VITAMIN B_6 AND CHOLESTEROL METABOLISM IN THE CHICKEN

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Vitamin B_6 and Cholesterol

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ABSTRACT

Research in vitamin B_6 and lipid metabolism has recently received a great impetus by its possible effect on cholesterol metabolism. Many studies have been reported, using chickens and several other animals, on different aspects of this interrelationship. No final conclusions, however, have been arrived at as yet.

Results of the first experiment in the present study indicate that cholesterol is adequately absorbed from the intestinal tract of the chicken, when fed in a purified, fat-free diet. The second experiment shows that, in the young chick, diets deficient in vitamin B_6, apart from reducing growth, increasing mortality and inducing other deficiency symptoms, had some mild hypercholesterolemic effect. Dietary cholesterol and fat significantly increased serum cholesterol when fed separately. Fat and cholesterol together, in the absence of vitamin B_6 were most effective in increasing serum cholesterol. Liver cholesterol followed the same trend as serum cholesterol. Dietary cholesterol and fat when fed either separately or together increased liver cholesterol.

Chicks fed a fat-free diet did not synthesize linoleic acid regardless of the presence or absence of vitamin B_6. Those fed diets adequate in fat did not convert linoleic acid to arachidonic acid, again regardless of the presence

or absence of vitamin B_6.

Results of the third experiment indicate that one percent dietary cholesterol significantly increased serum cholesterol of mature laying hens. Eight percent beef tallow, however, fed for a period of ten weeks had no effect on serum cholesterol. Vitamin B_6 injections, at levels fifty times the daily requirement, did not reduce serum cholesterol of the above two treatments.

In experiment four, the results indicate that the hypercholesterolemic effect of vitamin B_6 deficiency is probably not due to the lack of the vitamin itself but rather to conditions created by the deficiency, namely lower feed intake and reduced growth.

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INTRODUCTION

Agricultural research work in the last few decades has undergone a silent revolution, by which boundaries separating it from many other disciplines have gradually dissolved. No longer is an agriculturist limited to growing grains and raising cattle. He may now probe into minute details of cell chemistry or indulge himself in an intricate nutritional problem apparently far removed from any agricultural purpose. He works with complicated instruments and uses isotopes and atomic power in the solution of his problems. With this statement, it seems much easier to introduce the subject of vitamin B_6 and cholesterol metabolism as a thesis title for a student in agriculture.

Farm animals today are being pushed to their limits for more efficient production. As this "feeding for higher efficiencies" becomes more and more developed by findings in the field of nutrition, nutrient interrelationships will tend to stand out as limiting factors in the achievement of such efficiencies.

Vitamin B_6 and lipid interrelationship in the chicken may seem completely irrelevant as to its immediate application to poultry, but may have far reaching consequences in the distant future when more exact nutritional knoweldge will be applied in feeding poultry. Moreover,

there has been some evidence indicating that pyridoxine[1] and cholesterol may be related to atherosclerosis, a highly predominant disease in man.

The chicken, although anatomically markedly different from higher mammals, has been found to be an ideal experimental animal, especially in the field of nutrition. Of the vitamins, thiamin, vitamin K, and vitamin B_{12} were discovered through the use of this species. Knowledge of exact nutritional requirements of chickens is a further asset in favor of using this animal in research and experimentation.

In the present work, an attempt has been made to investigate possible interrelationships between vitamin B_6 and lipid metabolism in the chicken. Experiment one was designed to determine whether cholesterol is adequately absorbed from the digestive tract of chicks fed a purified, fat-free diet. In the second experiment, the objectives were to study the effect of dietary cholesterol and fat on serum and liver cholesterol in the presence or absence of pyridoxine, using young chicks. In the third experiment, the effect of dietary fat and cholesterol on the serum cholesterol of older birds and subsequent injections of large doses of pyridoxine, have been studied. Experiment four was

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[1] Pyridoxine and vitamin B_6 have been used interchangeably throughout this thesis.

designed to confirm or eliminate a direct relationship between vitamin B_6 and cholesterol metabolism. In this experiment, having observed that pyridoxine deficient birds feed and grow to a lesser extent than pyridoxine adequate ones, it was reasoned that if these conditions were simulated in pyridoxine adequate birds one could, following the serum cholesterol of these birds and comparing it with vitamin B_6 deficient ones, decide whether it is pyridoxine deficiency <u>per se</u> that is responsible for higher cholesterol values or rather the state of pyridoxine deficiency that is responsible for higher serum cholesterol values in deficient birds.

REVIEW OF LITERATURE

Fatty Acids and Vitamin B_6

Sherman (1950) after having reviewed the problem of vitamin B_6 and fat metabolism, concluded that a biochemical relationship must exist between the two even though the nature of this interrelationship is not clear. Mueller (1964) in a symposium indicated that the above statement remains essentially true after fourteen years and many experiments. Vitamin B_6 apparently does play a role in lipid metabolism but its specificity and importance have been seriously questioned in the last decade.

Interest in vitamin B_6 and lipid metabolism started with the original observation of Birch and Gyorgy (1936) who showed that in rats many vitamin B_6 deficiency symptoms are similar to those of essential fatty acid (EFA) deficiency. In each case, poor growth, scaly and erythematous skin rash about the paws and tails are prominent deficiency symptoms. In the case of fat deficiency, inclusion of fat in the diet, particularly unsaturated fat, will completely cure the rat. Vitamin B_6, however, will only improve the dermal symptoms, but will not restore growth. EFA will "spare" the vitamin by delaying the manifestation of dermatitis and also by decreasing its severity. Pyridoxine, however, is necessary

for complete reversion to normal.

Olson (1959) concluded that the role of vitamin B_6 as a coenzyme in protein metabolism is firmly documented. No such enzymatic role for this vitamin in lipid metabolism has been reported. For these reasons, many authorities seriously think that this vitamin plays only a secondary role in fat metabolism and perhaps only indirectly through its effect on protein metabolism. It is interesting, however, to mention the study by Wakil (1961) who reported the existence in mitochondria of a pyridoxal phosphate dependent enzyme essential for elongation of fatty acids.

Witten and Holman (1952) reported that rats deficient in vitamin B_6 and essential fatty acids synthesized less arachidonic acid when given linoleic acid, than those with only EFA deficiency. The addition of pyridoxine to the diet improved this conversion. Similarly, pyridoxine deficiency impaired the mechanism involving conversion of linoleic acid to a more unsaturated fatty acid, which again could be corrected by addition of pyridoxine to the diet. Holman (1960) reports comparable findings in mice, using deoxypyridoxine, a pyridoxine antimetabolite.

