46)	33.0(55)	0.7
27 days	33.4(45)	57.0(52)	30.4(40)	55.6(54)	1.1
Feed Consumption					
Feed/bird day, g	3.2	5.3	2.8	5.1	
Gain/feed	0.28	0.34	0.28	0.35	
Arginase[3]					
Unactivated	1514(18)	1800(23)	1526(23)	1532(22)	202
Activated[4]	9623(20)	11505(24)	10383(24)	9105(24)	1252
Liver Weight					
Fresh, g	1.60(38)	1.68(43)	1.38(25)	2.10(43)	0.04
% Body wt	4.94(38)	2.94(43)	4.52(25)	3.76(43)	0.11
Hematocrit, %	41.5 (10)	43.3 (13)	44.3 (13)	42.1(8)	1.1
Hemoglobin, g/100 ml	9.5 (9)	10.9 (9)	10.1 (8)	9.1(8)	0.5
Total Plasma Protein, g/100 ml	4.46(20)	4.58(30)	4.10(14)	4.23(26)	0.08

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]μ moles urea/g fresh tissue/hour.
[4]Activated with Mn^{+2} for 30 minutes.

an insufficient level of protein, an imbalancing effect of the supplemental tyrosine or a combination of the two.

The quail receiving supplemental arginine consumed more feed and utilized it somewhat more efficiently than birds receiving the arginine-deficient diets.

No significant treatment differences were observed in the arginase activities of the four groups of birds. The attempt to increase arginase activity was without success as the values obtained were even lower than those obtained in the previous experiments. It is possible that the high protein level used in the previous trials caused the high activities as high protein levels have been shown to increase arginase activity (Lightbody and Kleinman, 1939; Mandelstam and Yudkin, 1952; Ashida and Harper, 1961; Schimke, 1962a). Thus, the removal of 10 percent of the casein could have caused such a reduction in the arginase activities to have alleviated any increasing effect of the supplemental tyrosine. Equally as likely is that tyrosine was added at too low a level to have been effective in increasing the arginase activity of the birds.

Supplemental arginine and an arginine-nickel interaction resulted in significantly ($P < .005$) heavier liver weights. The increase was equally as significant when expressed as a percent of body weight.

No significant differences were observed for the hematocrit and hemoglobin values. The quail receiving the low-nickel diet had significantly ($P < .05$) higher total plasma protein levels.

Therefore, the main effect brought about by the changes in the diet for experiment V was a decreased growth rate which was probably due to the decreased level of dietary protein. The attempt to increase arginase activity was unsuccessful.

Experiment VI

All dietary conditions were the same for this experiment as for the previous one, with one exception. The manganese content of the diet was lowered from 90 to 40 ppm in an attempt to make manganese limiting and induce nickel to function as an *in vivo* activator of arginase. The results of experiment VI are presented in table 17.

Nickel supplementation of the diets resulted in a significant ($P < .01$) growth depression for the first week of the experiment indicating that nickel might have been activating arginase at this time. A significant ($P < .05$) growth depressing nickel-arginine interaction was evident for the first two weeks of the experiment. A highly significant ($P < .005$) positive effect of arginine supplementation was observed at all weigh periods subsequent to the initial one,

TABLE 17. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT VI.

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg	
Concentration of Ni, ppb	71	71	4660	4660	
Supplemental Arginine, %	0	1.13	0	1.13	
Concentration of Mn, ppm	40	40	40	40	
Weights, g					±S.E.[1]
Initial	6.7(62)[2]	6.7(62)	6.5(62)	6.5(62)	0.1
7 days	9.1(61)	12.1(59)	9.1(58)	11.3(58)	0.2
14 days	12.9(59)	22.9(58)	13.1(46)	21.1(50)	0.4
21 days	20.2(56)	37.8(58)	20.8(46)	38.4(50)	0.7
26 days	26.0(48)	49.4(52)	26.6(43)	49.6(50)	1.0
Feed Consumption					
Feed/bird day, g	2.28	3.84	2.14	4.01	
Gain/feed	0.31	0.42	0.32	0.40	
Arginase[3]					
Unactivated	1891(30)	2551(33)	2073(25)	2885(32)	197
Activated[4]	9398(29)	14672(33)	8704(25)	15658(32)	1122
Liver Weight					
Fresh, g	0.97(46)	2.05(50)	0.93(40)	1.79(46)	0.05
% Body wt	3.58(46)	4.05(50)	3.54(40)	3.62(46)	0.05
Hematocrit, %	41.1(15)	37.5(11)	40.1(16)	44.1(11)	1.1
Hemoglobin, g/100 ml	10.5(12)	9.3(12)	9.7(12)	10.7(12)	0.4
Total Plasma Protein, g/100 ml	3.58(10)	4.42(20)	3.48(8)	4.51(18)	0.09

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]μ moles urea/g fresh tissue/hour.
[4]Activated with Mn^{+2} for 30 minutes.

and feed consumption data indicate that the quail receiving supplemental arginine utilized their feed more efficiently.

Arginase activities were significantly ($P < .005$) increased by supplemental arginine, but the dietary level of nickel was without significant effect. Liver weights were significantly ($P < .005$) increased by supplemental dietary arginine but no significant difference was obtained when liver weight was expressed as a percent of body weight, indicating that the larger livers were simply due to larger birds.

Significant ($P < .01$) increases in hematocrit due to nickel and a positive nickel-arginine interaction were observed. A significant ($P < .05$), positive nickel-arginine interaction was responsible for an increase in the hemoglobin levels. Arginine supplementation was without significant effect on either hematocrit or hemoglobin values but did significantly ($P < .005$) increase total plasma protein levels.

