EFFECT OF RIBOFLAVIN ON EFFICIENCY OF DIETARY ENERGY AND PROTEIN UTILIZATION AND, THE SYMPTOMS OF RIBOFLAVIN DEFICIENCY IN GROWING CHICKS

by

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ABSTRACT

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Restricted feeding regimens were used in isonitrogenous isonutrient rations (semi-purified diet) to study the effect of dietary energy per se on riboflavin (flavin) requirement of early growing broiler cockerels (from one-day old to 19 days of age). Similarly, isocaloric-isonutrient rations were used to study the effect of dietary protein per se on flavin requirement of chicks. Since graded levels of flavin were used in combination with above feeding regimens, the effect of flavin intake per se on efficiency of energy and protein utilization was determined by carcass analyses at the end of the 16-day feeding period.

Reducing dietary energy to 80, 70 or 60% of that which chicks would consume ad libitum, reduced the amount of dietary flavin required by chicks for maximal performances: growth, feed intake, feed conversion, and prevention of deficiency symptoms.

Similarly, when dietary energy was restricted to 70%, a concomitant restriction of protein intake to 85 or 70% of that which chicks would consume, dietary flavin requirement was found to be parallel to the level of protein intake.

In severe flavin deficiency with chicks fed ad libitum, when flavin concentration of the ration was 60% of a marginal level (3.775 mg/kg), efficiency of utilization of both energy and protein was decreased significantly ($P < 0.05$).

Efficiency of energy utilization was significantly ($P < 0.01$) decreased by milk flavin deficiency when chicks were fed ad libitum. Efficiency of protein utilization was also significantly ($P < 0.05$) decreased due to a mild flavin deficiency when chicks received 80, 70 or 60% energy-restricted ration. Neither energy nor protein utilization was affected by a very mild flavin deficiency when both energy and protein were restricted to 70% of that which chicks would consume. However, this very mild flavin deficiency manifested characteristic flavin deficiency symptoms, viz., increased percentage of heterophils in white blood cells, decreased percentage of reticulocytes in the circulating blood, and decreased the concentration of liver flavin.

Plasma hydroxypurines and serum uric acid concentrations were not affected appreciably by flavin deficiency. A more sensitive analytical method may be necessary to elucidate possible changes of plasma hydroxypurines.

Significant ($P < 0.05$) accumulation of hydroxypurines (hypoxanthine and xanthine) occurred in both free and bound forms in the livers of flavin deficient chicks at the ages between 15 to 20 days. The injection of hydroxypurine derivative (sodium inosinate, 200 mg per 100 g of body weight per injection, once or twice daily) induced an increase in percentage of heterophils in white blood cells, increase in hematocrit, but a decreased percentage of reticulocytes in the circulating blood, and decreased concentration of liver flavin. These observations strongly support the hypothesized mechanism of

flavin deficiency, i.e. a decrease in xanthine dehydrogenase activity in the tissues of flavin deficient chicks resulting in an accumulation of hydroxypurines in the tissues. The presence of unusual amount of hydroxypurines in the tissues caused increased production of heterophils and decreased production of reticulocytes.

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INTRODUCTION

During the past three decades numerous studies have been conducted on the requirement of riboflavin (flavin) in relation to dietary energy or protein. Several workers (Chu et al. 1964; Appelgate and Potter, 1963; Reiser and Pearson, 1949 and Mannering et al. 1941) found that dietary energy governed the flavin requirement, while others (Kaunitz et al. 1954; Oldham et al. 1947; and Sarett et al. 1943; 1942) reported that dietary protein determined the requirement of flavin. These controversial results seemed to be due mainly to the difference in experimental conditions used by various investigators. Composition of the rations and the methods of feeding probably played an important role in producing these results.

A limited amount of information is available concerning biochemical and hematological changes which occur during flavin deficiency. Therefore studies in biochemical and hematological variation are necessary in order to properly describe the function of flavin in growing chicks.

The experiments described herein were designed to study:

(A) Experimental variables among dietary treatments by using restricted feeding regimens with (1) isonitrogenous-isonutrient rations to study the effect of energy per se on flavin requirement, (2) isocaloric-isonutrient rations to study the effect of protein per se on flavin requirement. Since graded levels of flavin were used in above feeding regimens, the effects of flavin intake upon efficiency of energy and protein utilization were

determined by carcass analysis.

(B) Sensitivities of various criteria to flavin deficiency.

(C) Certain hematological and biochemical changes associated with flavin deficiency and possible mechanisms of action.

REVIEW OF LITERATURE

Dietary energy and protein in relation to riboflavin requirement

The relation between riboflavin (flavin) requirement and dietary energy and/or protein has been studied by numerous workers with various animals. Reiser and Pearson (1949) reported that the flavin requirement of chicks was 300 to 325 microgram (µg) per 100 gram (g) of feed. However, when a high fat diet was used, the requirement increased to 400 µg per 100 g of feed. Chu et al. (1964) and Appelgate and Potter (1963) found that the flavin requirement was more highly correlated with the energy content of the ration or to the daily intake of flavin than to an amount of flavin per pound of feed for turkey poults. According to Mannering et al. (1941), ad libitum feeding experiments with flavin deficient young rats, using rations containing 25% or 40% fat, indicated a deleterious effect of the high fat level on growth that could be corrected by flavin supplementation. An extensive review of literature by Bro-Rasmussen (1958) on flavin requirements of various animals (horse, adult human, calf, pig, child, dog, fox, hen, turkey poult, chick, duckling, rat and mouse) revealed a similarity in requirement of the vitamin when the requirements were expressed as mg per 1000 kilocalories of energy intake.

Czaczkes and Guggenheim (1946) reported that the protein and fat of the diet of growing rats affected the flavin content of the organs (liver, kidney and muscle) and the excretion of the vitamin

in the urine. Mitchell et al. (1950) showed that increased levels of protein or fat in a diet of natural feeds depressed the output of flavin in the urine, indicating an increased requirement of the vitamin under conditions of increased assimilation of protein and fat in growing pigs. The studies of Kaunitz et al. (1954) on dietary protein, flavin and galactoflavin supplements indicated that the utilization of protein and of flavin are mutually limiting. High dietary protein levels cannot be utilized if flavin is rigidly restricted and vice versa.

Results from experiments thus far cited indicate that an increase in protein or fat density in the ration raises the requirement of flavin when expressed in terms of weight of the vitamin per unit weight of ration consumed. Thus, the requirement of the vitamin is proportional to the fat and/or protein consumed.

Two reasons for this apparent increase in flavin requirement have been postulated:

(1) Fat and/or protein depressed intestinal synthesis of flavin and thus altered the total flavin available to meet the requirement (Czaczkes and Guggenheim, 1946; and Mannering et al. 1944).

(2) Animals required flavin for energy (fat and carbohydrate) and protein metabolism (Kaunitz et al. 1954; Czaczkes and Guggenheim, 1946; and Potter et al. 1942).

In supporting the first postulation, Taylor et al. (1942) and, Mitchell and Isbell (1942) proved with rats that flavin synthesized by intestinal bacteria contributed to the body's supply of this vitamin. Mannering et al. (1944) showed that dextrin and corn starch

stimulated bacterial synthesis of flavin in the rat intestine, and reduced the dietary flavin requirement. "Nevertheless it can be argued that the flavin may be synthesized in a region of the intestine from which it cannot be absorbed. Selye (1943) studied the absorption of flavin from the various sections of the tract, separated by ligatures, using nephrectomized rats. He concluded that in the small intestine flavin is both absorbed and excreted, while, in the cecum and colon, injected flavin is rapidly destroyed with little, if any, absorption of the vitamin. Whether or not minute amounts of flavin are absorbed under normal conditions from the cecum and colon remains a question (Mannering et al. 1944)".

Intestinal synthesis of B-vitamins in birds has also been studied. Couch et al. (1950) reported that the cecal content of the turkey and chicken had a higher value for the five vitamins, flavin, niacin, pantothenic acid, folic acid and biotin than did small intestinal material taken from the same birds. Coates et al. (1951) found that the presence of the antibiotics had no effect on the degree of deficiency of flavin, pyridoxine or pantothenic acid, lessened that of biotin or folic acid and increased that of nicotinic acid. Shrimpton (1954) observed a marked increase of flavin in the eggs of hens on a diet low in flavin when maize starch was replaced by raw potato starch. These results indicate the possibility of intestinal synthesis of flavin in the birds. However, the significance of this in relation to dietary requirement of flavin has not been elucidated.

In substantiating the second postulation, Oldham et al. (1947)

found that in the adult human, daily flavin excretion varied inversely with the coexistent nitrogen balance. They represented from 40 to 60% of the flavin intake when the nitrogen balances were decidedly negative and approximately 7% of the intake when the nitrogen balances were strongly positive. Further to this finding, an inverse relationship between protein intake and the urinary excretion of flavin in dogs and rats was demonstrated by the studies of Sarett et al. (1942, 1943). In addition, Lockhart et al. (1966) found significant positive correlations between flavin intake and the percentage retention of dietary nitrogen or efficiency of metabolizable energy utilization by growing chicks.

Further evidence of relationship between flavin requirement and protein and/or fat gain in chicks and rats was noted by means of carcass analysis technique (Kleiber and Jukes, 1942; Voris and Moore, 1943; and Sure and Dichek, 1941).

Metabolism of carbohydrate, fat and protein in relation to riboflavin containing enzymes in birds and mammals

Metabolism of carbohydrate

The main function of ingested carbohydrate is to provide a source of energy to the host organism. Carbohydrate metabolism is essentially the metabolism of glucose and of substances related to glucose in their metabolic processes. Glucose metabolism may follow one of the following pathways, i.e. (1) be degraded to generate energy in the form of ATP, via glycolysis, tricarboxylic cycle, and the electron transport chain where ATP is the direct energy source for energy requiring metabolic processes. (2) enter the pentose

phosphate cycle with the result that reduced TPN is formed. (3) be stored as glycogen in liver and muscles or as fat in adipose tissues.

Physiologically the pentose phosphate cycle represents a mechanism for the formation of pentose (for nucleotide synthesis) and for the interconversion of pentoses and hexoses. One of the most important functions of the pentose phosphate cycle is to provide TPNH which serves as a reducing agent in the synthesis of fatty acids, steroids and various other substances.

Glycogen is the chief storage form of carbohydrate in tissues. The tissues involved in glycogen synthesis and storage appear to be mainly the liver and skeletal muscle.

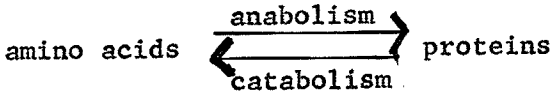
Metabolism of fat (lipid)

Like glucose and amino acids, fats are transported by the blood, especially in the plasma to all parts of the body. Fats exist in the animal body in at least two forms: tissue fats or those which appear to be an integral part of cell structure, and adipose tissues which may contain as much as 90% body fat (triglycerides). Fat is a very concentrated form of energy, having a caloric value more than twice that of carbohydrate. It appears that there is a continual metabolism of body fat, with anabolism and catabolism proceeding simultaneously. The complete breakdown of fat in the body ultimately leads to carbon dioxide and water, and the liberation of energy. The mechanisms by which fat is metabolized involve a long series of successive processes in which the glycerol and fatty acid components are dealt with in different ways. Glycerol is metabolized in a manner similar to that of carbohydrates, while fatty acids

are broken down through "β-oxidation" into two-carbon units as acetyl CoA.

Metabolism of protein or other nitrogenous compounds

The metabolism of protein essentially is the metabolism of amino acids. Dietary proteins are digested to amino acids in the gastrointestinal tract, and these amino acids then pass into the blood stream and circulate through various tissues of the body. Animals synthesize the non-essential amino acids by combining the nitrogen of the excess amino acids with the appropriate non-nitrogenous organic acids. Also, tissue protein catabolism adds amino acids to the blood. Blood amino acids therefore arise from absorption, synthesis and tissue catabolism, and represent an amino acid pool which can be drawn upon for all purposes of protein metabolism. Each tissue of the body takes from this pool the specific amino acids in the proper proportions and synthesizes them into the kinds of proteins required for growth, maintenance, and proper functions. The processes of tissue protein synthesis and breakdown go on simultaneously. Although both processes are constituted of many stages, the overall operation may be represented:



Amino acids in excess of the requirement for the formation of structural tissue proteins, enzymes, hormones, etc., are deaminized in the liver to form ammonia and keto acids. The ammonia is converted to uric acid in birds or urea in most mammals, and is excreted in the urine. The liver converts some of the keto acids to

ketone bodies, and some to glucose. These ketone bodies enter the metabolic pathway of fat. Thus, metabolism of protein is linked to both fat and carbohydrate metabolism.

The metabolism of the nucleic acids yields phosphate and purine bases, adenine and guanine, which are also converted to uric acid in birds and excreted as such in the urine.

The roles of riboflavin in the metabolic pathways

Riboflavin (flavin) in animal tissues forms a part of two coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Bessey et al. (1949) reported that in rats, of 21 different tissues studied, free flavin was found to be present in quantitatively insignificant amounts, while, FAD accounted for 70 to 90% of the total flavin.

Flavin in the form of one or another of these two coenzymes is required for various chemical reactions which are involved in the metabolism of carbohydrate, fat and protein. Since the metabolism of carbohydrate, fat and protein are interrelated, it is difficult to separate the flavin-containing enzymes into three distinct groups which are responsible for carbohydrate, fat and protein metabolism respectively. However, it is possible to divide flavin-containing enzymes into three groups. Those enzymes within each group generally share either similar enzymatic properties or serve a related metabolic goal. The enzymes in group I are involved in the generation of energy (ATP) through glycolysis, the tricarboxylic acid cycle, and the electron transport chain. Group II enzymes are involved in metabolism of nitrogenous compounds which

includes metabolism of protein, nucleic acids and other nitrogenous compounds. Group III enzymes are related to fatty acid synthesis or breakdown. This group of enzymes is mainly responsible for energy conservation (storage) as depot fat or the utilization of energy from depot fat.