Sherman (1947) had previously observed that fat from pyridoxine deficient rats contains proportionately more arachidonic acid than that from pyridoxine supplemented rats, even though deficient rats had a smaller amount of total unsaturated fatty acids. Kirschman and Coniglio (1961)

repeated this work using pair fed rats, and observed roughly similar trends. Johnston et al. (1961) equalized the feed intake of pyridoxine deficient and supplemented groups, but noted no differences in the proportion of arachidonic acid in carcass fat, although there was a significant increase in stearic and a decrease in palmitoleic acids in the pyridoxine deficient rats. In another experiment one hundred milligrams of linoleic acid was fed daily to rats deficient in both fat and pyridoxine for three weeks. Carcass fatty acids were then analyzed and no significant differences could be detected between the tetraenes of pyridoxine deficient and supplemented groups. In an attempt to determine whether absorption and utilization of the linoleic acid could be a limiting factor in such doubly deficient animals, these workers then fed linoleic acid-1-C^{14} and followed its disposition. Despite tremendous variations they observed that deficient animals absorbed more than the supplemented ones, and that equal amounts were oxidized by both groups. Liver linoleic and arachidonic acids were proportionally higher in the pyridoxine deficient animals but the total amounts were less owing to smaller livers of deficient animals. The percentage of absorbed radioactivity appearing in arachidonic acid was much lower in deficient animals. The same was true for linoleic acid. The percentage radioactivity was greater in arachidonic acid than in linoleic acid, thus possibly indicating conversion of linoleic to

arachidonic acid.

Williams et al. (1961, 1962) in pursuing this problem further, showed that a complete vitamin B_6 deficiency reduced the extent of conversion of linoleate to arachidonate, but that suboptimal doses of this vitamin corrected this reduction in conversion. Swell et al. (1961) reported significant decline in arachidonic acid in the triglyceride fraction of serum and liver in pyridoxine deficient rats as compared to pair fed controls.

Kahn (1964) observed that ethyl esters of the polyunsaturated fatty acids of cod-liver oil reduced the hypercholesterolemia produced in rats fed diets supplemented with coconut oil and cholesterol.

Greenberg and Moon (1961) carried out a critical experiment on the total fatty acid patterns in serum and red blood cells in groups of EFA-deficient, EFA-pyridoxine-deficient, pyridoxine deficient and control monkeys. The addition of pyridoxine deficiency to EFA deficiency did not alter the marked reduction of linoleic and arachidonic acids in both serum and red blood cells. A more important finding was that in two monkeys deprived only of pyridoxine no significant change was found in any of the fatty acid patterns over a seven months period.

Dam et al. (1958) studied the effect of vitamin B_6 deficiency on fatty acid metabolism of chicks maintained on fat-free and fat supplemented diets. Pyridoxine deprived

chicks on a fat-free diet had more heart and liver arachidonate than pyridoxine supplemented ones. When 10% peanut oil was added, pyridoxine supplementation resulted in significantly increased amounts of tetraenoics. They interpreted this as confirming a role for pyridoxine in unsaturated fatty acid interconversions.

It may be observed from the above review that the problem of pyridoxine and fatty acid interrelationships is not by any means settled and a great deal of experimental work is needed before final conclusions could be made.

Cholesterol and Vitamin B_6

The role of vitamin B_6 in cholesterol metabolism has been under investigation for a considerable period of time. Goswami and Sadhu (1961) reported hypercholesterolemia in pyridoxine deficient rats. Maggi *et al.* (1959) found increased cholesterol and phospholipid synthesis from labeled acetate in the liver and adrenal glands of rats fed deoxypyridoxine.

Shah *et al.* (1960) studied the effect of pyridoxine deficiency on the *in vivo* synthesis of cholesterol from acetate and mevalonic acid and on the excretion of cholesterol 4-C^{14} via bile in bile cannulated rats. The results indicated that pyridoxine deficiency enhanced the incorporation of labeled acetate into liver cholesterol. This enhanced

incorporation of labeled acetate into cholesterol in deficient rats could be reversed by injecting pyridoxine. The synthesis of cholesterol from mevalonic acid, and excretion of cholesterol 4-C^{14} were not altered by pyridoxine deficiency. From the above observations these workers assumed that the rather remarkable uptake of acetate reflected an increase in available 2-carbon fragments as a result of alterations in energy balance.

Williams and Pertel (1964), assuming that the increased incorporation of labeled acetate into liver cholesterol in pyridoxine deficient and the control used by Shah was based on ad libitum feeding, claimed that no comparisons were made with pair fed or pair weighed controls to evaluate possible effects of decreased food intake and growth of the deficient rats. These workers then set out to compare the incorporation of acetate-2-C^{14} into liver lipids in vivo by pyridoxine deficient rats with pair fed, pair weighed, and ad libitum fed controls. In addition, acetate-2-C^{14} incorporation was studied with liver slices from pyridoxine deficient rats and from pair fed and ad libitum fed controls. The results indicated that, in vivo, pyridoxine deficient rats incorporated significantly more C^{14} into sterol than any of the control groups. Liver slices from deficient rats also incorporated more C^{14} into sterol than did slices from the ad libitum control. Pair fed controls, however, incorporated more C^{14} than the

pyridoxine deficient ones.

Greenberg and Rinehart (1951) showed that feeding of cholesterol to pyridoxine deficient monkeys resulted in higher serum cholesterol values than when feeding even two to four times more cholesterol to non-deficient animals. In addition, the pyridoxine deficient monkeys showed evidence of a vascular disease similar to human atherosclerosis. Mann et al. (1953) were unable, however, to produce an elevation of serum cholesterol in cholesterol fed, pyridoxine deficient, rhesus monkeys. Emerson et al. (1960) observed that free and total plasma cholesterol increased with increasing levels of dietary pyridoxine irrespective of the dietary fat, although the saturated fat induced higher cholesterol levels.

It is interesting to note that three independent studies resulted in three varying results. This clearly illustrates the present lack of knowledge regarding this aspect of vitamin B_6 metabolism.

According to Beeles et al. (1962) chicks fed a synthetic diet containing 20 percent hydrogenated coconut oil and 1 percent cholesterol developed atherosclerotic lesions in twenty weeks. Serum cholesterol and aorta cholesterol esters were higher in comparison with birds receiving no dietary cholesterol with a low fat diet (0.5%). Plasma cholesterol was not elevated by feeding dietary cholesterol.