In this experiment as in experiment V, the attempts to stimulate arginase were largely unsuccessful.

An attempt was made to determine the nickel concentration in the livers of the quail from experiment VI. Livers from 5 to 10 birds which had received the same dietary treatment were pooled such that the final weight of each

pooled sample was 10 to 15 g. Nickel was determined by atomic absorption spectroscopy following wet digestion of the livers, with nitric and perchloric acids, and following a solvent extraction procedure as described by Mulford (1966).

The nickel concentration of the livers ranged between 0.055 and 0.346 ppm on a fresh basis. No correlation was observed between the dietary nickel level and the concentration of nickel in the liver.

Unsuccessful attempts were made to determine the nickel concentration of the kidney homogenates used for the arginase assay and of blood plasma. The amount of sample available in each case was so small as to make a meaningful analysis for nickel impossible by the methods employed. Tissue analyses were not attempted for any of the other experiments.

Experiment VII

The quail used for this experiment consisted of the third generation of the nickel-low and nickel-supplemented birds and were offspring of the quail used in experiment IV.

Weights and feed use data for experiment VII are presented in table 18. The initial weights of the two groups were significantly ($P < .05$) different but hatching weight had not been depressed by the feeding of a purified diet for two generations. No other significant difference in weights

TABLE 18. WEIGHTS AND FEED USE DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT VII.

Dietary Nickel, ppb	74	1780	
Weights, g			±S.E.[1]
Initial	6.6(47)[2]*	6.3(78)	0.1
7 days	17.9(35)	17.6(67)	0.3
14 days	40.4(35)	38.9(65)	0.5
21 days	64.1(34)	63.0(63)	0.8
28 days	84.2(28)	85.0(29)	1.1
35 days	97.2(28)	97.2(29)	1.6
Adult, 190 days	126.8(27)	131.3(25)	2.3
Males[3]	118.0(17)		2.9
Females	133.9(35)***		2.7
Feed Consumption			
Feed/bird day (to 35 days)	6.4	7.5	
Gain/feed (to 35 days)	0.35	0.41	

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]Statistical comparisons of males and females were conducted without regard to treatment.
*Significantly greater than lower value ($P < .05$).
***Significantly greater than lower value ($P < .005$).

was observed, except, once again, females were significantly ($P < .005$) heavier than males.

At 28 days into the experiment, the number of birds was reduced to 28 or 29 per treatment with a ratio of one male to three females in each treatment.

Feed consumption data indicate that the quail receiving supplemental nickel consumed slightly more feed per day and utilized it somewhat more efficiently.

No tissues were taken when the birds were killed so no arginase or hematological values were obtained.

Eggs were collected from the birds for the production of the quail used in experiments IX and X. A record of the reproductive performance of the third generation is presented in table 19. Average egg weight was about the same

TABLE 19. REPRODUCTIVE DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT VII.

Dietary Nickel, ppb	74	1780
First egg, days	43	40
Total eggs laid	1553	1278
Ave. wt, g	8.76	8.63
Eggs/female bird day	0.59	0.46
No. eggs set[1]	144	129
Net (uncracked)	114	82
No. fertile	103	72
% Hatch (of total set)	38.9	30.5
% Hatch (of net)	49.1	47.6
% Fertile (of total)	71.5	56.2
% Fertile (of net)	90.4	87.8
% Hatch (of fertile)	54.4	54.2

[1]Eggs collected when birds were 17 to 19 weeks of age for production of fourth generation, experiment IX.

as for the second generation. The number of eggs per female bird day appeared to decrease in this trial, but most of this decrease can be attributed to the failure to collect the eggs soon after they were laid. Such a delay allowed for more breakage of eggs, and therefore, the record for eggs laid is rather inaccurate for this trial.

Fertility and hatchability data were collected from the eggs set for production of the quail to be used in experiment IX but not experiment X. Eggs were collected when the birds were 17 to 19 weeks of age. The fertility data obtained show that fertility of the eggs was higher than for the two previous generations. Hatchability of the fertile eggs was higher for the third than the second generation for eggs collected at comparable stages of production. No difference in fertility or hatchability was observed due to nickel treatment; both groups received 1.13 percent supplemental arginine.

The eggs collected for the production of the quail used for experiment X were collected when the experiment VII birds were 27 to 29 weeks of age. Although no record was made of the fertility and hatchability of these eggs, the hatchability of the eggs was very low. Of course, this could have been due to an excessive number of cracked eggs, but the age of the laying birds undoubtedly had some effect.

Eggs from each of the first three generations were stored at least two weeks at 15° C before incubation. Eggs from the second generation were stored for approximately three weeks, due to the small number of surviving females in the low-nickel group. Hatchability of the eggs stored longer than two weeks was greatly reduced.

Experiment VIII

A 2 x 2 factorial design was used in this experiment as in experiments II, III, V and VI. The casein level was increased from the 25 percent level used in experiments V and VI to 35 percent, as used in experiments II and III. No supplemental tyrosine was included, arginine was supplemented at 1.13 percent of the diet and the nickel level of the supplemented diet was reduced back to the 1780 ppb level used in experiments II and III. Washing of the minerals and cellulose, as in experiments V and VI, was not carried out for the present experiment. The final diets were formulated to be identical to the diets used in experiment III, in an attempt to duplicate the feathering difference noted in that trial. The only difference in the two experiments involved the use of third generation quail (offspring of experiment IV) in the present trial.