1. Group I enzymes

The most important feature of this group of enzymes is that they are structural as well as functional components of the respiratory chain localized in mitochondria. The coenzyme (FAD) is very tightly bound to this group of enzymes and they show a high degree of specificity toward both substrate and electron carrier. This group of enzymes is as follows: DPNH, succinic, α-glycerophosphate, and choline dehydrogenases. Electron-transferring flavoproteins, pyridine nucleotide cytochrome c reductase, and quinone reductase. Only two enzymes in this group, DPNH and succinic dehydrogenases, have been studied in relation to flavin deficiency. DPNH dehydrogenase had the highest activity in tissues studied (brain, heart, liver and kidney) and showed no decrease in moderate flavin deficiency in rats. In severe deficiency it showed a downward trend (Burch et al. 1956). Burch et al. (1960) found that DPNH dehydrogenase content remained unchanged in mitochondria prepared from livers of flavin deficient rats. Succinic dehydrogenase activity of rat liver was reported to be decreased in flavin deficient rats (Burch et al. 1960; Axelrod et al. 1942a; and Axelrod et al. 1942b).

2. Group II enzymes

Dissociable FAD was observed in at least three enzymes, (i.e. glycine, D-amino acid, and glycolate oxidases) by Burch et al. (1956). The enzymes are not as specific for their substrates and electron acceptors as Group I enzymes. In addition, these enzymes appeared to be very sensitive to flavin deficiency.

Since numerous studies have been carried out on this group of enzymes, some details are given as follows:

a. Xanthine oxidase

Highly purified preparations of the enzyme from milk have been shown to catalyze the oxidation of hypoxanthine, xanthine, aldehydes, DPNH and several pterins. In addition, many compounds are known to be effective acceptors of hydrogen, viz., molecular oxygen, methylene blue (and other oxidation-reduction dyes), cytochrome c, ferricyanide, nitrates and quinones. Although less work has been done with the mammalian and avian liver enzymes, available evidence indicates that they, too, are capable of activating the same substrate and transferring hydrogen to diverse acceptors (De Renzo, 1956). Little is known about the biological function of xanthine oxidase, however, since aldehydes do not occur in substantial quantities in animals, and since the oxidation of hypoxanthine and xanthine by molecular oxygen is among the most rapid of the reactions catalyzed by the enzymes, it is reasonable to assume that these are biologically the most significant (Bray, 1959). Bauer and Bradley (1958) reported that xanthine oxidase activity rose markedly during virus multiplication. However, other

reports have shown that the enzyme activity is relatively low in various tumors (Colter et al. 1957; and Lewin et al. 1957).

al-Khalidi and Chaglassian (1965) found xanthine oxidase in 21 different tissues from cow, sheep, dog and cat. The tissues studied were liver, lung, kidney, skin, heart, muscle, pancreas, intestine (whole), intestinal mucosa, colon, thyroid, adrenal, uterus, diaphragm, ovary, spleen, bone marrow, adipose, mesentery, brain and blood.

The enzyme of bird tissues is called xanthine "dehydrogenase" because, it is not autoxidizable and a hydrogen carrier must be added to allow the reaction to proceed (Richert and Westerfeld, 1951; and Stirpe and Corte, 1965). It is hypothesized that xanthine dehydrogenase in avian liver and/or kidney is responsible for converting hypoxanthine and xanthine to uric acid, the end product of nitrogen catabolism (Richert and Westerfeld, 1951; and Stirpe and Corte, 1965).

According to Westerfeld et al. (1962), in both chicks and turkeys, the liver xanthine dehydrogenase activity increased in a relatively linear relationship with the total dietary protein, and was five to six times higher at 50% dietary protein than at 20%.

The xanthine oxidase activity of mammalian organs was influenced by dietary flavin. Burch et al. (1956), reported that liver xanthine oxidase activity from rats fed a flavin deficient diet for six weeks was reduced to 33% of the original level. Decker and Byerrum (1954), observed that liver enzyme activity depended on the flavin intake. When rats were fed a purified diet, xanthine

oxidase activity increased with increased intake of flavin. Doisy and Westerfeld (1952) and Axelrod and Elvehjem (1941), also reported a decrease in liver xanthine oxidase activity in flavin deficient rats.

b. L-Amino acid oxidase

Boulanger and Osteux (1956), discovered an L-amino acid oxidase in turkey liver which appeared to be specific for the basic amino acids; they also showed that the enzyme attacks the α-amino groups of lysine and ornithine (Boulanger et al. 1958, 1957).

Although the enzyme isolated from rat liver and kidney attacks a variety of monoamino-monocarboxylic acids of L-configuration (Blanchard et al. 1944), the function of the enzyme in mammalian tissues is not yet clear. Meister et al. (1960) noted that the low L-amino acid oxidase activity of various mammalian tissues gives indirect support to the belief that deamination of amino acids takes place mainly by coupled action of glutamic dehydrogenase and glutamic transaminase as suggested by Braunstein and Bychkov (1939). Nevertheless, the available evidence does not exclude the occurrence of oxidative deamination of certain amino acids. Transamination of lysine has not been demonstrated in mammalian tissues, and it is possible that this amino acid is oxidatively deaminated, perhaps by an enzyme similar to the basic amino acid oxidase described by Boulanger and Osteux (1956).

In a study on flavin deficient rats, Burch et al. (1956) found that L-amino acid oxidase of liver and kidney decreased in activity.

c. D-Amino acid oxidase

Since D-amino acids are rather rare in natural products, the occurrence of a powerful enzyme oxidizing these substances presents a puzzle. Neims and Hellerman (1962) suggested that the oxidation of D-amino acids may be an incidental property of glycine oxidase. In addition, Meister et al. (1960) noted that there were at least four categories of explanations for the presence of this enzyme:

(1) D-amino acid oxidase may be an artifact of isolation.

(2) The enzyme may have a natural substrate not yet discovered.

(3) The enzyme has no physiological function but exists in mammals as a vestigial enzyme.

(4) The physiological function of D-amino acid oxidase is to destroy D-amino acids which are (i) ingested by the mammal; (ii) formed by the mammal's bacterial flora; or (iii) formed in mammalian tissues by other reactions.

The same authors suggested that perhaps the fourth possibility is most plausible.

Reduction of D-amino acid oxidase activity in the liver of flavin deficient rats was reported by Hawkins (1952), Decker and Byerrum (1954) and Ramakrishnan et al. (1961). Further to this finding, Axelrod et al. (1940) and Burch et al. (1956) found that flavin deficiency in the rat resulted in a lowering of the D-amino acid oxidase content of liver and kidney. The effect was more pronounced in the liver than in the kidney.

d. Glycine oxidase

Glycine oxidase was found by Ratner et al. (1944) to catalyze oxidation of glycine. Krebs (1933) reported that rat liver and kidney slices slowly deaminated glycine.

Glycine oxidase activity was found to be reduced in flavin deficient rat liver and kidney (Burch et al. 1956).

e. Glycolate oxidase

According to Dohan (1940) the enzyme promotes consumption of oxygen by glycolic acid, was found in rat and rabbit livers, but not in similar preparations of rat kidney, heart and spleen or rabbit kidney, brain and pancreas.

Glycolate oxidase activity in rat liver decreased to 6% of original activity after six weeks on a flavin deficient diet (Burch et al. 1956).

f. Histaminase

In a number of papers Zeller and his coworkers (Zeller et al. 1939 and Zeller, 1938) advanced evidence that histaminase acts on various diamines such as cadaverine, putresine, agmatine and spermine, and suggested that this enzyme should be called diamine oxidase. Swedin (1943) suggested that the enzyme was a flavoprotein. Kapeller-Adler (1949) further suggested that hog kidney histaminase is a flavoprotein with FAD as its prosthetic group. It was shown that hydrogen peroxide is formed when histaminase acts on histamine.

g. Amine oxidase activity of the rat liver was about half in flavin deficiency in a study using tyramine as substrate by Hawkins (1952). He suggested a possibility that in flavin deficiency protein

utilization is affected and this factor is responsible for the low amine oxidase activity in the deficient animals.

h. Pyridoxamine phosphate oxidase

Conclusive evidence was presented by Wada and Snell (1961) that the primary pathway of pyridoxal phosphate formation from pyridoxine or pyridoxamine in animal tissues is phosphorylation by pyridoxal kinase followed by action of pyridoxamine phosphate oxidase. Thus, the dependence of pyridoxal phosphate (active form of pyridoxine) formation upon the flavin-containing enzyme, pyridoxine phosphate oxidase, is apparent. Since pyridoxal phosphate is associated with most of the non-oxidative metabolic changes of amino acids (West et al. 1966) and the synthesis of δ-amino-levulinic acid, a requisite intermediate in porphyrin synthesis (Granick, 1958), the importance of the enzyme in relation to protein metabolism is obvious.

3. Group III enzymes

A limited amount of research has been reported on this group of enzymes. Based on the research of Mahler et al. (1954) it appears that an enzyme in this group, acyl CoA dehydrogenase, has a dissociable coenzyme, FAD. Both TPNH dehydrogenase and acyl CoA dehydrogenase, which belong to this group, were found to be relatively sensitive to flavin deficiency. Burch et al. (1960) reported that TPNH dehydrogenase activity decreased in rat liver mitochondria to one-half of normal after the rats were fed a flavin deficient diet for six weeks. Acyl CoA dehydrogenase activity was shown to be reduced in a flavin deficient rat liver mitochondria preparation

(Burch et al. 1960).

Discussion

As already mentioned, the metabolism of carbohydrate, fat and protein is interrelated. However, there seems to be three general metabolic routes in the body in operation. These three metabolic routes can be related to the above mentioned three groups of enzymes as follows:

Metabolic route I: "Formation of ATP to meet the energy requirement of animal". Enzymes from Group I are required for this metabolic route.

Metabolic route II: "Maintenance and/or growth of tissues". This metabolic route requires enzymes belonging to Group II.

Metabolic route III: "Storage of excess energy as depot fat". This metabolic route requires enzymes belonging to Group III.

It is interesting to note that although flavin-containing enzymes are involved in many metabolic processes, the activities of these enzymes in tissues of flavin deficient animals were affected to a varying extent. Based on the work of Burch et al. (1956) and Burch et al. (1960), it appears that the enzymes involved in metabolic route I are least sensitive to flavin deficiency among the enzymes studied. In other words, this group of enzymes was found to be the last one to show the decrease in activity when the animal suffered from deficiency of the vitamin. This finding seems to be reasonable from the physiological point of view since ATP is the immediate energy source for numerous chemical reactions and physical activities in animals. Formation of ATP thus becomes a prerequisite

for all other metabolic processes, and its continued synthesis needs to be maintained even at the expense of other metabolic processes.

The difference in sensitivity to flavin deficiency may be explained on a chemical basis. Glycine, D-amino acid, and glycolate oxidases are most sensitive to flavin deficiency and are readily dissociable with FAD or FMN. Xanthine, L-amino oxidases and DPNH dehydrogenase are not as sensitive to flavin deficiency and they have non-dissociable coenzymes.

No quantitative comparison among the enzymes belonging to Group II and III (which are sensitive to flavin deficiency) can be made from the literature available. Therefore, the relative sensitivity to flavin deficiency between the two groups of enzymes involved in metabolic routes II and III is not clear.

Since proteins are the chief organic compounds of cellular structure and organization, maintenance and/or growth of tissues depend largely on metabolism of protein and other nitrogenous compounds. As already mentioned, the group of enzymes involved in this metabolic route showed a decrease in activity during flavin deficiency. Therefore, it may be concluded that "flavin deficiency affects the efficiency of protein utilization". This conclusion is substantiated by the work of Kleiber and Jukes (1942) and Lockhart et al. (1966).

After satisfying energy requirements for metabolic routes I and II, animals store excess energy as depot fat. This storage of energy is a safety measure for the well-being of the animal. Thus, the importance of fat deposition for an animal seems to be secondary

to metabolic routes I and II. Since fat deposition is the least important metabolic route, the enzymes involved in this process would be expected to be sensitive to flavin deficiency. Therefore, flavin deficiency would affect the efficiency of fat deposition relatively soon during inadequate intake of this vitamin. Ultimately, flavin deficiency would result in a poor efficiency of dietary energy utilization. This conclusion is supported by the work of Sure and Dichek (1941) and Voris and Moore (1943).

Utilization of protein seems to have priority over fat deposition for growing animals. Nevertheless, this theory is in conflict with the results obtained by Kleiber and Jukes (1942), viz., "efficiency of protein utilization but not energy utilization was affected by flavin deficient chicks". While, Lockhart et al. (1966), Sure and Dichek (1941) and Voris and Moore (1943) found that both protein and energy utilization were affected adversely by flavin deficiency. These controversial results seem to be due to differences in experimental conditions used by various investigators. In order to solve this controversial problem, well controlled experiments are needed to clarify how flavin deficiency influences the efficiency of energy and/or protein utilization.

Symptoms of flavin deficiency

1. Gross symptoms

a. Retardation of growth

When chicks were fed a diet deficient in flavin, they grew very slowly, became weak and emaciated. The leg muscles were flabby and withered. The skin of the chicks was dry and harsh (Norris et al. 1929;

Bethke et al. 1931; Lepkovsky and Jukes, 1936; Heuser et al. 1938 and Bethke and Record, 1941).

b. Depression of feed consumption

Decreases in feed consumption and efficiency of feed utilization were observed when chicks were fed a flavin deficient diet. The minimum requirement of the vitamin for optimal feed consumption and efficient feed utilization approximated those for optimum growth (Lepkovsky and Jukes, 1936; Bethke and Record, 1942; and Boucher et al. 1942).

c. Curled-toe paralysis (C.T.P.)

Curled-toe paralysis involves the legs and feet and occurs in two stages, viz., a preliminary stage in which the chicks do not walk except when forced to do so. The second stage is characterized by the sudden appearance of chicks walking on their hocks, with toes curling inward. Spontaneous recovery occurred in a marginal deficiency (Norris and Ringrose, 1930; Bethke et al., 1931 and Bethke and Record, 1941). According to Culton and Bird (1940) and Bethke and Record (1942) the flavin requirement for the prevention of C.T.P. was above that for maximum growth.