Dam and co-workers (1958) reported a significant increase in plasma and aorta cholesterol and a decrease in heart cholesterol in vitamin B_6 deficient chicks. The same findings were essentially confirmed by Daghir and Balloun (1962). These workers in addition observed that nicotinic acid did not alter the picture.

March et al. (1963) observed that there are marked individual differences in the hypercholesterolemia response of cockerels to high cholesterol diets. They demonstrated that differences in the amount of cholesterol absorbed from the intestines are primarily responsible for differences in blood cholesterol level of cockerels fed 1 or 2 percent cholesterol in the diet; cholesterol absorption being related to the efficiency with which dietary fat is absorbed by individual birds.

Adamson (1961) observed that serum cholesterol concentrations were increased only when both fat and cholesterol were fed. The increase was not inhibited by dietary unsaturated fats. Cholesterol when fed with corn oil, vegetable shortening, or butter fat, caused greatly increased liver cholesterol and lipid concentration. Endogenous cholesterol synthesis decreased with age and appeared to be independent of the degree of saturation of dietary fat. It was strongly inhibited by diets containing both fat and cholesterol. Hegsted (1960) observed that fats high in saturated fatty acids promote hypercholesterolemia in the

chick and that this effect is counteracted by polyunsaturated fatty acids.

Daghir et al. (1960) observed that feeding 12 percent soybean oil to laying hens resulted in a significant decrease in serum cholesterol levels, while 12 percent grease did not increase serum cholesterol concentrations.

In summary, evidence seems to support a possible interrelationship between vitamin B_6 deficiency and hypercholesterolemia. The exact mechanism involved is not yet elucidated, but lack of direct effect on synthesis or degradation suggests that pyridoxine may affect other aspects of lipids such as transport of cholesterol perhaps through its effect on fatty acids. Another hypothesis could be that cholesterol transport is altered by changes in the protein moiety of lipoproteins secondary to pyridoxine deficiency.

Many other aspects of the problem such as sex differences in response to vitamin B_6 deficiency and cholesterol have been reported by different workers. In this connection it is interesting to mention the recent work by Shue and Hove (1965) who reported that, in weanling rats fed purified diets supplemented with cholesterol and cholic acid for 20 weeks, the blood cholesterol levels of females were two to three times higher than the males. This effect was especially evident when the diet contained a severely hypercholesterolemic fat (coconut oil) and least so when it contained cottonseed oil.

A great deal of research and experimentation is needed before final conclusions regarding vitamin B_6 and cholesterol could be made.

MATERIALS AND METHODS

Animals and Management

Vent-sexed day-old broiler type chicks (Cornish male x Plymouth Rock female) used in these studies were obtained from a commercial hatchery. The laying hens used were of the Babcock's strain raised at the Agricultural Research and Education Center. All chick experiments were carried out either in 5-deck battery brooders equipped with wire floors and thermostatically controlled electric heating elements, or in unheated 4-deck growing batteries. The heat of the battery brooders was adjusted to the comfort of the chicks from an initial temperature of 100°F. The temperature of the brooding house was maintained within the range of 60-70°F.

In experiments I, II and III, feed and water were provided ad libitum. Waterers were scrubbed and washed daily to keep microbial synthesis of vitamin B_6 at a minimum. Rations for experiments I, II and IV were prepared from purified ingredients while in experiment III natural ingredients were used.

Compounds and Ingredients

Pure, crystalline pyridoxine hydrochloride (vitamin B_6)

was used in all experiments. Dextrose was obtained in 100 lb. bags from the Corn Products Company of New York. Promine-R, an isolated soybean protein containing 97% protein on a moisture free basis, was obtained from Central Soya, Chicago. Alphacel, a non-nutritive fiber, was used as a filler for balancing energy levels of diets. Brigg's mineral mixture, whose composition is shown in Table 1, was used in all experiments. Choline chloride used was a 70% aqueous solution. Pure DL-methionine and glycine were used to supplement the soybean protein. Pure, synthetic cholesterol from General Biochemicals Incorporation was used in all experiments. Mazola, a pure corn oil, was used for fat supplementation in experiments I, II and IV. Beef tallow used in experiment III was prepared by grinding and heating beef fat followed by stabilization with the addition of the antioxidant, Santoquin.

Records and Data

Chicks were wing banded and weighed on gram scales while layers were leg banded and weighed on a pound scale and the figures later converted to grams. Daily mortality records were kept, vitamin B_6 deficiency symptoms were observed and dead birds autopsied and abnormalities recorded. In the laying hen experiment, eggs were collected daily and feed consumption measured weekly. Data of experiments

II, III and IV were statistically analyzed according to Snedecor (1956) and Duncan's multiple range test was applied in experiment III.

Collection of Samples

Blood was obtained by heart puncture using a 5 cc glass syringe and a 21 gauge needle. The method used in obtaining blood is a modification of the method described by Hofstad (1950). The bird was placed flat on its back and the needle inserted on the left side through the upper sternal notch at about a 60° angle. One to two cc of blood were drawn for each sample. The blood was immediately transferred to a clean, dry centrifuge tube and covered with a cork stopper. Coagulated blood was then centrifuged in a Piccolo centrifuge at approximately 3000 R.P.M. for 15 minutes. The serum was immediately placed in a clean, dry, labeled vial and frozen until analyzed. Abdominal fats were collected by cutting small amounts of fatty tissue and placing them in jars in the freezer. In collecting feces, droppings were accumulated for a period of four days. They were then scraped off the tray, dried, and sampled for analysis. Eggs for cholesterol determinations were randomly collected from each treatment.

Chemical Analyses

1. Serum Cholesterol Determination

Determinations of total serum cholesterol were made according to a modification of the method described by Zlatkis *et al.* (1953). The reagents used for this determination were: (a) Standard cholesterol solution (1 mg/ml), prepared by dissolving 100 mg of pure, dry, ash-free cholesterol in 100 cc of 100 percent glacial acetic acid; (b) Ferric chloride solution, prepared by dissolving 10 grams of reagent grade, ferric chloride in 100 cc of 100 percent glacial acetic acid; (c) Color reagent, prepared by diluting 1 cc of the ferric chloride solution to 200 cc with C.P. concentrated sulfuric acid. This color reagent, being unstable, was prepared for every run.