Weights and analytical data of the experiment VIII quail are presented in table 20. Arginine significantly ($P < .005$) increased the growth rate for all weigh periods subsequent to the initial weigh period. Supplemental nickel significantly ($P < .05$) increased growth rate for the six and 20 day weigh periods, and approached significance at the same level for the 13 day weight. From table 20, it is quite evident that supplemental nickel had a greater growth promoting

TABLE 20. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT VIII.

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg	
Concentration of Ni, ppb	74	74	1780	1780	
Supplemental Arginine, %	0	1.13	0	1.13	
Weights, g					±S.E.[1]
Initial	5.9(27)[2]	6.1(27)	5.9(27)	5.7(27)	0.1
6 days	11.5(25)	18.3(23)	13.7(24)	18.5(26)	0.5
13 days	21.1(24)	40.3(23)	26.3(23)	38.7(26)	1.0
20 days	38.7(22)	64.0(23)	44.8(22)	61.4(25)	1.5
27 days	58.0(22)	85.0(23)	67.4(22)	80.4(25)	2.2
Feed Consumption					
Feed/bird day, g	4.5	6.5	5.1	6.0	
Gain/feed	0.41	0.44	0.43	0.45	
Arginase[3]					
Unactivated	3240(17)	4096(20)	2738(20)	4954(21)	308
Activated[4]	23946(17)	28772(20)	18340(20)	29430(21)	1793
Liver Weight					
Fresh, g	2.21(20)	2.49(21)	2.39(19)	2.47(23)	0.05
% Body wt	3.76 (20)	2.94(21)	5.34(19)	3.08(23)	0.16
Hematocrit, %	41.2(13)	38.0(12)	40.6(7)	38.2(7)	1.4
Hemoglobin, g/100 ml	10.5(12)	10.3(10)	10.9(10)	10.3(10)	0.4
Total Plasma Protein g/100 ml	3.82(10)	3.36(14)	3.26(10)	3.50(10)	0.06

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]μ moles urea/g fresh tissue/hour.
[4]Activated with Mn^{+2} for 30 minutes.

effect for the quail receiving the arginine deficient diets than the arginine supplemented diets. This difference could be accounted for by a significant ($P < .05$) growth depressing arginine-nickel interaction at all weigh periods.

If one postulates that the growth depressing interaction was due to an increase in arginase activity due to the activation of arginase by nickel, the growth increase observed in the arginine deficient group would not be expected. In essence, it appears as if the nickel is actually improving arginine utilization on the arginine deficient diet. The only significant effect on arginase activity was due to the addition of arginine to the diets, while the effect of nickel did not approach significance. The inverse relationship between arginase activity and protein quality, reported by Kean (1967) for the rat, was not evident in this or any of the earlier trials where an arginine deficient diet was fed. It is interesting to note that the quail receiving the arginine deficient diets were considerably heavier in experiment VIII than in experiment III at 26 to 27 days of age. Earlier weights for the arginine deficient groups of both trials exhibited very little difference except that nickel supplementation of the arginine deficient diet improved growth more in experiment VIII than in experiment III. Based on only this one comparison, it appears that the third generation birds used for experiment VIII had, at some point,

developed a greater tolerance to an arginine deficient diet which by 20 days of age allowed them to grow as rapidly as the birds receiving supplemental arginine.

Feed utilization figures indicate that there was no difference in feed utilization between the arginine deficient and arginine supplemented birds. The same result was observed in experiment III. However, in experiments V and VI, in which the dietary protein content was lowered and one percent of tyrosine added to the diet, the arginine deficient quail did not utilize their feed as efficiently as the arginine supplemented quail. These results suggest that a simple arginine deficiency decreases feed intake but not feed utilization, and that an arginine deficiency compounded by low protein level and excess tyrosine, in some manner, results in decreased feed utilization.

No difference in fresh liver weights was observed, but nickel supplementation significantly ($P < .005$) increased liver as a percent of body weight, and arginine supplementation and an arginine-nickel interaction significantly ($P < .005$) decreased liver as a percent of body weight.

No significant differences were observed in hematocrit or hemoglobin values. The addition of arginine to the diets significantly ($P < .05$) decreased total plasma protein levels. No difference in feathering was observed in this experiment

under conditions identical to experiment III where such a difference was observed.

Experiment IX

The quail used in this experiment were fourth generation birds (progeny of the experiment VII birds). The original purpose of this experiment was to use the birds for the production of the fifth generation. Because of the significant differences in growth (table 21) observed, the trial was terminated at 27 days and tissues removed for analyses. The only significant ($P < .05$) difference observed between the two groups was an increase in the liver as a percent of body weight in the low-nickel group.

A comparison of the arginase values obtained in experiment IX, and in the previous experiments, with the values obtained for the chicken by Tamir and Ratner (1963a) and by Nesheim (1968c) reveals that the figures obtained in the unactivated arginase assays for Japanese quail roughly correspond to the values obtained with activated assays for chick arginase. Values of 6880 and 7000 μ moles of urea per gram of fresh tissue per hour were obtained by Tamir and Ratner (1963a) and by Nesheim (1968c), respectively. The latter author reported that a value of 10,600 was obtained when a high arginine requiring strain of White Leghorns was fed an arginine deficient diet.

TABLE 21. WEIGHTS AND ANALYTICAL DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT IX.