Similar gross symptoms as mentioned above were also observed in turkey poults (Lepkovsky and Jukes, 1936; Heuser et al., 1938; and Patrick et al., 1944), ringnecked pheasants (Scott et al., 1959), and ducks (Hegsted and Perry, 1948). In mammals, in addition to the gross symptoms, an abnormal gait, similar to that of C.T.P. in birds, was observed in pigs (Wintrobe et al., 1944), mice (Lippincott and Morris, 1942), rats (Potter et al., 1942; and

Street et al., 1941), and monkeys (Day, 1934; and Shaw and Phillips, 1941).

2. Biochemical and hematological changes

a. Flavin content of liver and other tissues

The liver was found to be highest in flavin content among tissues of the body and also was observed to be most sensitive to flavin deficiency. Flavin concentration of liver was decreased in flavin deficient chicks (Bolton, 1944; 1947a and 1947b), hens (Stamberg et al., 1947), and rats (Bessey et al., 1958; Czaczkes and Guggenheim, 1946; and Supplee et al., 1942). According to Bolton (1944), the minimum requirement of flavin which gave the maximum content of the vitamin in the liver was higher than the requirement for preventing C.T.P. Flavin content of other tissues was also found to be decreased during flavin deficiency. The tissues studied were kidney, heart, leg muscle, breast muscle, (Bolton, 1947a; 1947b; and Stamberg et al., 1947). Bessey et al. (1958) reported that in rats, maximum growth occurred with tissue flavin concentrations that were about 75% of the maximum levels attainable.

b. Changes in the blood

1. Erythrocytes

According to Goff et al. (1953), flavin deficiency in White Leghorn cockerel chicks resulted in increased hematocrit, increased mean corpuscular volume, and decreased hemoglobin concentration. However, mean corpuscular hemoglobin was not affected noticeably. The deficiency had no effect on the total count of erythrocytes.

In humans, a dramatic drop in reticulocyte count (Lane et al.,

1964; and Foy et al., 1961) and a significant increase in serum iron levels (Foy et al., 1961) was observed in flavin deficiency.

Various types of anemia have been reported in flavin deficient animals, i.e. in baboons, hypochromic type (Foy et al., 1964); in humans, normocytic, normochromic type (Foy et al., 1961); in pigs, normocytic type (Wintrobe et al., 1944); and in dogs, microcytic, hypochromic type (Spector et al., 1943).

2. Leukocytes

Goff et al. (1953) reported that increases in total leukocyte count and the percentage heterophils in differential count of leukocytes were found in flavin deficient chicks. On the basis of the total and the differential leukocyte counts, it appeared that the primary change was a marked increase in the concentration of heterophils. This change was sufficient to account for the entire increase in the total leukocyte count.

Similarly, Mitchell et al. (1950) reported an increase in neutrophilic granulocytes in flavin deficient pigs. Jones et al. (1947) reported granulocytosis in monkeys, and Musser and Heinle (1958) reported granulocytosis in rats suffering from flavin deficiency.

Mechanism of flavin deficiency

1. Disturbance in erythrocyte formation

Based on the findings that flavin deficiency resulted in (a) decreased reticulocyte counts (Lane et al., 1964; and Foy et al., 1961); (b) decrease in cells of erythroid series in bone marrow (Endicott et al., 1947); and (c) increase in serum iron level

(Foy et al., 1961), it appeared that erythrocyte formation was blocked in flavin deficiency. This interpretation seems to be supported by the work of Lascelles (1957), who found that the synthesis of porphyrins from δ-aminolevulinic acid cell suspensions of Tetrahymena vorax was decreased in the absence of flavin.

2. Cause of granulocytosis

According to Goff et al. (1953), the mechanism of increase in heterophil concentration may be concerned with enzymes involved in the metabolism of hydroxypurines (hypoxanthine and xanthine). In other words, an accumulation of hypoxanthine and xanthine results from a decrease in xanthine dehydrogenase in birds or xanthine oxidase in mammals suffering from flavin deficiency. This hypothesis is substantiated by the following findings:

(a) The presence of xanthine dehydrogenase was reported by Westerfeld et al. (1962), Stirpe and Corte (1965) and Strittmatter (1965) in liver and by Landon and Carter (1960) and Strittmatter (1965) in kidney of chickens. The function of xanthine dehydrogenase, catalyzing the formation of uric acid from hypoxanthine and xanthine was studied in vitro by Landon and Carter (1960) and Strittmatter (1965) and, in vivo by Stirpe and Corte (1965).

(b) A decrease in liver xanthine oxidase activity in flavin deficient rat was reported by Axelrod and Elvehjem (1941) Doisy and Westerfeld (1952), Decker and Byerrum (1954) and Burch et al. (1956).

(c) A number of nucleic acid derivatives are capable of producing a neutrophilic leukocytosis when injected into man and animals. Reznikoff (1930) reported that the intravenous injection of adenine

and guanine in a human with agranulocytosis caused an increase in polymorphonuclear neutrophils. Jackson et al. (1931) and Jackson and Parker (1935) obtained similar clinical results following the injection of pentnucleotide intramuscularly or intravenously. Neyman (1917) reported that injection of a sodium nucleinate solution caused an increase in the number of leukocytes in both rabbits and cats.

MATERIALS AND METHODS

Day-old commercially available broiler cockerels were used in all trials. The chicks were placed in electric battery brooders having raised wire-mesh floors and thermostatically controlled heating units. Water was given ad libitum. The water troughs were cleaned at least once every three days. Light was provided continuously.

Semi-purified rations were used in all trials. The compositions of the rations are shown in Table 1. All chicks were fed a riboflavin (flavin) deficient ration (no supplemented flavin in the ration) for three days and then assigned to experimental groups on the basis of body weight. The extremes in body weight were discarded.

Six feeding trials were conducted with a total of 1100 chicks. Trials 1165, 666 and 1266, mainly dealt with the effect of flavin on energy and protein utilization. Trials 367, 1766 and 1866 were designed to study hematological and biochemical aspects of flavin deficiency.

Trial 1165

Ad libitum feed intake

Fifteen ration treatments involving three levels of metabolizable energy (2862, 3145 and 3428 kcal per kg of ration) and five levels of flavin (0.922, 1.635, 2.348, 3.062 and 3.775 mg per kg of ration) in a factorial arrangement were used. All 15 rations were given ad libitum to three replicate pens of five chicks each.

TABLE 1

COMPOSITION OF RATIONS (SEMI-PURIFIED)

Ration	R_1-R_5	R_6-R_{10}	R_{11}-R_{15}	C_{11}-C_{15}
Soybean protein	27.50(%)	27.50(%)	27.50(%)	27.00(%)
Glycine	0.22	0.22	0.22	0.22
Methionine	0.78	0.78	0.78	0.78
Dextrose	30.00	30.00	27.50	14.00
Sucrose	-	-	-	14.00
Corn starch	27.00	26.50	23.50	24.00
Soybean oil	1.00	1.00	1.00	1.00
Lard	1.50	5.00	10.50	10.00
Vitamin premix[1]	1.00	1.00	1.00	1.00
Mineral premix[2]	6.00	6.00	6.00	6.00
Alphacel	5.00	2.00	2.00	2.00
	100.00	100.00	100.00	100.00
Metabolizable energy kcal/kg	2862	3145	3428	3342

[1]Vitamin premix supplied the following in mg/100 g of ration

Biotin	0.02	Thiamine-HCl	0.40	PABA	4.00
Ca-pantothenate	2.00	Vitamin A	1000 IU	Vitamin B_{12}	0.002
Folic acid	0.20	Vitamin D_3	200 ICU	Choline	200.00
Inositol	100.00	α-tocopherol acetate	15 IU	Ascorbic acid	22.00
Menadione	0.10	Niacin	8.00	Santoquin	30.53
Pyridoxine-HCl	0.60				

[2]Mineral premix supplied the following in mg/100 g of ration

Defluorinated phosphate	3.50	$FeSO_4$	0.006	H_3BO_3	0.0009
$MnSO_4.4H_2O$	0.10	KCl	0.30	$KAl(SO_4)_2 \cdot 12\ H_2O$	0.00094
$ZnSO_4.7H_2O$	0.024	K_2CO_3	0.20	$Na_2MoO_4 \cdot 2H_2O$	0.00011
$CuSO_4.5H_2O$	0.002	KI	0.002	$Mg_2Si_3O_8 \cdot 5H_2O$	0.75
$CoCl_2.6H_2O$	0.00015	$MgSO_4.7H_2O$	0.50	$Al(OH)_3$	0.25
		NaCl	0.50		

Pooled body weight of the chicks in a pen and feed consumption data were recorded weekly. Incidence of curled-toe paralysis was recorded daily. The feeding period was three weeks.

Trial 666

Isonitrogenous-isonutrient rations

Fifteen ration treatments involving three levels of metabolizable energy and five levels of flavin in a factorial arrangement were used. High energy rations (3342 kcal per kg of ration) were fed ad libitum. Medium and low energy rations were given on a restricted basis. Since ration C_{15} (with adequate level of flavin) was used as a control, the average daily feed intake of the three pens of chicks fed ration C_{15} was used in calculating the amount of feed allowed to the restricted groups. Energy intake was restricted to 80% or 60% of that consumed by the ad libitum fed groups. This restriction of energy was done by reducing the amount of sucrose and corn starch in the ration. However, all chicks consumed about equal amounts of protein, minerals, fiber and vitamins, except flavin, regardless of level of energy intake. Thus, the difference among energy treatment groups was in metabolizable energy intake only, while the differences among treatment groups within each energy level was in flavin intake only. Chicks receiving energy restricted rations were fed once daily at the same time each day, and the quantity of the feed for each group was placed in a single feeder of sufficient size to provide free access to all chicks. Wasted feed and the remaining feed were weighed daily.

Rations were given to three replicate pens of five chicks each.

Pooled body weights were recorded once every four days. Immediately before the experiment began 30 chicks were fasted for 17 hours to permit emptying of the digestive tract. The 30 chicks were killed and the carcasses analyzed to supply data on initial carcass composition. At the end of the trial three chicks from each pen of the selected treatments were killed and the carcasses were analyzed to determine gains in carcass protein, fat and ash. Liver samples obtained from three chicks per experimental pen were frozen until subsequent analyses for moisture, protein, ether extract, ash and flavin could be done. Microbiological assay for flavin content of liver samples and feed samples was carried out according to the methods described by A.O.A.C. (1960).

In preparation for analysis, carcasses of chicks were frozen and cut into inch cubes with a Hobart Meat Saw (Model 5012-D) and then homogenized in a Gallon-size Waring Blender (Model, CB4). A weighed amount of distilled water equal to two times the weight of carcass material was added to facilitate homogenization. A large aliquot of homogenate was dried in a freeze-dryer (Virtis Freeze-Mobile with tray drying chamber). Analyses for protein (Macro-kjeldahl nitrogen x 6.25), ether extract, ash and residual moisture were made on the dry material by standard laboratory methods (A.O.A.C., 1960).

Tissue energy gains were obtained by two methods:

(1) Combustion in a Parr Adiabatic Bomb Calorimeter or

(2) Calculation from gains in tissue fat and protein using energy coefficients of 9.35 kilocalories (kcal) per g for fat and 5.66 kcal

per g for protein (Carew and Hill, 1964). Data on weight gains, tissue gains, and the percentage retention of energy and protein were treated statistically by analysis of variance (Snedecor, 1956) and the treatment means were compared by Duncan's multiple range test (Duncan, 1955).

Trial 1266

Isocaloric-isonutrient rations

Fifteen ration treatments involving three levels of protein and five levels of flavin in a factorial arrangement were used. Each ration was given to three replicate pens each containing five chicks.

Ration C_{15} from Trial 666 was used as a control ration fed ad libitum to three replicate pens. The average feed intake of the control chicks was used in calculating the amount of feed which would supply the desired quantities of nutrients to those chicks receiving other treatments. Metabolizable energy intake was restricted to 70% of that consumed by the control chicks. Protein intake was 100, 85 or 70% of the control group, while fiber, mineral and vitamin intakes were identical for all treatments. Thus the difference among the protein treatment groups was in protein intake only, and the difference within each protein treatment group was in flavin intake only. Other experimental procedures were the same as in Trial 666, except that at the end of the experimental period, brachial veins of the chicks (random sampling of three chicks from each pen) were punctured and blood samples were obtained in heparinized capillary tubes for hematocrit determination. Also blood smears

were prepared for taking differential counts of leukocytes. Giemsa stain was used for staining the blood smears.

Trial 367

Determination of hydroxypurines

A total of 60 chicks was divided into two groups. Forty chicks were fed a flavin deficient ration which contained 1.888 mg per kg of ration. This is one-half of the marginal requirement of flavin for the chick. Twenty chicks were fed a ration with a high level (15.100 mg per kg) of flavin. Both rations were fed ad libitum. Individual body weight and feed consumption data were recorded every four days. Blood samples were collected for determination of uric acid in serum when the chicks fed the flavin deficient ration started showing curled-toe paralysis symptoms at 12 days of age. At the end of an 18-day feeding period, chicks were sacrificed and livers were removed and frozen immediately. Determination of hydroxypurines (free and bound forms of hydroxypurines) in the liver was made spectrophotometrically with a Unicam Spectrophotomer (Model SP800) using the combined methods from Plesner and Kalckar (1956), Jorgensen and Poulsen (1955) and Kalckar (1947). Xanthine oxidase used was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA. Uric acid concentration in serum was determined by the method of Bittner et al. (1963) with Oxford Uric Acid Reagents obtained from Oxford Laboratories, San Mateo, California, USA. Liver samples were also analyzed for moisture, nitrogen, ether extract and ash content.

Trial 1766

Sodium inosinate injection: part I

One hundred chicks were divided into 20 pens of five chicks each. Twelve pens of chicks were fed a semi-purified ration (ration C_{11} - C_{15} table 1) supplemented with marginal amounts of flavin (3.775 mg per kg of ration) starting at four days of age. Eight pens of chicks were given a commercial chick starter ration. Replicate pens of chicks received sodium inosinate injection intramuscularly (breast muscle) starting at ten days of age.