A standard curve for cholesterol was obtained in the following manner: Standard cholesterol solution (0.1, 0.2, 0.3 and 0.4 cc of standard cholesterol solution) was pipetted into clean, dry, 30 ml test tubes and each was diluted with glacial acetic acid to 5.0 cc. Distilled water (0.1 ml) was added to each standard and mixed thoroughly. A blank was prepared which contained 5.0 ml of glacial acetic acid and 0.1 ml of distilled water. The color reagent (2.0 cc) was then pipetted into the sample by carefully allowing it to flow down the side of

the test tubes, thus producing two layers. The tubes were then shaken vigorously to effect mixing and heat distribution. When the tubes had cooled to room temperature, the absorbancy (optical density) was measured in a Beckman spectrophotometer at 560 mu using one cm cuvettes. The optical densities were then plotted against cholesterol concentrations.

To determine serum cholesterol, 0.1 ml of serum was pipetted into a 30 ml test tube containing 5.0 ml of glacial acetic acid. The color reagent was added and mixed as explained above for the standard. The optical density of the solution was then measured after it had come to room temperature and total cholesterol was determined from the calibration curve.

2. Liver Cholesterol and Fat Determinations.

Livers were first individually homogenized in a mortar and then dried in a vacuum oven at 90°- 100° C and a 25 mm mercury vacuum for approximately 10 hours. The dry homogenate was then ground and 2 gram samples were extracted in a Soxhlet extractor for 16 hours using diethyl ether. Percent lipids was calculated from the weight loss. Extracts were diluted to 200 ml with the solvent and 1 ml sample was evaporated to dryness for total cholesterol determination as outlined above for serum cholesterol determinations.

3. Egg Yolk Cholesterol

Eggs were boiled for 10 minutes and yolks were then removed, smashed and oven dried. The fats were then extracted in a Soxhlet extractor as described above. Extracts were diluted to 200 ml with the solvent and 1 ml sample was evaporated to dryness and total cholesterol determined as outlined above for serum cholesterol.

4. Gas Chromatography of Abdominal Fats

Ten cc of a 2:1 chloroform methanol mixture was added to the fat sample and crushed, with acid washed celite as a grit, in a mortar and pestle. Distilled water was then added to the mixture to make separation of the lipid phase possible. The mixture was filtered into a separatory funnel. The bottom layer was taken and washed once more with distilled water and then dried over anhydrous sodium sulfate for two hours. Chloroform was removed afterwards by bubbling nitrogen gas into the tube containing the sample.

The sample was then cross-esterified with 20 ml methanol and 0.5 ml concentrated sulfuric acid. The mixture was refluxed on a water bath for a period of two hours. The solution was then cooled and transferred to a separatory funnel, the sulfuric acid was neutralized with a 5 percent solution of sodium bicarbonate and

petroleum ether was added to effect separation of the fatty esters. The mixture was then shaken and the bottom layer discarded. The upper layer was washed twice with distilled water and finally the ether extract was dried over anhydrous sodium sulfate powder, and put in the freezer, to be run through the gas chromatographer.

The petroleum ether was evaporated by bubbling nitrogen gas into the tube containing the sample. Samples of 0.01 ml were applied, using a micropipette, to a polyethylene glycol adipate column, of a Pye gas chromatographer, using argon as carrier gas, with a flow of 30-50 ml per minute and a column temperature of exactly 174.5°C. The graph paper rolled at a speed of 12 inches per hour and each sample was allowed between two to three hours of running time. The machine was operated in medium to high sensitivity at 1500 voltage. From the graphs, percent of each fatty acid was then calculated as methyl ester.

Experiment I

Purpose

Sections of the present work necessitated the feeding of a fat-free, cholesterol supplemented diet. Some reports have indicated that cholesterol is not as well absorbed from a fat-free diet as from a fat adequate one. The present experiment was therefore conducted to evaluate the extent of this absorption.

Procedure

Two groups of ten chicks each were fed the two rations shown in Table 1. These are a fat-free cholesterol supplemented, and a fat adequate cholesterol supplemented diets. The two rations were identical except for the fat content. The chicks were kept on their respective diets for a period of two weeks, at the end of which serum samples were collected from nine birds in each group. Serum samples from every three birds were pooled, thus obtaining three different samples per treatment. Feces collected from the last four days were sampled and analyzed for cholesterol content. Body weight gains were also recorded.

Results

Data on growth are presented in Table 2. Both rations supported normal growth and did not differ much from each

Table 1 - Composition of diets used in experiment I (%).

Ingredients	1	2
Dextrose	68.00	62.00
Soybean protein (Promine-R)	22.00	22.00
Corn oil	0.00	4.00
Alphacel	1.75	3.75
Mineral mixture*	5.30	5.30
Choline chloride	0.25	0.25
DL-Methionine	0.50	0.50
Glycine	0.20	0.20
Vitamin mixture**	1.00	1.00
Cholesterol	1.00	1.00

* Mineral mixture composition: calcium carbonate 16.6%; calcium phosphate 47.3%; copper sulfate 4.017%; ferric citrate 0.333%; magnesium sulfate 5%; manganese sulfate 0.417%; potassium chloride 11.6%; potassium iodide 0.017%; sodium chloride 6.6%; sodium phosphate dibasic 11.6%; zinc carbonate 0.217%.

** Vitamin mixture contributed the following to the pound of ration: 4550 I.U. of vitamin A; 800 I.C.U. of vitamin D_3; 10 I.U. of vitamin E; 60 mg inositol; 1.5 mg folic acid; 30 mg P.A.B.A.; 40 mg niacin; 10 mg calcium pantothenate; 4 mg riboflavin; 2 mg thiamin; 2 mg menadione; 100 mg ascorbic acid; 10 mcg B_{12}; 100 mcg biotin and 4 mg pyridoxine hydrochloride.

other, even though the fat supplemented diet resulted in slightly higher weights.

Table 2 - Body weights in grams at 0, 1 and 2 weeks of age - Experiment I.

Weeks	Group 1 (fat-free)	Group 2 (fat-adequate)
0	46.2*	46.3
1	87.8	89.2
2	118.2	126.9

* Values are averages of 10 birds each.

Data on serum and feces cholesterol are presented in Table 3. As had been expected, more cholesterol was excreted from the chicks receiving the fat-free diet as compared to the fat-adequate diet. The serum cholesterol values, however, were not too different even though the fat supplemented diet resulted in slightly higher serum cholesterol values. The amount of cholesterol absorbed from a fat-free diet seems to be adequate enough to induce hypercholesterolemia in the chick.

Table 3 - Cholesterol content of feces (mg/100 grams dry feces) and serum cholesterol (mg %) - Experiment I.

Dietary treatment	Feces cholesterol	Serum cholesterol
1 Cholesterol - fat	9588*	271**
2 Cholesterol + fat	6718	284

* Average of 2 determinations.
** Average of 3 determinations.