Dietary Nickel, ppb	74		1780	±S.E.[1]
Weights, g				
Initial	5.6(53)[2]		5.3(38)	0.1
8 days	14.0(40)		17.6(23)***	0.4
15 days	32.7(38)		36.6(23)***	0.7
22 days	62.8(37)		65.8(21)	1.0
27 days	76.3(36)		79.8(21)*	0.8
Feed Consumption				
Feed/bird day	5.4		5.4	
Gain/feed	0.47		0.45	
Arginase[3]				
Unactivated	7672(29)		7452(21)	259
Activated[4]	31963(29)		30855(21)	952
Males[5]		32747(23)		
Females		30102(26)		
Liver Weight				
Fresh, g	2.34(34)		2.31(21)	0.03
Males	2.32(20)		2.32(8)	0.04
Females	2.36(14)		2.30(13)	0.05
% Body wt	3.10(36)*		2.89(21)	0.04
% Dry matter	31.6 (36)		31.8 (21)	0.7
Hematocrit, %	42.5(14)		43.4(15)	0.6
Hemoglobin, g/100 ml	10.3(15)		10.9(15)	0.2
Total Plasma Protein, g/100 ml	3.87(18)		3.60(8)	0.08

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.
[3]μ moles urea/g fresh tissue/hour.
[4]Activated with Mn^{+2} for 30 minutes.
[5]Statistical comparisons of males and females were conducted without regard to treatment.
*Significantly greater than lesser value ($P < .05$).
***Significantly greater than lesser value ($P < .005$).

Almost all of the values obtained from the activated assays of Japanese quail arginase were higher, in some experiments by a factor of four, than the values reported for the chick. A number of explanations are possible; the most obvious is the possibility that the Japanese quail kidney has a higher concentration of arginase or a more active arginase per unit of the enzyme than the chick. Storage conditions of the kidneys or the homogenates until assayed apparently can be an important factor in preventing loss of activity, as discussed earlier. The use of a too concentrated solution of arginine substrate has been reported to decrease the color produced in the color reaction between urea and α-isonitrosopropiophenone (Van Slyke and Archibald, 1946).

It is noteworthy that no sex difference was observed in liver weights at 27 days of age. Therefore, the heavier livers from adult females, as observed in experiments I and IV, must be the result of the increased metabolism associated with egg production.

Experiment X

This experiment was conducted to fulfill the original purpose of experiment IX, that of providing the fifth generation. Growth and feed consumption data for the experiment are presented in table 22. No significant difference in

TABLE 22. WEIGHTS AND FEED USE DATA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENT X.

Dietary Nickel, ppb	74	1780	
Weights, g			±S.E.[1]
Initial	6.1(44)[2]	6.2(35)	0.1
7 days	14.6(37)	15.5(27)	0.2
14 days	38.3(36)	37.7(27)	0.5
21 days	58.9(36)	58.2(27)	0.8
28 days	81.0(36)	83.8(27)	1.0
Feed Consumption			
Feed/bird day, g	6.0	6.1	
Gain/feed	0.43	0.43	

[1]Standard error of the mean.
[2]Numbers in parentheses represent number of quail for each value.

weight was observed at any of the weigh periods. The one significant factor evident from this experiment was the fact that growth rates, after four generations on a purified diet, were still within the "normal" range for Japanese quail. The experiment was terminated at 28 days, but no tissues were removed from the quail.

General Discussion

Numerous reports relate the dietary amino acid levels to the plasma amino acid pattern observed after the diet is fed. Because of the apparent lack in the literature of any plasma amino acid values for the Japanese quail, and because of the lysine-arginine relationship in casein which resulted

in a relative arginine deficiency in the birds, plasma amino acid analyses were carried out on pooled blood samples from seven of the 10 experiments which were conducted. Analyses were also performed on three pooled plasma samples from a group of quail fed a commercial diet and housed under conventional conditions. The values obtained (table 23) were to be used as controls for evaluating the plasma amino acid patterns resulting from feeding of the purified diets. As discussed previously, growth rates on the commercial diet were not as rapid as on the purified diets, and the vitamin adequacy of the diet was questioned. Therefore, the plasma amino acid pattern which developed after the diet was fed does not necessarily reflect the "normal" plasma amino acid pattern of the Japanese quail. The plasma amino acid pattern of chicks fed a crystalline amino acid reference diet (Dean and Scott, 1966) is also presented in table 23. The plasma amino acid values for the quail fed the purified diets, expressed as μ moles of amino acid per 100 ml of plasma, are presented in tables 23 to 29. Total plasma amino acids, total essential and nonessential amino acids and the essential to nonessential amino acid ratio are presented for each experiment.

The data in table 23 vary considerably. The variation could be due to amino acid deficiencies or excesses in either

TABLE 23. PATTERNS OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED A COMMERCIAL DIET[1] AND OF CHICKS FED A CRYSTALLINE AMINO ACID REFERENCE DIET.

Amino Acid	μ moles/100 ml[2]		Amino Acid	μ moles/100 ml[2]	
Arginine	19	16[3]	Glutamic Acid	23	78[3]
Lysine	69	35	Aspartic Acid	9	--
Methionine	7	9	Serine	50	41
Threonine	40	10	Proline	29	--
Histidine	2	15	Alanine	22	196
Isoleucine	15	9	Cystine	4	19
Leucine	32	15	Tyrosine	12	10
Valine	59	28	Ornithine	19	--
Glycine	69	77	Total Amino Acids	490	568
Phenylalanine	10	10	Total Essential (EAA)[4]	345	302
			Total Nonessential (NEAA)	136	266
			EAA/NEAA	2.54	1.14

[1]Composition of commercial diet was as follows (in percent): ground yellow corn, 41.1; soybean meal, 37.0; alfalfa meal, 5.0; dried whey, 2.5; meat and bone meal, 2.5; Menhaden fishmeal, 2.5; ground limestone, 5.0; dicalcium phosphate, 1.5; iodized salt, 0.5; vitamin and trace mineral premix, 0.4; fat, 2.0; crude protein content was assayed to be 25.8 to 26 percent with arginine, lysine and glycine concentrations of 1.55, 1.46, and 1.39 percent, respectively. Nickel content assayed to be 4.2 ppm.