Injection period was 11 days. During the first five days each chick received 20, 40, 100 or 200 mg of sodium inosinate injection once a day at 1:30 p.m. During the following six days each chick received 20, 40, 100 or 200 mg of sodium inosinate injection twice daily at 11:00 a.m. and 11:00 p.m. Since during the first five days the chicks weighed approximately 100 g (range in body weight, 90-130 g), and the following six days the chicks weighed about 200 g (body weight range, 130-300 g), the dosage used was approximately 20, 40, 100 or 200 mg of sodium inosinate per 100 g of body weight per injection, respectively.

Individual body weight and feed consumption data were recorded every second day. At the end of the 11-day injection period, blood samples were taken for hematocrit determination and blood smears were prepared for differential counts of leukocytes and reticulocytes. Livers were removed for flavin determination as mentioned under Trial 666. Sodium inosinate used was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA.

Trial 1866

Sodium inosinate injection: part II

Six injection treatments involving two rations (the semi-purified ration C_{11} - C_{15} in table 1 supplemented with a marginal, 3.775 mg per kg of ration or a high, 15.100 mg per kg, level of flavin in the ration) and three dosages (no injection, 100 or 200 mg per injection) of sodium inosinate in a factorial arrangement were used. Duplicate pens of ten chicks each received injection starting at four days of age. The dosage was approximately 100 or 200 mg of sodium inosinate per 100 g of body weight of chicks per injection. Injections were performed four times daily at 4:00 and 10:00 a.m. and 4:00 and 10:00 p.m. Individual body weight and pooled feed consumption data were recorded every two days. At the end of the 12-day injection period, chicks were treated as described under trial 1766. In addition, blood samples were obtained for determination of hydroxypurines in plasma and uric acid in serum following the methods described under trial 367.

RESULTS AND DISCUSSION

Trial 1165 *Ad libitum* feed intake

Low levels of riboflavin (flavin) in the ration significantly ($P < 0.05$) depressed growth (gain in body weight), feed intake (consumption) and feed conversion (weight of feed consumed divided by body weight gain) of the chicks (Figure 1 and Table 2). There was no difference in requirement of flavin for the three rations differing in energy content, viz., 2862, 3145 and 3428 kilocalories (kcal) per kilogram (kg) of ration. Moderate flavin deficiency resulted in high incidence of curled-toe paralysis (C.T.P.). The data also show that the minimum flavin requirements for maximum performances, i.e. (a) growth, (b) feed consumption and (c) prevention of C.T.P. were identical, viz., 3.775 milligram (mg) of flavin per kg of ration. The finding that equal amounts of flavin were required for maximal growth and feed intake was in agreement with the work of Lepkovsky and Jukes (1936) and Boucher *et al.* (1942). However, Culton and Bird (1940) and Bethke and Record (1942) reported that the flavin requirement for prevention of C.T.P. was above that for maximum growth. This discrepancy in flavin requirement for prevention of C.T.P. seemed to be due to the relative difference in the flavin levels used.

Results obtained in the above experiments are subject to the following criticisms:

1. Requirement of flavin by chicks expressed as "unit weight of the vitamin per unit weight of ration" ignores the composition of

Figure 1. Feed consumption, body weight gain, and incidence of C.T.P. of chicks during the 3-week feeding period (Trial 1165).

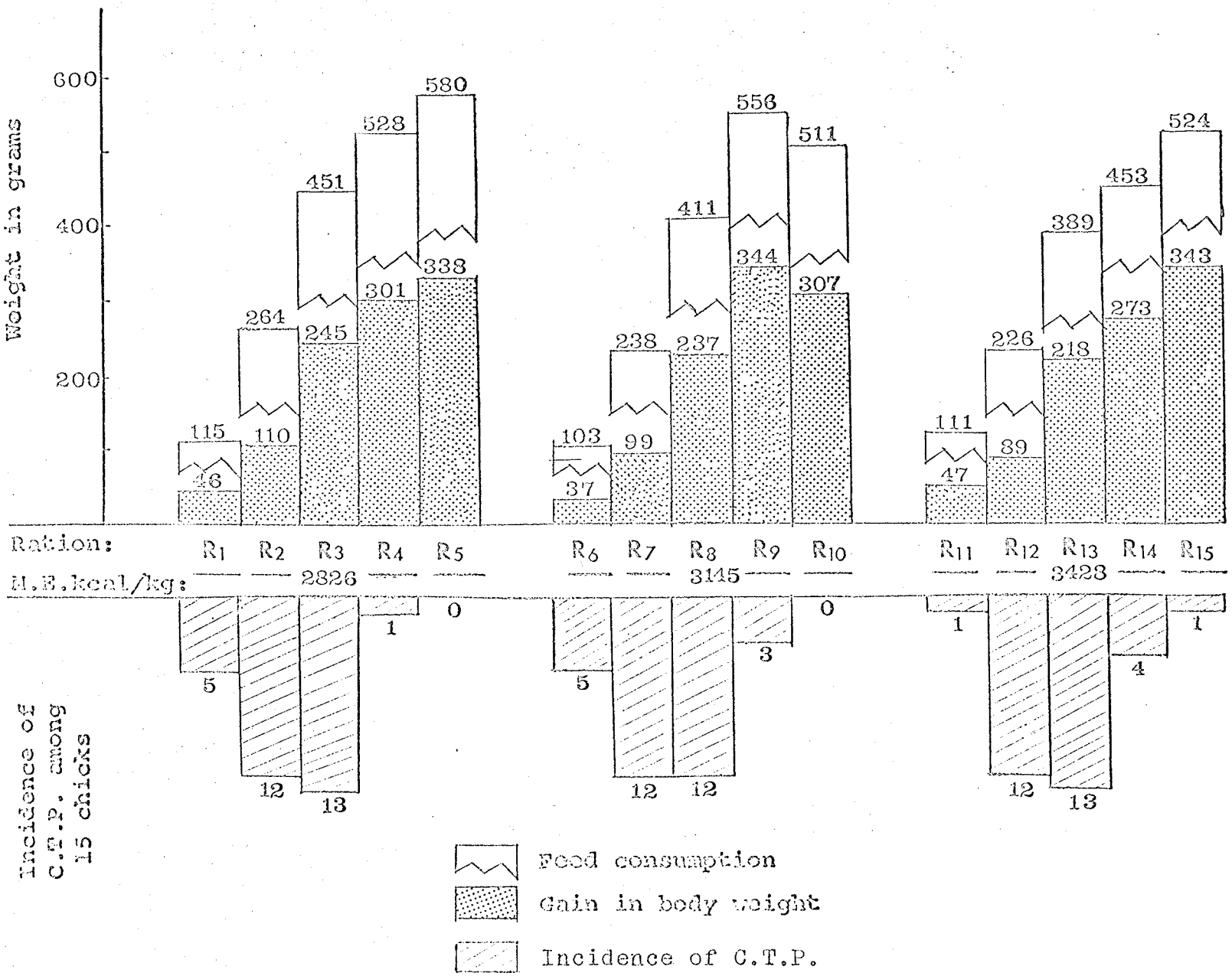

Weight in grams
600
400
200
580
528
451
264
115
110
46
245
301
338
556
511
411
344
307
238
237
103
99
37
524
453
389
343
273
226
218
111
89
47
Ration:
R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15
M.E.kcal/kg:
2826
3145
3428
0
1
0
3
1
4
1
5
12
13
5
12
12
12
13
Incidence of C.T.P. among 15 chicks
Food consumption
Gain in body weight
Incidence of C.T.P.

TABLE 2

FEED INTAKE, BODY WEIGHT GAIN, FEED CONVERSION, AND INCIDENCE OF CURLED-TOE PARALYSIS OF CHICKS AT THE END OF 3-WEEK FEEDING PERIOD (TRIAL 1165)

Ration	M. E. kcal/kg ration	Flavin mg/kg ration	Feed intake g/chick	Body weight gain g/chick	Feed conversion g feed/g gain	Incidence of C.T.P. among 15 chicks
R_1	2862	0.922	-	46 ± 6.6 a	-	5 b
R_2	2862	1.635	264 ± 16.2[1] a[2]	110 ± 8.6 a	2.418 ± 0.044 a	12 a
R_3	2862	2.348	451 ± 8.8 bcd	245 ± 3.4 bc	1.839 ± 0.016 b	13 a
R_4	2862	3.062	528 ± 40.1 de	301 ± 27.2 cd	1.757 ± 0.027 b	1 c
R_5	2862	3.775	580 ± 15.2 e	338 ± 6.9 d	1.713 ± 0.012 b	0 c
R_6	3145	0.922	-	37 ± 4.2 a	-	2 b
R_7	3145	1.635	238 ± 19.9 a	99 ± 15.8 a	2.473 ± 0.212 a	12 a
R_8	3145	2.348	411 ± 35.6 bc	237 ± 30.4 bc	1.754 ± 0.077 b	12 a
R_9	3145	3.062	556 ± 14.4 de	344 ± 10.4 d	1.618 ± 0.008 b	3 b
R_{10}	3145	3.775	511 ± 43.4 cde	307 ± 26.0 cd	1.662 ± 0.000 b	0 b
R_{11}	3428	0.922	-	47 ± 6.7 a	-	1 b
R_{12}	3428	1.635	226 ± 15.4 a	89 ± 10.2 a	2.564 ± 0.121 a	12 a
R_{13}	3428	2.348	389 ± 49.1 b	218 ± 40.4 b	1.843 ± 0.143 b	13 a
R_{14}	3428	3.062	453 ± 46.4 bcde	273 ± 30.8 bcd	1.731 ± 0.191 b	4 b
R_{15}	3428	3.775	524 ± 61.6 de	343 ± 54.8 d	1.529 ± 0.030 b	1 b

[1] Mean ± standard error

[2] Means not having common letter superscripts are significantly different at the 0.05 level of probability

the rations used. Composition of the ration (e.g. energy and/or protein levels) influences both feed consumption and body composition of chicks (Combs and Romoser, 1955; Hill and Dansky, 1954; Donaldson et al., 1956; Robel et al., 1956; Donaldson et al., 1958).

2. Body weight gain as a criterion of chick performance can be misleading, because the chicks fed rations varying in flavin content may have different body composition (Kleiber and Jukes, 1942; Sure and Dichek, 1941; Voris and Moore, 1943).

3. The causative factors of C.T.P. appear to be either insufficient intake of both flavin and total feed or insufficient intake of flavin per se.

In order to obtain more specific information on these facets of the problem, two trials were conducted using equalized feeding methods and chemical analysis of carcasses.

Trial 666 and 1266: Isonitrogenous and isocaloric feed intake

Effect of dietary energy and protein on flavin requirement

When a well balanced ration (C_{15}) was given ad libitum (control chicks), the dietary flavin requirement for maximal performances of the chick was 3.775 mg of flavin per kg of the ration (Figure 2 and Table 3). When energy intake alone was restricted to 80 or 60% of that consumed by the control chicks, chicks required only 80% of the flavin for relative maximum performances. This result shows that reduced energy intake per se was accompanied by a lower flavin requirement of the chicks. Thus, it is evident that flavin is required in increasing quantity as more dietary energy is utilized. This finding confirmed the suggestion made by Czaczkes and Guggenheim

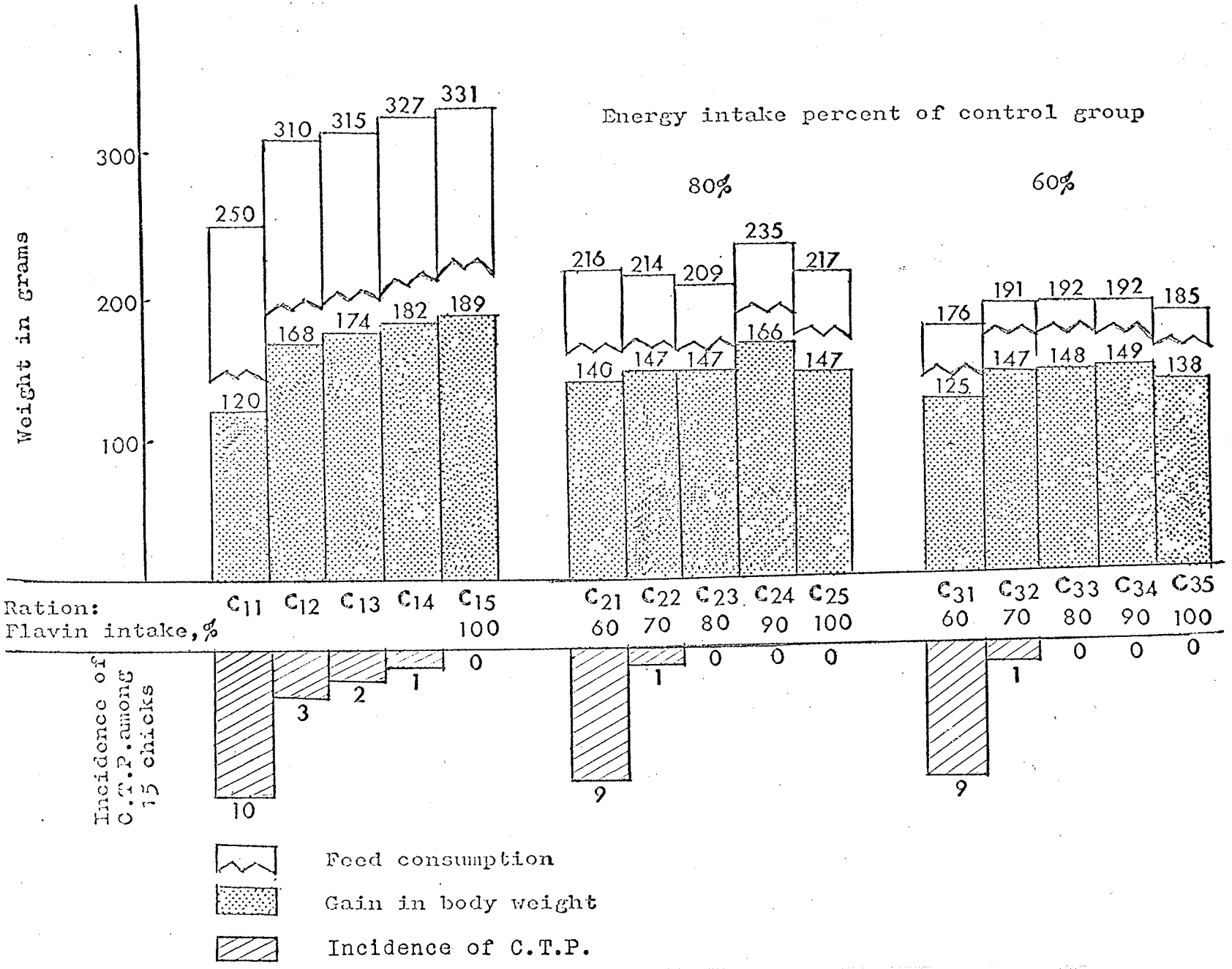

Energy intake percent of control group
80%
60%
Weight in grams
300
200
100
250
310
315
327
331
120
168
174
182
189
216
214
209
235
217
140
147
147
166
147
176
191
192
192
185
125
147
148
149
138
Ration:
Flavin intake,%
C11 C12 C13 C14 C15
100
C21 C22 C23 C24 C25
60 70 80 90 100
C31 C32 C33 C34 C35
60 70 80 90 100
Incidence of C.T.P. among 15 chicks
10
3
2
1
0
9
1
0
0
0
9
1
0
0
0
Feed consumption
Gain in body weight
Incidence of C.T.P.