Experiment II

Purpose

This experiment was conducted to investigate possible interrelationships of vitamin B_6, cholesterol and an unsaturated vegetable oil; and to single out the more significant interrelations to be further investigated. More specifically, it was hoped that this experiment would provide answers to some of the following questions:

1. Does vitamin B_6 deficiency cause hypercholesterolemia in the chick?

2. Do vitamin B_6 injections reduce hypercholesterolemia resulting from vitamin B_6 deficiency?

3. Does fat supplementation of a chick ration cause hypercholesterolemia?

4. Does incorporation of cholesterol at the rate of 1 percent of the ration cause severe hypercholesterolemia in the chick?

5. Is vitamin B_6 related to fatty acid metabolism in the chick?

Procedure

A total of eighty, day-old, male chicks were used. The experiment included eight treatments of ten chicks each. The treatments were further divided into two replicates of five birds each. Chicks were categorized according to weight

and then uniformly distributed among the treatments. A completely randomized design was used. Individual chick weights were recorded weekly and average body weights calculated. Feed consumption was calculated as the average number of grams per chick per day. Mortality was recorded daily and calculated at the end of the experiment as the total number of deaths per treatment. Death in vitamin B_6 deficient chicks occurred as early as eight days after the start of the experiment and later it was necessary, in order to maintain an adequate number of chicks per treatment to the end of the experiment, to inject some of the vitamin B_6 deficient birds with small amounts of vitamin B_6 (1 cc injections of 0.2 mg/cc of vitamin B_6 solution administered twice, on August 18 and 21, 1964).

Dead birds were autopsied and observed for any pathological changes. At the end of the experiment, the chicks were sacrificed and livers collected from three birds in each replicate. Cholesterol content, percent fat, and percent moisture of these livers were then determined. Blood samples for serum cholesterol determinations were collected from the same birds. Pieces of abdominal fat were also collected from the same birds. The three samples of each replicate were pooled and fats were analyzed for their fatty acid content by gas chromatography.

Table 4 presents the composition of the eight different rations used in this experiment. The number of these

experimental rations have been used in all tables in this experiment to designate the various treatments. A schematic representation of these eight treatments is also presented in Table 5.

Results

Growth: Data on growth rate are presented in Table 5. The lower growth rate due to vitamin B_6 deficiency is apparent starting with the first week of age regardless of the presence or absence of cholesterol. Fat in the diet, however, slightly improved growth rate. Chicks receiving the diets adequate in both vitamin B_6 and fat had the best growth. Chicks on vitamin B_6 adequate diets had growth rates comparable to chicks receiving a practical starter ration, thus indicating the adequacy of the purified diets used.

Statistical analysis of body weights at 4 weeks of age indicates that reduction in growth due to vitamin B_6 deficiency is significant at the 1 percent level of probability. The increase in weight due to fat supplementation is significant at the 5 percent level of probability.

A record of feed consumed per bird per day is presented in Table 6. The feed consumption of vitamin B_6 deficient birds, regardless of the presence or absence of either fat or cholesterol, was drastically lowered as evidenced by small figures during the first and second weeks of the expe-

Table 4 - Composition of diets used in experiment II (%).

Ingredients	1	2	3	4	5	6	7	8
Dextrose	66.00	69.00	66.00	69.00	61.00	62.00	61.00	62.00
Soybean protein (Promine-R)	22.00	22.00	22.00	22.00	22.00	22.00	22.00	22.00
Corn oil	-	-	-	-	4.00	4.00	4.00	4.00
Alphacel	3.75	1.75	3.75	1.75	4.75	4.75	4.75	4.75
Mineral mixture*	5.30	5.30	5.30	5.30	5.30	5.30	5.30	5.30
Choline chloride	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-Methionine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Glycine	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin mixture**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	1.00	-	1.00	-	1.00	-	1.00	-
Vitamin B_6 (mg/lb)	4	4	-	-	4	4	-	-

* Same composition as shown in Table 1 page 22.
** Same composition as shown in Table 1 page 22 except pyridoxine hydrochloride omitted.

Table 5 - Average body weights in grams at 0, 1, 2 and 3 weeks of age - Experiment II.

Dietary treatment	1	2	3	4	5	6	7	8
Fat (4% corn oil)	-	-	-	-	+	+	+	+
Cholesterol (1%)	+	-	+	-	+	-	+	-
Vitamin B_6 (4 mg/lb)	+	+	-	-	+	+	-	-
Body weights at								
0 week	42*	42	42	42	42	42	42	42
1 week	74	78	70	66	76	85	73	71
2 weeks	143	155	72	85	163	179	88	81
3 weeks	232	248	123	164	248	288	188	155

* Values are averages of 10 birds except in treatments with mortality.

Table 6 - Feed consumption (grams consumed per bird per day at 1, 2 and 3 weeks of age) and mortality (number of deaths out of a total of ten birds started) - Experiment II.

	Dietary treatment	Feed consumption			Mortality
		First week	Second week	Third week	
1	Cholesterol + vitamin B_6	9	22	35	0
2	Vitamin B_6	10	21	30	0
3	Cholesterol	8	6	18	6
4	-	8	9	34	5
5	Fat + cholesterol + vitamin B_6	9	23	32	0
6	Fat + vitamin B_6	9	24	32	0
7	Fat + cholesterol	7	9	21	6
8	Fat	7	9	40	6

riment. Injection of small quantities of vitamin B_6 to the deficient birds restored feed consumption temporarily to a normal level. In this connection, it is interesting to note the rapidity with which vitamin B_6 affects feed consumption. Almost within one or two days after injection, there was a rise in feed consumption as evidenced by third week figures. This probably suggests a neuro-hormonal effect of this vitamin on the appetite center of the bird.

Mortality and deficiency symptoms: Mortality figures are presented in Table 6. Deaths occurred only in vitamin B_6 deficient birds. Mortality was independent of cholesterol or fat supplementation or a combination of both as evidenced by almost identical death rates in treatments three, four, seven and eight. All the vitamin B_6 deficient birds would have been lost, had it not been for the small injections of this vitamin. The injected birds, however, remained in a deficient state despite this treatment.