[2]Values given represent an average of an assay of three pooled plasma samples, each sample representing six quail at three weeks of age.

[3]Amino acid pattern of chicks fed a crystalline amino acid reference diet for six hours (Dean and Scott, 1966).

[4]Includes glycine and glutamic acid.

TABLE 24. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL - EXPERIMENTS I and IV.

Amino Acid	μ moles/100 ml (Expt. I)[1]		μ moles/100 ml (Expt.IV)[1]	
	74 ppb Ni	1780 ppb Ni	74 ppb Ni	1780 ppb Ni
Arginine	58	50	21	27
Lysine	133	95	34	43
Methionine	10	8	4	7
Threonine	17	16	12	17
Histidine	9	21	3	4
Isoleucine	15	14	13	14
Leucine	20	16	22	22
Valine	17	20	43	37
Phenylalanine	15	16	11	6
Glycine	27	15	53	49
Glutamic Acid	28	49	59	46
Aspartic Acid	2	3	19	16
Serine	41	36	60	28
Proline	11	21	30	36
Alanine	23	20	44	39
Cystine	3	2	4	5
Tyrosine	18	15	10	16
Ornithine	31	37	8	11
Total Amino Acids	478	454	450	423
Total Essential (EAA)[2]	349	320	275	272
Total Nonessential (NEAA)	129	134	175	151
EAA/NEAA	2.71	2.39	1.57	1.80

[1]Values represent an average of an assay of two pooled plasma samples, each sample representing three adult quail.
[2]Includes glycine and glutamic acid.

TABLE 25. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT II[1].

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg
Concentration Ni, ppb	74	74	1780	1780
Supplemental Arg, %	0	0.88	0	0.88
Amino Acid	μ moles/100 ml			
Arginine	17	15	15	14
Lysine	79	85	77	85
Methionine	3	4	4	4
Threonine	22	25	25	36
Histidine	2	2	2	3
Isoleucine	28	21	35	42
Leucine	46	32	63	87
Valine	62	41	63	95
Phenylalanine	9	7	7	9
Glycine	50	39	40	45
Glutamic Acid	18	10	13	10
Aspartic Acid	4	3	3	5
Serine	20	22	17	22
Proline	23	17	52	42
Alanine	28	35	33	64
Cystine	2	3	2	4
Tyrosine	14	12	12	13
Ornithine	12	8	12	16
Total Amino Acids	439	381	477	596
Total Essential (EAA)[2]	336	281	344	430
Total Nonessential (NEAA)	103	100	131	166
EAA/NEAA	3.26	2.81	2.63	2.59

[1]Values represent an average of an assay of two pooled plasma samples, each sample representing five quail at 16 days of age.
[2]Includes glycine and glutamic acid.

TABLE 26. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT III.[1]

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg
Concentration Ni, ppb	74	74	1780	1780
Supplemental Arg, %	0	1.13	0	1.13
Amino Acid		μ moles/100 ml		
Arginine	12	22	17	25
Lysine	84	62	79	61
Methionine	5	7	7	6
Threonine	28	46	66	58
Histidine	2	5	-	7
Isoleucine	16	19	24	18
Leucine	12	19	14	20
Valine	39	43	43	55
Phenylalanine	8	9	13	10
Glycine	52	56	71	57
Glutamic Acid	55	53	61	59
Aspartic Acid	12	3	5	6
Serine	43	99	54	98
Proline	26	13	31	25
Alanine	38	74	51	77
Cystine	4	4	3	3
Tyrosine	33	22	28	19
Ornithine	9	15	165	18
Total Amino Acids	478	571	732	622
Total Essential (EAA)[2]	313	341	395	376
Total Nonessential (NEAA)	165	230	337	246
EAA/NEAA	1.90	1.48	1.17	1.53

[1]Values represent an average of an assay of two pooled plasma samples, each sample representing three quail at 25 days of age.
[2]Includes glycine and glutamic acid.

TABLE 27. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL, TWO LEVELS OF ARGININE AND ONE PERCENT OF SUPPLEMENTAL TYROSINE - EXPERIMENT V.[1]

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg
Concentration Ni, ppb	71	71	4660	4660
Supplemental Arg, %	0	1.13	0	1.13
Concentration Mn, ppm	90	90	90	90
Amino Acid	μ moles/100 ml			
Arginine	102	101	77	216
Lysine	487	229	586	754
Methionine	1	1	1	2
Threonine	21	12	42	16
Histidine	99	28	32	54
Isoleucine	5	3	5	7
Leucine	7	7	11	7
Valine	22	19	24	16
Phenylalanine	10	7	12	10
Glycine	71	37	67	71
Glutamic Acid	58	38	73	50
Aspartic Acid	6	3	6	3
Serine	30	25	67	52
Proline	58	11	23	35
Alanine	49	34	61	68
Cystine	2	2	5	2
Tyrosine	39	8	17	23
Ornithine	52	40	29	80
Total Amino Acids	1119	605	1138	1466
Total Essential (EAA)[2]	883	482	930	1203
Total Nonessential (NEAA)	236	123	208	263
EAA/NEAA	3.74	3.92	4.47	4.57

[1]Values given represent an average of an assay of two pooled plasma samples, each sample representing three quail at 27 days of age.
[2]Includes glycine and glutamic acid.