TABLE 3

FEED INTAKE, BODY WEIGHT GAIN, FEED CONVERSION AND THE INCIDENCE OF CURLED-TOE PARALYSIS OF CHICKS FED ISONITROGENOUS-ISONUTRIENT RATIONS WITH VARYING LEVELS OF ENERGY AND FLAVIN AT THE END OF 16-DAY FEEDING PERIOD (TRIAL 666)

Ration	M. E. intake % of control	Flavin concentration mg/kg ration	Flavin intake % of control	Feed intake g per chick	Body wt. gain g per chick	Feed conversion g feed/ g gain	Incidence of C.T.P. among 15 chicks
C_{11}	ad lib.	2.265	ad lib.	250 ± 3.1[1]A[2]	120 ± 6.5A[3]	2.08 ± 0.077A	10A
C_{12}	ad lib.	2.643	ad lib.	310 ± 11.3	168 ± 7.6b	1.84 ± 0.006	3
C_{13}	ad lib.	3.020	ad lib.	315 ± 8.2	174 ± 6.1	1.81 ± 0.025	2
C_{14}	ad lib.	3.398	ad lib.	327 ± 4.5	182 ± 2.4	1.80 ± 0.029	1
C_{15}	100	3.775	100	331 ± 6.5	189 ± 3.2	1.75 ± 0.033	0
C_{21}	80	2.786	60	216 ± 4.8	140 ± 5.4	1.54 ± 0.030	9A
C_{22}	80	3.250	70	214 ± 17.6	147 ± 16.9	1.46 ± 0.055	1
C_{23}	80	3.715	80	209 ± 18.3	147 ± 15.4	1.42 ± 0.051	0
C_{24}	80	4.179	90	235 ± 6.9	166 ± 7.4	1.42 ± 0.021	0
C_{25}	80	4.643	100	217 ± 18.9	147 ± 14.6	1.48 ± 0.007	0
C_{31}	60	3.624	60	176 ± 5.5	125 ± 6.6	1.41 ± 0.029	9A
C_{32}	60	4.228	70	191 ± 0.6	147 ± 2.0	1.30 ± 0.013	1
C_{33}	60	4.832	80	192 ± 4.4	148 ± 6.7	1.30 ± 0.042	0
C_{34}	60	5.436	90	192 ± 0.8	149 ± 2.7	1.30 ± 0.018	0
C_{35}	60	6.040	100	185 ± 9.0	138 ± 8.6	1.34 ± 0.029	0

[1] Mean ± standard error
[2] Significantly different at the 0.01 level of probability
[3] Significantly different at the 0.05 level of probability

(1946) and Mitchell et al. (1950) that supplemented dietary fat (energy) requires extra flavin for the metabolism by rats and pigs, respectively.

Since no decrease in flavin requirement was observed when energy intake only was further reduced from 80 to 60%, it appears that the flavin requirement of chicks was also influenced by certain dietary components other than dietary energy.

There was a tendency for feed conversion to become poorer as flavin intake was decreased. However, statistical analyses indicate that the change in feed conversion due to insufficient flavin intake was not as sensitive as the incidence of C.T.P.

It is interesting to note that feed conversion was improved when energy intake was reduced from 100 to 80% or 80 to 60%. This seems to indicate that the smaller amount of energy consumed the more efficiently the feed was utilized for growth.

When three groups of chicks were separately fed three isocaloric rations, reduced protein intake per se resulted in a lowering of the flavin requirement of the chicks (Figure 3 and Table 4). Reduction in flavin requirement was parallel to the amount of protein consumed by chicks when incidence of C.T.P. was used as a criterion of flavin adequacy. Thus, protein intake and the subsequent metabolism have an effect on dietary flavin requirement. This finding is in agreement with the work of Czaczkes and Guggenheim (1946), Mitchell et al. (1950) and Kaunitz et al. (1954) who found that the amount of flavin required by the animals was determined in part by protein content of the ration.

Figure 3. Feed consumption, body weight gain, and incidence of C.T.P. of chicks fed isocaloric-isonutrient rations during the 16-day feeding period (Trial 1266).

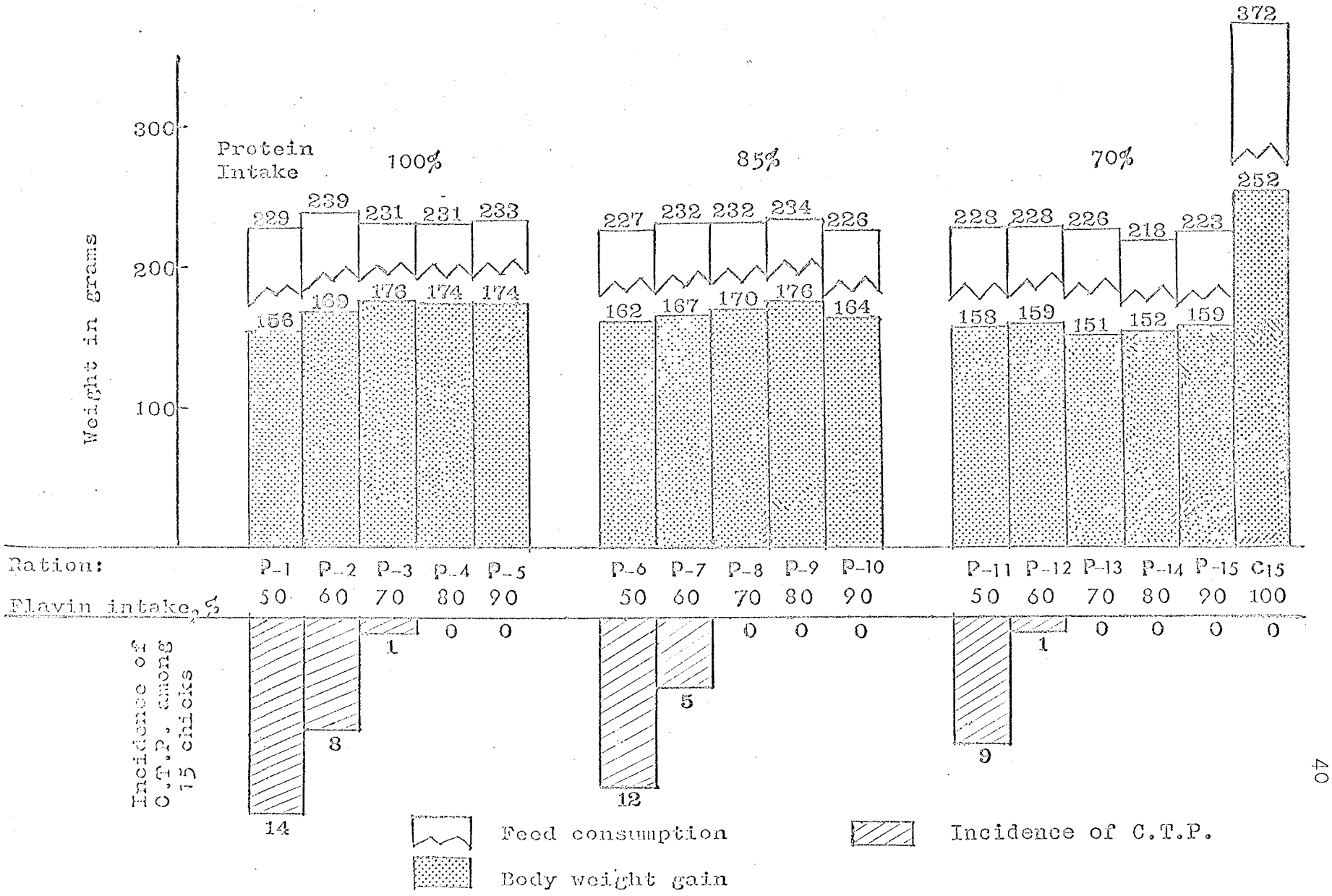

Weight in grams
300
200
100
Protein Intake
100%
85%
70%
229 239 231 231 233
227 232 232 234 226
228 228 226 218 223 252 372
156 169 176 174 174
162 167 170 176 164
158 159 151 152 159
Ration: P-1 P-2 P-3 P-4 P-5 P-6 P-7 P-8 P-9 P-10 P-11 P-12 P-13 P-14 P-15 C15
Flavin intake, γ 50 60 70 80 90 50 60 70 80 90 50 60 70 80 90 100
Incidence of C.T.P. among 15 chicks
14 8 1 0 0
12 5 0 0 0
9 1 0 0 0 0
Feed consumption
Body weight gain
Incidence of C.T.P.

TABLE 4

FEED INTAKE, BODY WEIGHT GAIN, FEED CONVERSION, AND THE INCIDENCE OF CURLED-TOE PARALYSIS OF CHICKS FED ISOCALORIC-ISONUTRIENT RATIONS WITH VARYING LEVELS OF PROTEIN AND FLAVIN AT THE END OF 16-DAY FEEDING PERIOD (TRIAL 1266)

Ration	M.E. intake % of control	Protein intake % of control	Flavin content mg/kg ration	Flavin intake % of control	Feed intake g per chick	Body weight gain g/chick	Feed conversion g feed/g gain	Incidence of C.T.P. among 15 chicks
P-1	70	100	2.625	50	229	156	1.46	14 a[1]
P-2	70	100	3.150	60	239	169	1.40	8 cd
P-3	70	100	3.675	70	231	176	1.31	1 e
P-4	70	100	4.200	80	231	174	1.33	0 e
P-5	70	100	4.725	90	233	174	1.34	0 e
P-6	70	85	2.708	50	227	162	1.40	12 b
P-7	70	85	3.250	60	232	167	1.39	5 d
P-8	70	85	3.791	70	232	170	1.37	0 e
P-9	70	85	4.333	80	234	176	1.32	0 e
P-10	70	85	4.874	90	226	164	1.38	0 e
P-11	70	70	2.796	50	228	158	1.44	9 bc
P-12	70	70	3.356	60	228	159	1.43	1 e
P-13	70	70	3.915	70	226	151	1.49	0 e
P-14	70	70	4.474	80	218	152	1.44	0 e
P-15	70	70	5.033	90	223	159	1.41	0 e
Control	100	100	3.775	100	372	252	1.48	0

[1]Values not having common letter superscripts are significantly different at the 0.05 level of probability

Results from trials 666 and 1266 (Figure 2 and 3) show that chicks consuming rations C_{21}, C_{31}, P-1, P-2, P-6, P-7 and P-11 had very similar body weight gains in relation to the chicks receiving more dietary flavin, yet they had significantly ($P < 0.05$) higher incidences of C.T.P. These results illustrate that the flavin requirement of chicks for maximal growth was less than the requirement for prevention of C.T.P. Similar results were reported by Culton and Bird (1940) and Bethke and Record (1942).

The insensitiveness of feed conversion as a criterion of flavin adequacy was also obvious from the results of trial 1266 (Figure 3 and Table 4). For example, the chicks fed two low flavin rations, P-6 and P-11 had comparable feed conversion to that of their counterparts receiving adequate flavin yet they had a very high incidence of C.T.P.

Feed conversion data from chicks fed isocaloric-isonutrient rations show that a decrease in protein intake resulted in a poor conversion of feed into tissues when flavin intake was adequate.

Efficiency of dietary energy and protein utilization as influenced by dietary flavin level

In severe flavin deficiency (i.e. chicks fed ration C_{11}), retention (percentage recovery of energy and protein in carcass) of both non-protein energy and protein was lower than that of chicks fed ration C_{15} (Figure 4 and Table 5). This observation is in agreement with the work of Sure and Dichek (1941) and Voris and Moore (1943). However, these findings are complicated because flavin was very deficient and feed intake of the chicks decreased drastically.

Figure 4. Efficiency of energy and protein utilization of chicks fed ad libitum during the 16-day feeding period (Trial 666).

Energy
Protein
Kilocalories
800
400
674
849
895
145
200
268
61.4
77.3
81.4
23.9
35.1
36.3
80
40
Weight in grams
Ration:
C11
C13
C15
C11
C13
C15
Flavin mg/kg:
2.265
3.020
3.775
2.265
3.020
3.775
Retention (%)
15
30
21.5
23.6
29.9
38.9
45.4
44.6
20
40
Retention (%)
Energy or protein consumption
Energy or protein retention
Energy or protein retention (%)

TABLE 5

EFFECT OF FLAVIN INTAKE ON EFFICIENCY OF ENERGY (NON-PROTEIN) AND PROTEIN UTILIZATION OF CHICKS FED AD LIBITUM (TRIAL 666)

Ration	Flavin in ration mg/kg	Energy retention %	Energy intake kcal/chick	Energy retention kcal/chick	Carcass fat % of D.M.	Moisture content of carcass %
C_{11}	2.265	21.5±1.48[1] B[2]	674	145	33.29	67.37
C_{13}	3.020	23.6±4.84 B	849	200	32.41	66.58
C_{15}	3.775	29.9±3.02 A	895	268	38.71	65.09
		Protein retention %	**Protein intake g/chick**	**Protein retention g/chick**	**Carcass protein % of D. M.**	
C_{11}	2.265	38.9±1.79 a[3]	61.4	23.9	59.20	
C_{13}	3.020	45.4±1.28 b	77.3	35.1	58.52	
C_{15}	3.775	44.6±0.76 b	81.4	36.3	53.39	

[1] Mean ± standard error

[2] Means not having common superscripts (capitalized letter) significantly different at the 0.01 level of probability

[3] Means not having common superscripts (small letter) significantly different at the 0.05 level of probability

Therefore, the decrease in energy and protein retention may be partly due to flavin deficiency per se and partly due to limited feed consumption.