Vitamin B_6 deficiency is virtually unknown to a commercial poultryman, because of the abundance of this vitamin in most feed ingredients. In fact, only by the use of purified rations and under very clean conditions can vitamin B_6 deficiency be induced in birds, and then only very young birds should be used for clear cut clinical symptoms. All the above conditions prevailed in this experiment, and therefore provided a chance to closely observe

deficiency symptoms of this vitamin. The most pronounced symptom in the growing bird is, of course, reduced growth. Within a week, clinical deficiency symptoms were observed. The birds were hyperexcitable despite a general droopy mood. This hyperexcitability advanced by time into intermittent convulsions, whereby the birds would run aimlessly around the cage hitting the walls, feeders, and other birds and finally undergo tremors of very short duration and then into a droopy mood again. Death usually occurred at the end of the second or third convulsion.

Decreased feed consumption was evident within three to four days. The incidence of gizzard erosion, even though not measured, was obvious in many of the dead birds. Pendulous crops which were atrophic, hanging, and filled with a thin paste were observed in some of the deficient birds.

Apart from the above vitamin B_6 deficiency symptoms, some abnormalities were observed in birds receiving fat-free or cholesterol supplemented diets; the livers of the former group being pale and fatty, and the gall bladders of the latter enlarged, necrotic, and sticking sideways to the liver causing necrotic lesions on the liver at the site of attachment.

Serum cholesterol: Because of necessity to inject some of the vitamin B_6 deficient birds, serum cholesterol levels were determined twice - before and after injections.

The figures are presented in Table 7. The inequality between the number of birds bled for cholesterol determination is due to the fact that mortality in vitamin B_6 deficient birds reduced the number in some treatments. Also because of extreme weakness and fear of losing the birds, some were bled only after they had been injected with vitamin B_6 and therefore accounting for the variability in the number of birds used for cholesterol determination before and after vitamin B_6 injections.

Cholesterol values for the first four treatments both before and after vitamin B_6 injections, averaged lower than the last four treatments, thus indicating that fat supplementation of the diet increased serum cholesterol. Birds in treatment two, receiving a fat-free, cholesterol-free and vitamin B_6 adequate diet had the lowest serum cholesterol values. Treatment seven containing cholesterol, fat and no vitamin B_6 resulted in highest serum cholesterol values. The analysis of variance indicates that only dietary cholesterol significantly increased serum cholesterol. Comparing treatments one and three, it is observed that vitamin B_6 has had some mild hypocholesterolemic effect. In the presence of fat, however, this effect is not observed as can be seen by comparing treatments five and seven. It is also observed that vitamin B_6 injections reduced serum cholesterol to a slight extent. The hypercholesterolemic effect of vitamin B_6 deficiency was not consistant.

Table 7 - Serum cholesterol (mg %) before and after injections of vitamin B_6 - Experiment II.

	Dietary treatment	Before injections		After injections	
		No. of birds	Av. serum cholesterol	No. of birds	Av. serum cholesterol
1	Cholesterol + vitamin B_6	5	171	6	157
2	Vitamin B_6	6	80	5	148
3	Cholesterol	3	254	4*	214
4	-	3	229	5*	164
5	Fat + cholesterol + vitamin B_6	6	251	6	378
6	Fat + vitamin B_6	6	165	6	192
7	Fat + cholesterol	2	216	3*	400
8	Fat	3	353	4*	149

* Injected twice with 0.2 mg of vitamin B_6.

Liver cholesterol, fat and moisture: Liver cholesterol values are presented in Table 8. Birds receiving ration two which was cholesterol-free, fat-free and adequate in vitamin B_6 had the lowest liver cholesterol, while those receiving ration seven which was fat adequate, cholesterol adequate but deficient in vitamin B_6 had the highest liver cholesterol. The effect of vitamin B_6 in reducing liver cholesterol values was variable. There was a tendency, however, for pyridoxine adequate birds to have lower liver cholesterol values. This effect was not statistically significant. Dietary cholesterol increased liver cholesterol and the effect was significant at the 1 percent level of probability. Fat supplementation of the diets also increased liver cholesterol significantly ($P < 0.01$). Diets containing both cholesterol and fat increased liver cholesterol significantly ($P < 0.01$).

Liver fat and moisture values are also presented in Table 8. Cholesterol supplementation significantly ($P < 0.05$) increased liver fat while fat supplementation significantly ($P < 0.05$) reduced liver fat. Liver moisture values indicate that fat supplementation increased liver moisture, as compared to the fat-free diets ($P < 0.05$), and cholesterol supplementation slightly reduced liver moisture.

Fatty acid composition of abdominal fats: The compositions of abdominal fat from the different treatments are presented in Table 9. Fat from chicks receiving treatments

Table 8 - Liver cholesterol (mg/100 grams dry tissue); liver fat (percent on dry weight basis) and moisture of the different treatments - Experiment II.

Dietary treatment		Liver cholesterol	Liver fat	Liver moisture
1	Cholesterol + vitamin B_6	2103*	24	70
2	Vitamin B_6	1250	19	72
3	Cholesterol	1863	41	66
4	-	1623	19	71
5	Fat + cholesterol + vitamin B_6	3270	19	71
6	Fat + vitamin B_6	1568	14	72
7	Fat + cholesterol	4708	19	71
8	Fat	1368	15	73

* Each value represents an average of two determinations from two different pooled samples of 3 livers.

Table 9 - Fatty acid composition of abdominal fat (percent of total methyl esters) - Experiment II.

	Dietary treatment	Palmitic acid C16:0*	Palmitoleic acid C16:1	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2
1	Cholesterol + vitamin B_6	27**	10	6	56	0
2	Vitamin B_6	27	8	6	58	0
3	Cholesterol	30	9	12	48	0
4	-	28	9	7	55	0
5	Fat + cholesterol + vitamin B_6	25	7	5	45	16
6	Fat + vitamin B_6	26	6	6	44	15
7	Fat + cholesterol	24	6	7	44	18
8	Fat	25	6	7	48	14

* C16:0 = fatty acid with 16 carbons and no double bond etc.
** Each value is an average of two replicates, and each replicate represents a pooled sample of fat from three birds.

one to four did not contain any linoleic acid, thus indicating that in the absence of a dietary source of fat, regardless of the presence or absence of vitamin B_6, the chicks did not synthesize linoleic acid. Apart from this difference, the pattern of distribution of other fatty acids remained unchanged. In fats containing no linoleic acid there was a general increase in the level of other fatty acids to compensate, percentage-wise, for the missing fatty acid. None of the fat samples yielded any amount of arachidonic acid detectable by the gas chromatographer used. It was expected that fat from chicks receiving a fat and vitamin B_6 adequate ration, would contain small amounts of arachidonic acid.