TABLE 28. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL, TWO LEVELS OF ARGININE AND ONE PERCENT OF SUPPLEMENTAL TYROSINE - EXPERIMENT VI.[1]

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg
Concentration Ni, ppb	71	71	4660	4660
Supplemental Arg, %	0	1.13	0	1.13
Concentration Mn, ppm	40	40	40	40
Amino Acids		μ moles/100 ml		
Arginine	46	265	70	199
Lysine	761	609	538	499
Methionine	1	1	1	29
Threonine	32	12	27	3
Histidine	29	62	44	32
Isoleucine	7	5	6	1
Leucine	9	7	9	5
Valine	34	23	24	1
Phenylalanine	34	--	--	21
Glycine	97	57	64	51
Glutamic Acid	61	67	52	4
Aspartic Acid	23	12	21	14
Serine	61	22	48	39
Proline	108	58	72	--
Alanine	60	43	49	65
Cystine	2	1	1	--
Tyrosine	34	15	34	--
Ornithine	32	135	31	87
Total Amino Acids	1431	1394	1091	1050
Total Essential (EAA)[2]	1111	1108	835	845
Total Nonessential (NEAA)	320	286	256	205
EAA/NEAA	3.47	3.87	3.26	4.12

[1] Values given represent an average of an assay of two pooled plasma samples, each sample representing four quail at 26 days of age.
[2] Includes glycine and glutamic acid.

TABLE 29. PATTERN OF FREE AMINO ACIDS IN BLOOD PLASMA OF JAPANESE QUAIL FED TWO LEVELS OF NICKEL AND TWO LEVELS OF ARGININE - EXPERIMENT VIII.[1]

Diet	-Ni-Arg	-Ni+Arg	+Ni-Arg	+Ni+Arg
Concentration Ni, ppb	74	74	1780	1780
Supplemental Arg, %	0	1.13	0	1.13
Amino Acid		μ moles/100 ml		
Arginine	30	72	20	27
Lysine	155	82	76	64
Methionine	10	10	17	14
Threonine	30	24	65	48
Histidine	25	14	12	10
Isoleucine	25	32	16	23
Leucine	40	59	26	45
Valine	58	89	81	78
Phenylalanine	17	14	41	25
Glycine	84	77	100	100
Glutamic Acid	41	44	48	49
Aspartic Acid	26	34	36	19
Serine	45	55	71	66
Proline	68	74	75	93
Alanine	73	59	134	73
Cystine	5	6	7	4
Tyrosine	24	27	23	28
Ornithine	22	17	7	16
Total Amino Acid	778	789	855	782
Total Essential (EAA)[2]	515	517	502	483
Total Nonessential (NEAA)	263	272	353	299
EAA/NEAA	1.96	1.90	1.42	1.62

[1]Values given represent an average of an assay of two pooled plasma samples, each sample representing three quail at 27 days of age.
[2]Includes glycine and glutamic acid.

or both of the diets fed, to the type of diet fed or to a species difference. The high essential to nonessential amino acid ratio of the chick plasma is due mainly to the unusually high plasma level of alanine. However, the amino acids of most interest in the present study, arginine, lysine and tyrosine, are in relatively close agreement between the two species and will be used as reference levels.

Amino acid analyses were carried out on plasma from the adult birds of experiments I and IV and the immature quail of experiments II, III, V, VI and VIII. In all experiments, the birds had continuous access to feed prior to exsanguination.

The plasma concentration of both arginine and lysine were higher in the adult birds of experiment I (table 24) than in the three week old quail fed the commercial diet (table 23). The values obtained for the adult birds of experiment IV are comparable to those of table 23 but exhibit a somewhat narrower lysine to arginine ratio. No explanation can be given for the differences observed between the two experiments, as both groups of birds were receiving identical diets containing 1.13 percent supplemental arginine at the time the plasma samples were obtained. Total plasma amino acid concentrations for the two experiments were similar. It appears very unlikely that any of the variation in plasma amino acid concentrations in the two trials was due to the dietary

nickel concentration although nickel supplementation of the diets appeared to result in a decrease of the essential to nonessential amino acid ratio.

Plasma amino acid values from experiment II are presented in table 25. Arginine was supplemented at a level of 0.88 percent of the diet to one-half of the birds of this trial. However, the dietary arginine level had no effect on the plasma arginine concentration. The plasma arginine level of chicks has been reported not to increase until after the dietary arginine concentration was high enough to permit maximum weight gain (Zimmerman and Scott, 1965; Scott, 1967). Therefore, it is likely that the 0.88 percent supplemental arginine did not meet the requirement for arginine, and as a result, no increase in the plasma arginine level occurred. A dietary amino acid deficiency is also indicated by the high essential to nonessential amino acid ratio observed (Dean and Scott, 1966). No relationship between dietary arginine and plasma threonine was evident. An inverse relationship between the two amino acids has been reported by Morrison et al. (1961). Plasma lysine concentration was unaffected by arginine supplementation.

Arginine supplementation at the level of 1.13 percent of the diet in experiment III caused an increase in the plasma arginine concentration, indicating that the require-

ment of the Japanese quail for supplemental arginine to a 35 percent casein diet is greater than the 0.88 percent level of experiment II and probably near the 1.13 percent level of the present experiment. Arginine supplementation caused a reduction in the plasma lysine concentration and a reduction in the essential to nonessential amino acid ratio which, according to Dean and Scott (1966), represents the result of feeding a more balanced protein. As in experiment II, nickel supplementation decreased the essential to nonessential amino acid ratio, and the decrease was somewhat greater in the groups not receiving supplemental arginine in both experiments.