A decrease in efficiency of dietary energy (of non-protein origin) utilization was observed in mild flavin deficiency when chicks had free access to the feed of adequate energy and protein content. Chicks fed rations C_{13} and C_{15} consumed similar amounts of energy but the percentage retention of energy in carcass as fat (Table 5) was significantly ($P < 0.01$) different, viz., 23.6% versus 29.9%, respectively (Figure 4 and Table 5). These results show that when flavin was not severely deficient fat deposition alone was reduced while the efficiency of protein utilization was not affected by this degree of flavin deficiency (percentage protein retentions of chicks fed ration C_{13} and C_{15} were 45.4% and 44.6%, respectively).

Efficiency of protein utilization was decreased by flavin deficiency when energy intake was restricted to 80, 70 or 60% of that consumed by the control chicks (Figure 5 and Table 6). Chicks fed rations C_{21}, P-1 and C_{31} retained a significantly ($P < 0.05$) lower percentage of dietary protein than their counterparts receiving adequate flavin. However, retention of energy by chicks fed rations C_{21}, P-1 or C_{31} (Figure 6) was not affected significantly by flavin deficiency. These results show that when the energy content of the ration was low, incorporation of dietary protein into tissue protein alone was adversely affected by insufficient flavin intake. Similar results were reported by Kleiber and Jukes (1942) using the paired feeding technique and flavin deficient chicks.

Figure 5. Efficiency of protein utilization of chicks fed isonitrogenous-isonutrient rations during the 16-day feeding period (Trial 666 and 1266).

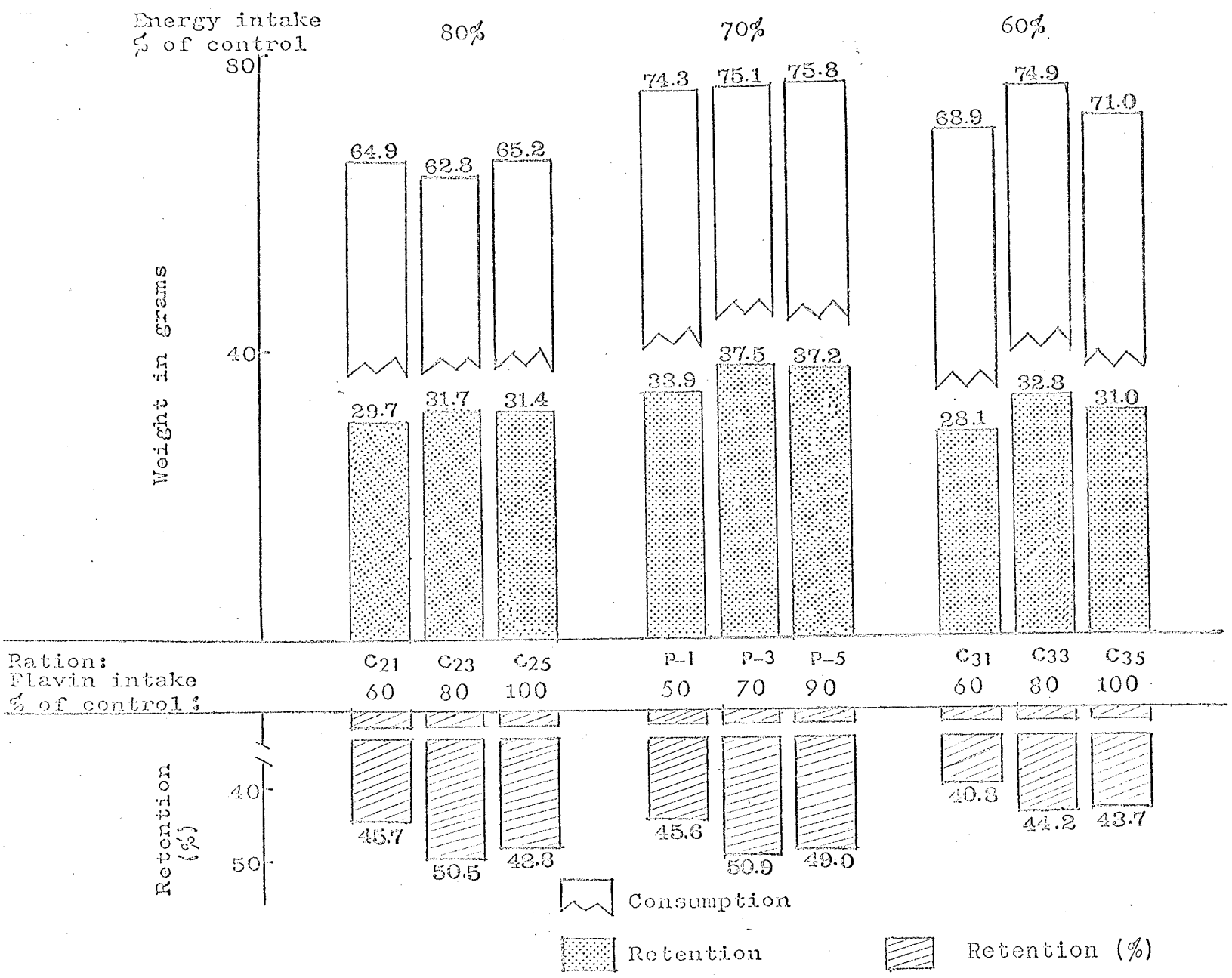

Energy intake
% of control
80%
70%
60%
80
40
Weight in grams
64.9
62.8
65.2
74.3
75.1
75.8
68.9
74.9
71.0
29.7
31.7
31.4
33.9
37.5
37.2
28.1
32.8
31.0
Ration:
Flavin intake
% of control:
C21
C23
C25
P-1
P-3
P-5
C31
C33
C35
60
80
100
50
70
90
60
80
100
Retention
(%)
40
50
45.7
50.5
48.3
45.6
50.9
49.0
40.3
44.2
43.7
Consumption
Retention
Retention (%)

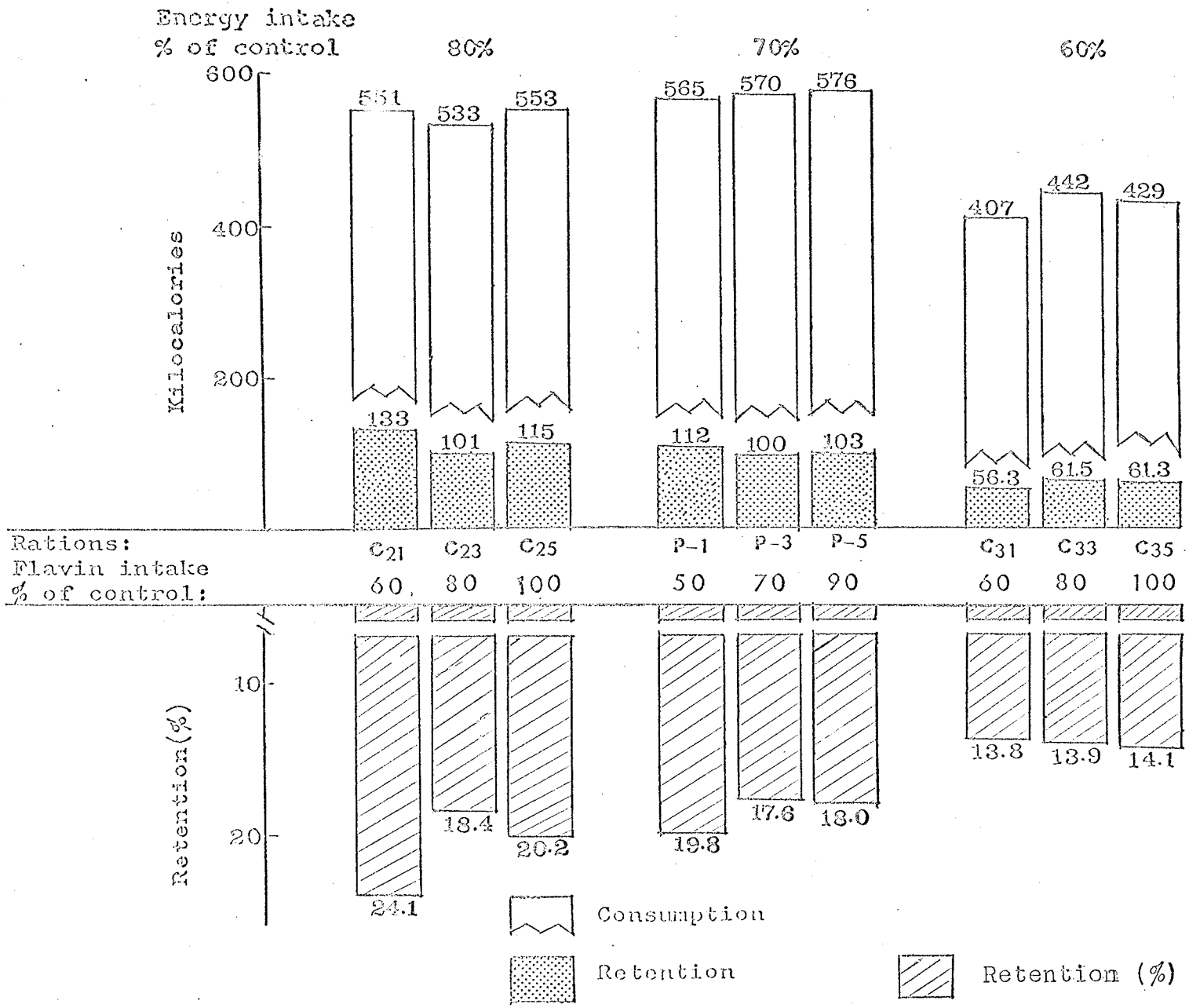
TABLE 6

EFFICIENCY OF ENERGY (NON-PROTEIN) AND PROTEIN UTILIZATION OF CHICKS FED ISONITROGENOUS-ISONUTRIENT RATIONS WITH VARYING LEVELS OF ENERGY AND FLAVIN (TRIAL 666 and 1266)

Ration	M. E. intake % of control	Flavin intake % of control	Energy retention %	Protein retention %	Carcass dry matter %	Carcass fat % of D.M.	Carcass protein % of D.M.	Incidence of C.T.P. among 15 chicks
C_{21}	80	60	24.1±2.17[1]	45.7±2.08a	31.6	28.1	63.2	9A[3]
C_{23}	80	80	18.4±3.96	50.5±1.64b	30.5	22.1	66.3	0
C_{25}	80	100	20.2±3.95	48.8±2.94b	31.2	23.6	64.8	0
P-1	70	50	19.8±1.46	45.6±0.83a[2]	31.1	22.6	65.2	14A
P-3	70	70	17.6±2.08	50.0±1.16b	29.6	19.4	68.2	1
P-5	70	90	18.0±1.01	49.0±1.69b	29.9	19.9	67.9	0
C_{31}	60	60	13.8±1.27	40.8±1.38a	29.2	14.7	71.4	9A
C_{33}	60	80	13.9±1.24	44.2±1.10b	29.2	15.4	71.4	0
C_{35}	60	100	14.1±2.96	43.7±0.54b	28.8	15.3	71.2	0

[1] Mean ± standard error
[2] Means not having common superscripts significantly different at the 0.05 level of probability
[3] Significantly different at the 0.01 level of probability

Figure 6. Efficiency of energy (non-protein origin) utilization of chicks fed isonitrogenous-isonutrient rations during the 16-day feeding period (Trial 666 and 1266).


Energy intake
% of control
80%
70%
60%
600
400
200
Kilocalories
551
533
553
565
570
576
407
442
429
133
101
115
112
100
103
56.3
61.5
61.3
Rations:
Flavin intake
% of control:
C21
C23
C25
P-1
P-3
P-5
C31
C33
C35
60
80
100
50
70
90
60
80
100
10
20
Retention (%)
24.1
18.4
20.2
19.8
17.6
18.0
13.8
13.9
14.1
Consumption
Retention
Retention (%)

There were differences in the amounts of protein consumed by chicks fed the 80, 70 or 60% energy restricted ration (Figure 5). For example, chicks restricted to an energy intake of 80% of ad libitum consumed the least amount of protein by the end of the 16-day feeding period. This was due to the fact that during the last few days of the feeding period, chicks fed 80% energy rations were considerably smaller in body size and were unable to consume all the feed given. Since this discrepancy in protein intake occurred during the last few days of the 16-day feeding trial, various comparisons were made, ignoring the minor differences in protein intake.

Neither energy nor protein retention was decreased by a mild flavin deficiency (Table 7). Chicks fed ration P-6 and P-11 appeared to utilize both dietary energy and protein efficiently even though a majority of the chicks fed these two rations suffered from C.T.P. This indicates that in a very mild case of flavin deficiency, certain metabolic disturbances induce C.T.P. and this metabolic disturbance seems to be independent of overall utilization of dietary energy or protein by the chicks.

It is interesting to note that a high incidence of C.T.P. but no decrease in efficiency of utilization of dietary energy and protein was observed when energy intake was restricted to 70% together with concomitant restriction of protein intake to 85 or 70% of that consumed by control chicks fed ad libitum.