Experiment III

Purpose

In this experiment, laying hens were used instead of growing chicks to achieve the following objectives:

1. Induce hypercholesterolemia in layers by feeding a ration containing 1 percent supplementary cholesterol or one containing a highly saturated animal fat (beef tallow) at 8 percent of the ration.

2. Study the effect of vitamin B_6 injections, at fifty times the daily requirement, on the serum cholesterol of the above hypercholesterolemic layers and on egg yolk cholesterol.

Procedure

Three groups of ten mature, single comb, White Leghorn hens were used in two treatment groups and one control group. The birds were eighteen months old at the start of the experiment. The composition of the diets used is presented in Table 10. Diet one was a layer ration supplemented with 1 percent cholesterol. Diet two contained 8 percent beef tallow and diet three was a control. The beef tallow in ration two was added at the expense of corn, and wheat bran was used as a filler. The three rations were approximately isocaloric. To prevent rancidity, Santoquin, an antioxidant, was incorporated into ration two at the rate of 0.015% of the feed.

Table 10 - Composition of diets used in experiment III (%).

Ingredients	Treatments		
	1	2	3
Yellow corn	69.25	47.25	70.25
Soybean meal (44% protein)	17.00	17.00	17.00
Alfalfa meal (17% protein)	2.00	2.00	2.00
Fish meal (65% protein)	2.00	2.00	2.00
Salt	0.50	0.50	0.50
Vitamin mixture*	0.25	0.25	0.25
Limestone	5.00	5.00	5.00
Bone meal	3.00	3.00	3.00
Wheat bran	0.00	15.00	0.00
Beef tallow	0.00	8.00	0.00
Cholesterol	1.00	0.00	0.00

* Nopcosol M-4, a commercial vitamin mixture had the following composition per pound: Vitamin A 800,000 I.U.; vitamin D_3 300,000 I.C.U.; vitamin E 1500 I.U.; riboflavin 600 mg; niacin 400 mg; d-pantothenic acid 1000 mg; choline chloride 40,000 mg; vitamin B_{12} 2 mg; this mixture in addition provided the following as percentage: Mn 2.400%; Zn 1.100%; iodine 0.048%; Fe 0.800%; Cu 0.080% and Co 0.008%.

Each treatment was allocated to a floor pen with a deep litter system. The birds were leg banded and weighed at the start and end of the experiment. Feed and water were provided ad libitum. Feed consumption and egg production were recorded and pounds of feed per dozen eggs and percent production were then calculated.

Weekly blood samples were drawn from each bird in all treatments and serum cholesterol content determined. The experiment was divided into two periods of five weeks each. After the first five weeks, each bird was injected, three times weekly, intramuscularly with 1 cc of a 50 mg/cc solution of vitamin B_6. This provided the birds with approximately fifty times their daily requirement of this vitamin.

Total egg cholesterol content from each treatment was determined three times in each period. For every determination three eggs were collected and duplicate analyses were carried out.

Results

Body weight: Data on body weight are presented in Table 11. It is observed that birds maintained their body weights from the start to the end of experiment, despite the fact that they were bled weekly for a period of ten weeks. Layers receiving the beef tallow supplemented ration did not gain weight, contrary to expectation. This is partially explained on the basis that the diet was rendered less

Table 11 - Body weights in grams at the beginning and end of experiment III.

Dietary treatment	Body weights at	
	Start	End
1% cholesterol	1752*	1748
8% beef tallow	1824	1815
Control	1714	1722

* Values are averages of 10 birds each.

palatable, together with the fact that chickens eat to satisfy their energy requirements.

Egg production and feed consumption: Table 12 contains egg production and feed consumption figures. Layers receiving the cholesterol supplemented diet had the highest rate of egg production and consumed the least amount of feed per dozen eggs. Those receiving the beef tallow supplemented diet had the lowest rate of egg production and consequently ate the largest amount of feed per dozen eggs.

Egg yolk cholesterol: Data on egg yolk cholesterol content of the different treatments are presented in Table 13. There is a general increase in the egg yolk cholesterol from the first to the second period. This slight increase is apparent in all the three treatments, thus providing grounds to speculate that vitamin B_6 injections might have enhanced cholesterol excretion and its subsequent deposition in the egg yolk.

Layers receiving the cholesterol supplemented diet

Table 12 - Percent egg production and pounds of feed consumed per dozen eggs - Experiment III.

Weeks	Treatments					
	1% cholesterol		8% beef tallow		Control	
	% egg production	lbs/dozen eggs	% egg production	lbs/dozen eggs	% egg production	lbs/dozen eggs
1	79	4.4	53	6.4	66	4.6
2	75	4.6	56	5.8	64	4.8
3	69	4.5	51	6.3	61	4.8
4	73	4.4	47	5.7	56	5.6
5	60	5.3	44	7.2	48	6.0
6	57	5.2	56	5.4	51	5.6
7	49	5.7	51	5.0	54	5.2
8	56	5.5	49	5.3	50	4.8
9	43	6.6	47	4.9	44	8.0
Average	62	5.2	50	5.8	55	5.5

Table 13 - Egg yolk cholesterol (mg/100 grams fresh tissue) - Experiment III.

Period	Treatments		
	1% cholesterol	8% beef tallow	Control
Before vitamin B_6 injections	2706*	1843	2529
	2921	1740	2640
	2771	1293	2610
Average	2799	1625	2593
After vitamin B_6 injections	2883	1188	2566
	2823	2297	2733
	2976	1945	2844
Average	2894	1810	2714

* Each figure represents the average value for three eggs and each egg an average of two determinations.

had the highest egg yolk cholesterol values. The low values for those receiving the diet supplemented with beef tallow are contrary to expectation. The antioxidant added to prevent rancidity might, partially, be responsible for this result.

Serum cholesterol: Data on serum cholesterol are presented in Table 14 and in Figure 1. Feeding of a diet supplemented with 1 percent cholesterol caused a rather immediate elevation of serum cholesterol, which maintained itself at this high level without further increase. The diet supplemented with 8 percent beef tallow, contrary to expectation, did not cause an elevation of serum cholesterol. Vitamin B_6 injections at a level of fifty times the daily requirement did not reduce serum cholesterol. Statistical analysis for the first period indicated that only dietary cholesterol caused a statistically significant ($P < 0.05$) increase in serum cholesterol. The same holds true for the second period of the experiment. Vitamin B_6 injections were entirely ineffective in reducing serum cholesterol, as can be seen by comparing the averages of the first period with the respective averages of the second period for each treatment.

Table 14 - Serum cholesterol (mg %) - Experiment III.