Plasma amino acid patterns for experiments V and VI are presented in tables 27 and 28. Supplementation of the lower protein diets used in these experiments with tyrosine resulted in greatly increased levels of total amino acids and increased essential to nonessential amino acid ratios, indicating that the protein quality was lower than in experiment III. Most of the increase in total plasma amino acids was due to the great increase in plasma lysine. Plasma arginine levels were higher in both experiments than in experiment III, indicating that its use in protein synthesis was probably impaired. Arginine supplementation increased plasma arginine levels on both dietary nickel levels in experiment VI but only on the higher nickel levels in experiment V. Supple-

mental dietary tyrosine did not produce any definite increase in plasma tyrosine.

A more balanced diet was fed in experiment VIII as is indicated by the lower essential to nonessential amino acid ratio than obtained in experiments V and VI. Nickel supplementation again reduced this ratio. Arginine supplementation increased plasma arginine slightly and decreased plasma lysine. Total plasma amino acids were much lower than in experiments V and VI but slightly above those of experiments I, II, III and IV.

Therefore, the only effect which could possibly be attributed to nickel is the lowering of the essential to nonessential amino acid ratio as noted in experiments II, III and VIII. Such an effect, if real, would tend to implicate nickel in some manner in amino acid utilization, possibly in membrane transport of amino acids or in protein synthesis per se.

At the conclusion of each experiment, two quail, selected at random, were necropsied for microbiological and histopathological evaluation. No significant microbiological findings were reported. Tissues evaluated histologically included bone, cardiac and skeletal muscle, liver, skin and feather follicles, bursa of Fabricius, brain, spinal cord, sciatic nerve, spleen and thymus. In general, marked differences were not observed between treatment groups. The single ex-

ception was the tendency for the birds receiving the low-nickel diet, unsupplemented with arginine, to have very prominent nucleoli in the hepatic cells. The only other differences consistently noted were attributed to the difference in size of the birds, as the birds which received the arginine deficient diets were much smaller than those which received the arginine supplemented diets. Those differences attributed to size included a difference in thickness of the cortical bone of the tibia, differences in the stage of development of the feather follicles and minor differences in the size and development or state of atrophy of the bursa of Fabricius. The pathologist concluded that there were few, if any, real differences in the tissues from birds of the different groups.

Thus, some question arises concerning the observations reported by Newberne et al. (1960). The report is concerned with the lesions developed in chicks fed an arginine deficient diet, and one must question whether the effect of size was eliminated as a contributing factor in the lesions reported. It is also possible that chicks respond differently to an arginine deficiency and that the lesions reported were real.

The hematocrit, hemoglobin and total plasma protein values obtained in the 10 experiments compare closely with values for the Japanese quail which have been published

previously. Hemoglobin and hematocrit values of 8.4 to 10 g per 100 ml and 33.8 to 41.8 percent, respectively, were reported by Fox and Harrison (1964). A gradual increase with age, to 43 days, in both hematocrit and hemoglobin values has been reported by Atwal et al. (1964). They reported hematocrit and hemoglobin values of 42 percent and approximately 13.7 g per 100 ml, respectively, with male Japanese quail possessing a somewhat higher hemoglobin level than the females. A total plasma protein concentration of approximately 3.5 g per 100 ml was reported by the same authors. The value for hematocrit and total plasma proteins agree very closely with the 42 percent hematocrit value and 3.6 g per 100 ml total plasma protein value obtained by Jacobs et al. (1969).

The values obtained on these parameters in the present series of experiments were very close to the values just discussed. The total plasma protein values tended to be somewhat higher than the literature values, except in experiments VIII and IX where the values averaged very near to the 3.5 to 3.6 figures.

Analytical values for a number of the minerals and crude protein values were obtained on the purified diets as a measure of the uniformity of mixing of the diets and for comparison with the calculated values. The values obtained

for calcium, phosphorus, magnesium, manganese, iron, zinc, copper and crude protein are presented in table 30. The calculated values are also given for comparison.

Most of the values obtained by assay agreed quite closely with the calculated values. The calculated crude protein values include the nitrogen supplied by the supplemented amino acids, glycine, glutamic acid and arginine. The casein used in the diets was assayed to contain 77 percent protein. The mixed diets contained approximately 4100 kcal of gross energy per kg.

During the first four weeks of all the experiments, uninked newsprint was used to absorb the excreta. The nickel content of the newsprint was determined to be 0.38 ppm. Some consumption of the newsprint did occur during the course of the experiments, but the significance of the consumption of a very small amount of the paper could not be evaluated.

TABLE 30. ANALYTICAL DATA ON PURIFIED DIET.

Nutrient	Assayed concentration		Calculated concentration	
	Grower diet	Layer diet	Grower diet	Layer diet
Calcium, ppm	10900	25680	11950	28450
Phosphorus, ppm	9652	9652	10820	10820
Magnesium, ppm	1596	1596	1401	1401
Manganese, ppm	112[1](39)[2]	112[1](39)[2]	90[1](40)[2]	90[1](40)[2]
Iron, ppm	103	103	91	91
Zinc, ppm	100	100	89	89
Copper, ppm	12	12	12	12
Crude protein, %	34[3](35)[4]		35[3](33)[4]	
	28[5](26)[6]		27[5](25)[6]	

[1]Content of Mn in all experiments except V and VI.
[2]Mn content of diets for experiments V and VI.
[3]Crude protein (N x 6.25) content of 35% casein diet containing supplemental arginine.
[4]Crude protein (N x 6.25) content of 35% casein diet without supplemental arginine.
[5]Crude protein (N x 6.25) content of 25% casein diet containing supplemental arginine.
[6]Crude protein (N x 6.25) content of 25% casein diet without supplemental arginine.