TABLE 7

EFFICIENCY OF ENERGY (NON-PROTEIN) AND PROTEIN UTILIZATION OF CHICKS FED ISOCALORIC-ISONUTRIENT RATIONS WITH VARYING LEVELS OF PROTEIN AND FLAVIN (TRIAL 1266)

Ration	M.E. intake % of control	Protein intake % of control	Flavin intake % of control	Energy retention %	Protein retention %	Carcass D.M. %	Carcass fat % of D.M.	Carcass Protein % of D.M.	Incidence of C.T.P. among 15 chicks
P-6	70	85	50	19.5±1.95[1]	54.4±0.94	31.4	23.7	66.8	12
P-8	70	85	70	21.1±2.34	53.3±2.08	31.0	25.4	64.2	0
P-10	70	85	90	17.1±1.08	52.3±1.00	29.3	22.8	67.2	0
P-11	70	70	50	23.9±0.37	59.6±1.13	32.4	28.6	60.9	9
P-13	70	70	70	15.9±1.62	56.8±1.18	29.9	24.2	64.8	0
P-15	70	70	90	22.0±0.59	61.3±0.74	31.5	27.1	62.6	0

[1] Mean ± standard error

Biochemical and Hematological studies

Effect of dietary energy and protein on liver content of flavin

Results of microbiological assay for flavin content of the rations are shown in Table 8. There was no significant difference between the determined and the calculated flavin content of the rations.

Results from Trial 666 (Table 8) show that reduced energy intake concomitantly reduced the requirement of flavin necessary to maintain a given level of liver flavin. The response of liver flavin level to flavin deficiency appears to be more sensitive than that of body weight gain or protein retention.

These findings support the observation that the flavin requirement for saturation of organs was higher than that for maximum growth of chicks (Bolton, 1944; 1947a, and Stamberg et al., 1947) and rats (Lowry, 1952 and Schweigert et al., 1945).

Results from Trial 1266 (Table 9) show that reduced protein intake also concomitantly reduced the requirement of flavin to maintain a given level of liver flavin. However, the sensitivity of liver flavin concentration determined in this trial was not as high as that of Trial 666. Possible reasons for this low sensitivity of liver flavin to dietary flavin are (i) minor differences in obtaining the liver samples resulted in a relatively large percentage liver weight (of body weight), i.e. mean percentage liver weight of 4.01 ± 0.062 for Trial 1266 versus 3.69 ± 0.050 for Trial 666. Therefore, liver concentration of flavin would be expected to be lower. (ii) storage of the liver samples in a frozen state

TABLE 8

EFFECT OF FLAVIN INTAKE ON LIVER CONTENT OF RIBOFLAVIN AND THE INCIDENCE OF CURLED-TOE PARALYSIS (TRIAL 666)

Ration	M. E. intake	Flavin in the ration mg/kg calculated	Flavin in the ration mg/kg determined	Incidence of C.T.P. among 15 chicks	Liver flavin µg/g fresh liver	Liver weight % body weight
C_{11}	ad lib.	2.265	2.748	10 a	11.038±0.627[1] abc[2]	4.070
C_{12}	ad lib.	2.643	2.785	3	10.886±1.115 ab	3.678
C_{13}	ad lib.	3.020	3.051	2	13.210±1.903 ef	3.645
C_{14}	ad lib.	3.398	3.403	1	12.892±0.903 de	3.819
C_{15}	100%	3.775	3.705	0	14.033±0.917 fg	3.796
C_{21}	80%	2.786	2.763	9 a	10.713±0.807 a	3.678
C_{22}	80%	3.250	3.432	1	12.015±0.113 cd	3.139
C_{23}	80%	3.715	3.973	0	13.352±0.622 ef	3.582
C_{24}	80%	4.179	4.591	0	13.088±1.266 ef	3.668
C_{25}	80%	4.643	5.471	1	14.146±0.392 fg	3.664
C_{31}	60%	3.624	3.623	9 a	11.811±0.827 bc	3.765
C_{32}	60%	4.228	4.662	1	13.309±0.483 ef	3.575
C_{33}	60%	4.832	4.811	0	14.488±1.584 g	3.783
C_{34}	60%	5.436	5.535	0	15.925±1.187 h	3.743
C_{35}	60%	6.040	5.454	0	15.478±1.216 h	3.732

[1] Mean ± standard error

[2] Values or means not having common letter superscripts are significantly different at the 0.05 level of probability

TABLE 9

EFFECT OF FLAVIN INTAKE ON INCIDENCE OF CURLED-TOE PARALYSIS, LIVER CONTENT OF RIBOFLAVIN, AND HETEROPHIL COUNTS (TRIAL 1266)

Ration	M.E. intake % of control	Protein intake % of control	Flavin content mg/kg of ration	Flavin intake % of control	Incidence of C.T.P. among 15 chicks	Liver flavin µg/g Liver	Liver wt. % of body weight	Heterophil counts (Het./ Het. + Lym.) %
P-1	70	100	2.625	50	14 d	8.919±0.651[1]	4.01	47.3±1.78 a[2]
P-2	70	100	3.150	60	8 bc	8.759±0.318	3.92	37.6±3.94 abc
P-3	70	100	3.675	70	1	9.031±0.452	4.43	33.9±3.75 c
P-4	70	100	4.200	80	0	9.209±0.496	3.95	33.4±1.66 c
P-5	70	100	4.725	90	0	10.010±0.548	4.16	32.4±4.12 c
					23A[3]			
P-6	70	85	2.708	50	12 a	7.542±0.316	4.13	40.8±4.23 ab
P-7	70	85	3.250	60	5	8.060±1.162	4.26	32.7±3.58 c
P-8	70	85	3.791	70	0	8.022±0.995	3.81	34.5±1.71 bc
P-9	70	85	4.333	80	0	8.751±0.886	4.02	32.1±1.88 c
P-10	70	85	4.874	90	0	9.482±0.570	3.49	35.9±1.64 bc
					17B			
P-11	70	70	2.796	50	9 ab	7.727±1.263	4.17	45.4±2.81 abc
P-12	70	70	3.356	60	1	8.893±1.270	3.70	34.9±6.28 bc
P-13	70	70	3.915	70	0	9.333±0.888	4.18	41.1±1.40 abc
P-14	70	70	4.474	80	0	10.672±0.693	3.81	32.2±1.17 c
P-15	70	70	5.033	90	0	11.468±0.783	4.12	38.7±5.08 abc
					10C			

[1] Mean ± standard error

[2] Means not having common letter (small) superscripts are significantly different at the 0.05 level of probability

[3] Values with capitalized letters (A, B or C) are significantly different at the 0.01 level of probability

for approximately two months before the analysis was made for the flavin content in the case of Trial 1266. According to Kotschevar (1955) when liver was frozen for 60 days, there was substantial loss of flavin.

Curled-toe paralysis (C.T.P.) and liver flavin

Incidence of C.T.P. and lower concentration of liver flavin were found to be specific and sensitive criteria of dietary flavin adequacy. Results from Trial 666 and 1266 are shown in Table 8 and 9. It is evident that liver flavin concentrations responded more sensitively to varying levels of flavin intake than did incidence of C.T.P. A moderate decrease in liver flavin was associated with C.T.P. while there was a very low incidence of C.T.P. when liver flavin decreased only slightly. Since flavin content of tissues reflects the level of flavin-containing enzyme (Bessey et al., 1949), a decrease of certain enzymes in liver or in other tissues in flavin deficient chicks probably reflects a metabolic disturbance which results in C.T.P.

Percentage heterophil counts

Marked increases in percentage heterophil counts were observed as a result of flavin deficiency (Table 9). However, the sensitivity of this criterion of flavin deficiency was found to be less than that of liver flavin. This low sensitivity towards flavin deficiency was likely due to the difficulty of determining the types of leukocytes in blood smears which resulted in a high error of determination.

When data of percentage heterophil counts from the chicks fed

ration P-1, P-2, P-6, P-7, P-11 and P-12 (treatments which produced a high incidence of C.T.P.) were pooled those chicks suffering from C.T.P. had a mean percentage heterophils of 45.11 $\pm$ 2.37% versus 33.02 $\pm$ 2.44% for chicks from the same treatments not showing C.T.P. This difference in percentage heterophils was statistically significant ($P < 0.01$). Mean percentage heterophil counts of the chicks fed ration P-5, P-10 and P-15 (treatments with no incidence of C.T.P.) was 35.66 $\pm$ 2.21. Increased heterophil counts due to flavin deficiency in chicks was reported previously by Goff et al. (1953). The magnitude of increase in heterophil counts observed in the current study and that reported by Goff et al., (1953) was comparable.

Hematocrit

Pooled data for chicks fed ration P-1, P-2, P-6, P-7, P-11 and P-12 show there was no difference in mean hematocrit between the chicks showing C.T.P. (31.78 $\pm$ 0.429) and normal chicks from the same treatments (32.96 $\pm$ 0.558). Hematocrit was not influenced noticeably by the level of flavin in the ration (e.g. chicks fed rations P-5, P-10 and P-15 which were adequate in flavin had a mean hematocrit of 31.79 $\pm$ 0.603. This observation is contrary to that of Goff et al. (1953) who observed an increased hematocrit in flavin deficient chicks.

Trial 367 Determination of hydroxypurines

Flavin deficient chicks showing C.T.P. had a significantly ($P < 0.05$) higher concentration of hydroxypurines (hypoxanthine and xanthine) in both free and bound forms in the liver than chicks fed

adequate flavin (Table 10). The increase in hydroxypurines indicates a decrease in xanthine dehydrogenase activity in the liver. Since flavin in animal tissues forms a part of two coenzymes, FMN and FAD in various flavin-containing enzymes, the reduction in flavin concentration of liver (which was shown in Trial 666 and 1266) was a good indication that xanthine dehydrogenase activity decreased in flavin deficient chicks. Since the bound form of hydroxypurine is the immediate precursor of free hydroxypurines and a reversible reaction maintains equilibrium between the two forms, an accumulation of free hydroxypurines would also lead to an accumulation of bound hydroxypurine. The increase in concentration of both free and bound forms of hydroxypurines in livers of deficient chicks over those of normal chicks (44.58 and 44.44%, respectively) strongly supports this hypothesis.

The observed increase in concentration of liver nitrogen and the concomitant increase in liver weight supports the finding of an accumulation of free and bound hydroxypurines in livers. Accumulation of the hydroxypurines would lead to a higher nitrogen content of the liver and would contribute to an increase in liver weight. However, the amount of nitrogen which hydroxypurines alone contributed to the liver may not be great enough to cause such a large increase in nitrogen concentration and the liver weight. Thus, there is a possibility that precursors of bound hydroxypurine were also accumulated. The significant ($P < 0.01$) decrease in body weight of flavin deficient chicks observed in this trial is in agreement with those observed in Trial 1165 and 666.

TABLE 10

THE CHANGES IN COMPOSITION OF LIVER DUE TO FLAVIN DEFICIENCY (TRIAL 367)

	Normal chicks	Flavin deficient chicks	Percentage increase
No. of chicks used	6	10	
Incidence of C.T.P. (%)	0	100	
Free H.P.[1] in liver (µg/g)	91.7±8.74[2]	132.6±10.05*[3]	44.58
Bound H.P. in liver (µg/g)	61.1±4.62	88.2± 8.04*	44.44
Total H.P. in liver (µg/g)	152.8±11.22	220.8±10.91**[4]	44.53
Liver nitrogen (%)	3.438±0.0217	3.518±0.0135*	2.33
Liver wt. (% B.wt.)	2.395±0.2059	2.977±0.1267**	24.32
Liver wt. (g)	7.38±0.541	5.59±0.318**	
Body weight (g)	312.7±19.64	188.3±10.76**	
Liver moisture (%)	71.67±0.173	71.76±0.466	
Liver fat (% of D.M.)	9.70±2.012	7.02±0.700	
Liver ash (% of D.M.)	5.60±0.101	5.29±0.052	
Uric acid in serum (mg %)	5.423±0.0919	5.544±0.1638	

[1] H.P. Hydroxypurines (hypoxanthine and xanthine)
[2] Mean ± standard error
[3] Indicates the difference significant at the 0.05 level of probability
[4] Indicates the difference significant at the 0.01 level of probability

The percentage content of liver moisture, fat and ash was not affected by flavin deficiency in the chicks (Table 10). These data further support the above findings, namely, the increase in hydroxypurines and nitrogen concentration of liver.

Uric acid concentration of serum was not affected by flavin deficiency. A similar result was reported by Bolton (1947a).

Trial 1766 Sodium inosinate injection: part I

Flavin content of the semi-purified ration was calculated to be 3.775 milligram per kg of ration which was deemed a marginal dietary level. However, microbiological assay for the vitamin gave a value of 3.085 which according to Trial 1165 was a deficient level of flavin. Therefore, the occurrence of curled-toe paralysis among the chicks fed the semi-purified ration was not unexpected. Data from all the chicks exhibiting C.T.P. were pooled, regardless of the dosages of the sodium inosinate injection given to these chicks. Thereby biochemical and hematological changes observed with chicks suffering from C.T.P. were compared with chicks not having C.T.P. which received injections of 0, 20, 40, 100 or 200 mg of sodium inosinate per 100 g of body weight per injection.

Injection of the highest dosage of sodium inosinate (200 mg per 100 g of body weight) caused similar biochemical and hematological changes to those observed in chicks suffering from C.T.P. (Table 11). The changes observed were an increase in percentage heterophil counts (in differential counts of leukocytes), an increase in hematocrit, a decrease in liver flavin content and a slight decrease in reticulocyte counts (% of red blood cells). Statistical analyses

TABLE 11

HEMATOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY SODIUM INOSINATE INJECTION VERSUS CHICKS SHOWING C.T.P. (TRIAL 1766)

Ration fed	chick starter	Semi-purified ration					
Injection	None	None	Saline	Sodium inosinate mg/100 g 40	100	200	C.T.P. chicks
No. of chicks used	10	7	8	8	9	6	9
Hematocrit %	31.74±0.674[1]	--	--	31.08±0.901	36.00±0.643A[2]	35.10±1.62A	34.57±0.987A
Reticulocytes % of R.B.C.	2.470±0.452A	0.728±0.202	1.300±0.412	1.386±0.675	1.530±0.257	0.528±0.250	0.322±0.121
Heterophils % of W.B.C.	16.00±1.47	19.00±1.29	27.88±7.45a[3]	27.86±743a	24.89±2.78	46.71±6.61A	36.33±2.48A
Liver flavin µg/g	16.620	17.486	--	--	16.723	14.385a	12.282A
Body wt. gain g/chick/11 days	--	191±6.3	190±7.8	167±9.9	188±5.8	132±13.0A	183±9.5

[1] Mean ± standard error
[2] A: Indicates the difference significant at the 0.01 level of probability
[3] a: Indicates the difference significant at the 0.05 level of probability

show that relative sensitivity of these changes due to the injections were in the following descending order: hematocrit, heterophil counts and liver flavin content.