V. CONCLUSIONS

Within the limits of the experimental conditions employed, the following conclusions can be made:

1. Normal weight gain and reproduction of Japanese quail have been demonstrated to occur in a controlled environment, such as exists in a gnotobiotic isolator.
2. A purified diet was developed which supported normal weight gain, egg production, fertility, hatchability and chick weight at hatching.
3. In a casein diet, arginine is the first limiting amino acid for the Japanese quail as it is for the chicken. Supplementation of a 35 percent casein diet with 1.13 percent of L-arginine hydrochloride alleviated the growth limiting effects of the casein diet and increased the plasma arginine, an indication that the arginine requirement had been met.
4. The dietary vitamin requirement of the Japanese quail is greater than that of chickens.
5. Washing dietary ingredients with chelating agents is not an effective method for reducing the nickel content of the mixed diet below 75 ppb.

6. The Japanese quail has a higher concentration of kidney arginase than the chicken.
7. Attempts to demonstrate in vivo activation of arginase by nickel produced variable and inconclusive results, even when the dietary manganese concentration was reduced to 40 ppm.
8. Excess dietary tyrosine had no apparent effect on kidney arginase activity when added as one percent of the diet. It is possible that higher levels would stimulate arginase activity.
9. The dietary levels of nickel employed in this study had no effect on hematocrit, hemoglobin or total plasma protein values.
10. The addition of nickel to a diet deficient in arginine improved weight gain and decreased the plasma essential to nonessential amino acid ratio and may be an indication that nickel improved the efficiency of arginine utilization.
11. Nickel supplementation of an otherwise adequate diet was without effect upon the parameters measured. This suggests that the nickel requirement for the Japanese quail is less than 74 ppb.

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VI. BIBLIOGRAPHY

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APPENDIX

VII. APPENDIX

APPENDIX TABLE 1. COMPOSITION OF MINERAL PREMIX[1]

$CaHPO_4$	4440.0 g
$KHCO_3$	1200.0
$NaHCO_3$	1092.0
$4MgCO_3(Mg)\cdot H_2O$[2]	653.0
$CaCO_3$	300.0
KCl	195.5
$FeSO_4\cdot 7H_2O$	53.0
$ZnSO_4\cdot 7H_2O$	42.0
$MnSO_4\cdot H_2O$	33.3
$CuSO_4$	3.5
$NaMoO_4\cdot 2H_2O$	950 mg
Kl	950
NaF	264
$CoCO_3$	120
$CrK(SO_4)_2\cdot 12H_2O$	120
Na_2SeO_3	26.4

[1]Sufficient premix to make 120 kg of mixed basal diet.
[2]Assayed to contain 25.75% Mg.
[3]4850 g $CaCO_3$ added to mineral premix for layer diets.

APPENDIX TABLE 2. COMPOSITION OF VITAMIN PREMIX[1]

Nicotinic Acid	25.2 g
Calcium Pantothenate	10.8
Thiamine Mononitrate	3.6
Riboflavin	3.6
Pyridoxine·HCl	3.6
Folic Acid	1.4
Inositol	46.8
Ascorbic Acid	28.8
Para Amino Benzoic Acid	4.67
Menadione	432.0 mg
D-Biotin	39.6
Cholecalciferol	7.2
B_{12}[2] triturate (0.1% B_{12})	4.9
Total Vitamins	104.1 g
Glucose	895.9 g
Total	1000.0 g

[1]Sufficient premix to make 120 kg of mixed basal diet.

APPENDIX TABLE 3. PREPARATION OF NICKEL STANDARDS

1. Dissolve 1.0020 g of 99.8% nickel shot[a] in approximately 20 ml of concentrated HNO_3.

2. Dilute to 1000 ml = 1000 ppm standard.

3. Make appropriate dilutions to obtain working standards with concentrations of 0.030, 0.075, 0.150, 0.300, 0.500, 0.750 and 1.000 ppm nickel.

4. 10 ml of each of the working standards analyzed in duplicate through the analytical procedure along with the unknown samples.

[a]J. T. Baker Chemical Co., Phillipsburg, N.J.

APPENDIX TABLE 4. SOLUTIONS FOR ARGINASE ASSAY

0.1M glycine buffer, pH 9.5	11.15g glycine/liter, pH adjusted with NaOH
0.02M KH_2PO_4 buffer, pH 7.5	2.72g KH_2PO_4/liter, pH adjusted with NaOH
0.25M sucrose	85.6g sucrose/liter
0.1M $MnCl_2$	19.79g $MnCl_2$/liter
15% trichloroacetic acid (TCA)	150g TCA/liter
4% alcoholic α-isonitrosopropiophenone (α-INPP)	4g α-INPP/100 ml absolute ethanol
0.085M L-arginine hydrochloride, pH 9.5	1.791g L-arginine HCl/liter, pH adjusted with NaOH
Acid mixture made to contain 10^{-2}M Fe^{+3} ion	1 part water 1 part concentrated H_2SO_4 3 parts concentrated H_3PO_4 5.4g $FeCl_3 \cdot 6H_2O$/liter
Urea standards 150 μ moles urea/ml 300 600 900 1200 1500	90.09g urea/liter = 1500 μ moles/liter, appropriate dilutions made to obtain standards of lesser concentration