A decrease in liver flavin content and an increase in heterophil counts were also observed in Trial 666 and 1266, respectively. An increase in hematocrit was found to be a sensitive criterion of flavin deficiency in this experiment which is in agreement with the finding of Goff et al. (1953).

The changes in hematocrit, heterophil counts, liver flavin and reticulocyte counts induced by injection of sodium inosinate may be due to the primary effect of the presence of additional hydroxypurines in the tissues. This hypothesis was suggested by Goff et al. (1953) based on the work of Neyman (1917), Reznikoff (1930), Jackson et al. (1931) and Jackson and Parker (1935). Another possible explanation would be a secondary effect of the presence of additional hydroxypurines in the tissues, since xanthine dehydrogenase was suggested by Stirpe and Corte (1965) as an adaptive enzyme, its activity being regulated by inosine or by one of its metabolites. Utilization of an extra amount of flavin for this purpose may have resulted in partial depletion of flavin which then induced the increase in hematocrit and heterophil counts and reduction in liver flavin and reticulocyte counts.

Trial 1866: Sodium inosinate injection: part II

Injection of sodium inosinate (4 times daily, 100 mg per 100 g of body weight per injection) for a period of ten days to two groups of chicks fed a marginal (3.775 mg per kg of ration) and a high

(15.100 mg per kg) level of flavin in the ration had no significant effect on hematocrit, heterophil counts, reticulocyte counts and liver flavin concentration (Table 12). However, tendencies toward increased heterophil counts and a concomitant decrease in reticulocyte count were observed in injected chicks regardless of flavin content of the rations. These observed differences between injected and non-injected chicks were not statistically significant. These results support the hypothesis that the increase in heterophil counts and the decrease in reticulocyte counts were the primary effect of the presence of abnormal amounts of inosinate in the tissues.

A statistically significant ($P < 0.05$) difference among the treatment means was found in liver flavin content. Chicks fed the high flavin ration had a significantly ($P < 0.05$) higher liver content of flavin than did chicks fed the low flavin ration.

The hydroxypurines (hypoxanthine and xanthine) content of plasma and the uric acid concentration of serum are also shown in Table 12. There were no significant differences among the treatments in plasma concentrations of the hydroxypurines or serum uric acid concentration.

Chicks which received a high dosage of sodium inosinate (200 mg per 100 g of body weight per injection) suffered over 50% mortality between the 6th and the 8th day of injection. Post-mortem examination revealed lesions in many organs, particularly hemorrhages and severe congestion in the muscles, liver and lungs. These symptoms were similar to the symptoms of salt (sodium chloride)

TABLE 12

HEMATOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY SODIUM INOSINATE INJECTION (TRIAL 1866)

Flavin in ration	15.100 mg/kg		3.775 mg/kg	
Sodium inosinate injection	None	100 mg/100 g	None	100 mg/100 g
No. of chicks used	7	8	6	7
Hematocrit %	32.45±0.638[1]	32.33±0.388	32.89±0.607	32.64±0.544
Reticulocytes % of R.B.C.	9.93±1.41	7.76±1.44	10.00±1.22	7.35±1.26
Heterophils % of W.B.C.	19.00±1.27	26.10±2.76	23.60±3.19	26.80±3.11
Liver flavin µg/g	16.416±0.781	16.878±0.722	14.156±0.252a[2]	15.118±0.338a
Hydroxypurines in plasma µg/ml.	4.40±0.515	4.73±0.404	5.66±1.295	4.97±0.408
Uric acid in serum mg %	7.81±0.357	8.36±0.982	9.44±0.757	7.75±0.462
Body weight gain g/bird/12 days	139±5.3	125±8.5	139±6.7	119±9.4

[1] Means ± standard error
[2] Significant at the 0.05 level of probability

intoxication as described by Norris and Scott (1959). Therefore, all surviving chicks were discarded.

Four injections of 200 mg of sodium inosinate per 100 g of body weight daily contributed 0.5 g of sodium per kg of body weight to the chicks. This amount of sodium is equivalent to 1.26 g of sodium chloride which plus the daily intake from feed resulted in a total intake of 1.34 g of sodium chloride. This amount was still lower than the lethal dose of 4.00 g of sodium chloride per kg of body weight reported by Suffran (1909). However, continuous injections of a sublethal dose of sodium (as sodium inosinate) for six days may have resulted in a cumulative toxic effect.

GENERAL DISCUSSION

Effect of dietary composition on the requirement of flavin by chicks

The conventional way of expressing the flavin requirement for chicks, i.e. "unit weight of the vitamin per unit weight of ration" seems to be a very convenient measure for formulating a ration. However, when rations containing either a high level of energy or protein, or a high level of both energy and protein are to be used, a question arises whether the conventional way of expressing requirement of flavin is appropriate.

Since the composition of the ration energy and/or protein influence the amount of feed a chick would consume (Hill and Dansky, 1954; Donaldson *et al.*, 1956), the requirement of flavin by chicks in relation to dietary energy and protein becomes an interesting subject. Furthermore, since body composition of chicks is also influenced by the feed consumed, the possible effect of flavin on efficiency of dietary energy and protein retention needs consideration.

Effect of dietary energy or protein *per se* on flavin requirement of chicks was demonstrated in the current study with a restricted feed intake technique, i.e. reduction of energy and/or protein intake was accompanied by a lower requirement of dietary flavin. Because flavin is required for the metabolism of energy and protein, reduced energy and/or protein intake consequently reduced the requirement of the vitamin.

Feed conversion (unit weight of feed required for unit weight gain) indicating efficiency of feed utilization was found to be adversely affected by flavin deficiency. This inefficient utilization of feed was found to be due to an inefficient utilization of dietary energy when energy intake of the chick was not restricted. However, when energy intake was restricted to 80, 70 or 60% of the amount a chick fed ad libitum would consume, efficiency of protein utilization but not energy utilization was reduced.

Efficiency of fat deposition versus protein retention by flavin deficient chicks

Since the group of flavin-containing enzymes involved in generation of energy (ATP) were found by Burch et al. (1956) and Burch et al. (1960) to have preferential use of flavin in animal body when the animal suffers from flavin deficiency, efficiency of fat deposition and/or protein retention would be adversely affected first. When chicks were fed a ration which permitted rapid growth and generous storage of fat in the body, mild flavin deficiency was found to affect fat deposition but not protein retention. This seems to be a very logical sequence where proteins are the chief organic compounds of cellular structure and organization, with maintenance and/or growth of tissues depending largely on the metabolism of protein and other nitrogenous compounds. Conversely, storage of energy as depot fat is a safety measure for the well-being of the animal. Therefore, the importance of fat deposition for an animal is secondary to protein utilization.

However, when energy intake of the chicks was restricted to 80,

70 or 60%, protein utilization but not energy utilization was affected adversely by flavin deficiency. This seems to indicate that a certain minimum amount of energy retention as body fat is so important to the chick that flavin deficiency results in an inefficient utilization of protein.

Flavin deficiency symptoms of chicks

Results from concomitant restriction of energy and protein intake of chicks indicate that in a very mild case of flavin deficiency (no appreciable reduction in efficiency of energy and protein utilization) certain metabolic disturbances induce increases in heterophils and the incidence of C.T.P., decreases in liver flavin concentration and blood reticulocytes. Since these changes occurred before the overall efficiency of energy or protein utilization was appreciably reduced, the response in body weight gain or feed conversion to flavin deficiency was not as sensitive as were hematological and biochemical changes and the incidence of C.T.P.

Mechanism of flavin deficiency

According to Goff et al. (1953) the increase in heterophil concentration may be due to the decrease in activity of xanthine oxidase (dehydrogenase) in flavin deficient chicks. This results in an accumulation of hypoxanthine and xanthine in the tissues.

An accumulation of hydroxypurines (hypoxanthine and xanthine) was successfully demonstrated in flavin deficient chicks. Furthermore, injection of hydroxypurine (sodium inosinate) caused an increase in heterophil counts. These results indicate that the presence of an unusual level of hydroxypurines in the tissues is the

causative factor related to changes in heterophil counts of flavin deficient chicks. More detailed mechanisms involved in this general syndrome await further investigations.

The reduction of reticulocyte counts due to the hypothesized blockage in red blood cell formation might have been the direct effect of low flavin concentration in the tissues as reported by Lascelles (1957) where the synthesis of porphyrins (which are intermediates in the synthesis of protoporphyrin of heme) from δ-aminolevulinic acid was decreased in the absence of flavin. However, the reduction of reticulocyte counts appeared to accompany the increase in heterophil counts. These observations suggest that the mechanism involved in blockage of red blood cell formation may relate to the increase in heterophil counts. Since both red blood cells and heterophils are formed in bone marrows of chicks, there might be a possible relation between the blockage of red blood cell formation and the concomitant enhancement of heterophil formation.

The observed increase in hematocrit might be the result of heme synthesis in the red blood cells of chicks (Shemin et al., 1955; Shemin et al., 1948). Since the formation of reticulocyte in bone marrow was blocked by flavin deficiency, a compensatory effect of extra heme synthesis in the red blood cells of circulating blood seemed to be compulsory for the well-being of the chick.

The observation that manifestation of C.T.P. due to flavin deficiency followed a sequence after the reduction of liver flavin concentration, changes in heterophil and reticulocyte counts. These

results suggest that the mechanism involved in manifestation of C.T.P. may not be related to the above mentioned biochemical and hematological changes.

The mild flavin deficiency in chicks fed ad libitum adversely affected energy utilization before the chicks manifested C.T.P. However, the mild flavin deficiency when chicks were fed limited amounts of both energy and protein, chicks manifested C.T.P. before the efficiency of either energy or protein utilization was adversely affected. These results indicate that the mechanism involved in manifestation of C.T.P. and the efficiency of energy or protein utilization are independent.

The interrelations among the efficiency of energy and protein utilization and the activity of flavin-containing enzymes, and the sequence that these enzymes are affected in flavin deficiency have not been elucidated.

The observed plasma hydroxypurines and serum uric acid pattern show that no detectable change was induced by flavin deficiency. A similar serum uric acid pattern was reported by Bolton (1947a).

Since hydroxypurines were found to be accumulated in the liver of flavin deficient chicks, an increase in blood level of hydroxypurines seems to be a logical consequence if a renal threshold for hydroxypurines permits this. Nevertheless, a slight change in plasma concentration of hydroxypurines may have occurred yet the method used for the determination of hydroxypurines was not sensitive enough to detect this minor difference.

The observed constancy of serum uric acid level seems to suggest that there is a constant level of uric acid in serum despite the decreased rate of uric acid formation due to a decrease of xanthine dehydrogenase activity in chick liver in flavin deficiency. However, a slight change in concentration of uric acid in serum may have occurred which was undetectable by the method used in determining serum uric acid concentration.

SUMMARY AND CONCLUSIONS

With ad libitum feeding as a control, restricted feeding regimens were used in isonitrogenous, isonutrient rations (semi-purified diets) to study the effect of dietary energy per se on riboflavin (flavin) requirement of growing chicks. Similarly, isocaloric-isonutrient rations were used to study the effect of dietary protein per se on flavin requirement. Since graded levels of flavin were used in combination with the above feeding regimens, the effect of flavin intake upon efficiency of energy and protein utilization was determined by carcass analyses. Blood and tissue studies related to flavin deficiency were conducted. The pertinent observations are as follows:

1. The requirement of riboflavin (flavin) for chicks expressed as "unit weight of the vitamin per unit weight of ration" is not appropriate.
2. Reduced energy intake per se was accompanied by a smaller amount of flavin required by the chicks for the relative maximal performances (growth, feed intake, feed conversion and prevention of C.T.P.).
3. Reduced protein intake per se was accompanied by a smaller amount of flavin required by the chicks for the relative maximal performances (growth, feed intake, feed conversion, and prevention of C.T.P.).
4. Severe flavin deficiency resulted in an inefficient utilization of dietary energy when chicks were fed ad libitum on a diet

supplying liberal levels of energy and protein.

5. Mild flavin deficiency resulted in an inefficient utilization of protein when energy intake was restricted to 80, 70 or 60% of the amount of energy the chicks would consume.
6. Mild flavin deficiency resulted in an inefficient utilization of dietary energy when chicks were fed ad libitum on a diet supplying liberal levels of energy and protein.
7. In a very mild flavin deficiency, chicks manifested characteristic deficiency symptoms (i.e. increase in heterophils, decrease in liver flavin and incidence of C.T.P.) yet the overall efficiency of energy and protein utilization was not affected.
8. Among the symptoms of flavin deficiency studied, an increase in percentage heterophil count of white blood cells, decrease in percentage reticulocyte in the circulating blood, and the decrease in concentration of liver flavin appeared to be more sensitive than the incidence of C.T.P.
9. Hydroxypurines, in both free and bound forms, were found to be accumulated in the liver of flavin deficient chicks exhibiting the symptoms of C.T.P.
10. Plasma hydroxypurines and serum uric acid concentrations were not affected appreciably by flavin deficiency. A more sensitive analytical method may be necessary to elucidate possible changes of plasma hydroxypurines.
11. Injection of a hydroxypurine derivative (sodium inosinate) induced an increase in percentage heterophils, a decrease in

percentage reticulocytes and a concomitant decrease in concentration of liver flavin.

12. A possible reduction of xanthine dehydrogenase activity in chick liver due to flavin deficiency leads to an accumulation of hydroxypurines in the liver, and the presence of excess amount of hydroxypurines in the tissues causes the increase in heterophils and a decrease in reticulocytes in the blood.

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