	Weeks	Treatments		
		1% cholesterol	8% beef tallow	Control
Before B_6 injections	1	285 ± 23*	255 ± 28	284 ± 15
	2	364 ± 41	225 ± 16	248 ± 19
	3	349 ± 87	240 ± 21	180 ± 12
	4	322 ± 55	222 ± 13	189 ± 17
	5	390 ± 56	257 ± 35	276 ± 18
	Average	342 ± 25	240 ± 7	235 ± 7
After B_6 injections	6	351 ± 43	214 ± 17	233 ± 25
	7	356 ± 39	254 ± 18	283 ± 32
	8	397 ± 57	242 ± 22	274 ± 27
	9	349 ± 62	218 ± 18	269 ± 37
	10	312 ± 92	236 ± 11	185 ± 19
	Average	353 ± 20	233 ± 10	249 ± 12

* Each figure represents an average of 10 determinations ± standard error.

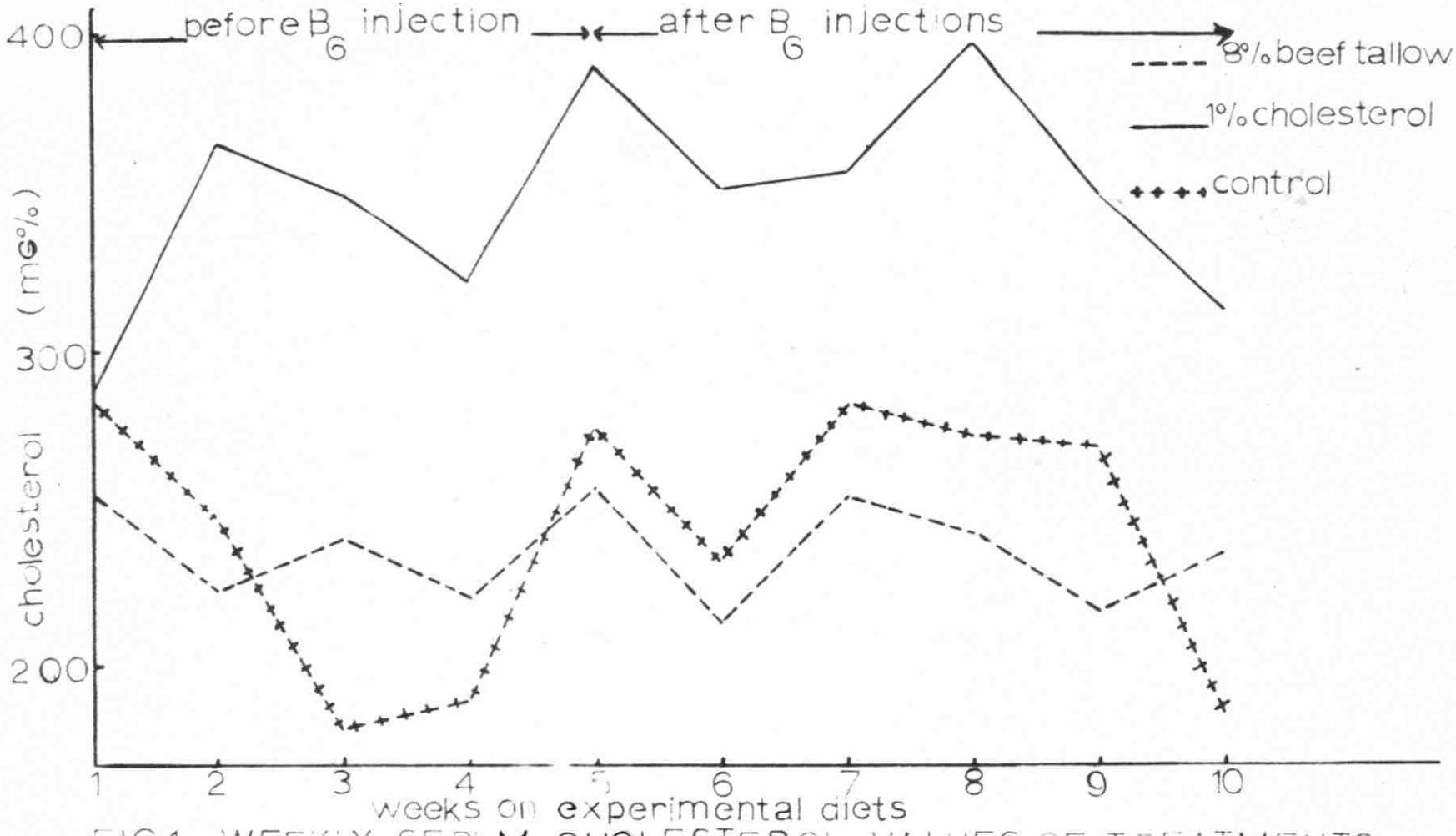

FIG.1 WEEKLY SERUM CHOLESTEROL VALUES OF TREATMENTS USED IN EXPERIMENT III

Experiment IV

Purpose

Having observed from the previous trials that vitamin B_6 does not seem to influence cholesterol metabolism directly, an attempt was made in this experiment to investigate the possibility of an indirect influence of this vitamin on cholesterol metabolism.

Since a vitamin B_6 deficient chick does not eat and grow to the same extent as a vitamin B_6 adequate one and in general has a higher serum cholesterol, it was postulated that may be these two conditions, i.e. lower feed intake and growth, could affect the balance of cholesterol metabolism in the chick. The objective of this experiment was therefore to study the cholesterol picture in vitamin B_6 adequate chicks subjected to similar growth and feed intake conditions as vitamin B_6 deficient ones.

Procedure

A total of thirty day-old male broiler chicks were used. They were all first grown on a vitamin B_6 adequate purified diet (Table 15) for a period of four weeks. This initial period was intended so that the chicks could withstand subsequent bleedings and also to reduce early mortality due to vitamin B_6 deficiency.

Table 15 - Composition of the diet used in experiment IV.

Ingredients	Percent
Dextrose	65.75
Soybean protein (Promine-R)	22.00
Corn oil	2.00
Alphacel	3.00
Mineral mixture*	5.30
Vitamin mixture**	1.00
Methionine	0.50
Glycine	0.20
Choline chloride	0.25
Vitamin B_6	4 mg/lb

* Same composition as in Table 1 page 22.
** Same composition as in Table 1 page 22.

At the end of four weeks, the chicks were divided into three groups of ten birds each. The groups were further divided into two replicates of five birds each. The replicates were then randomly housed in six compartments of a 4-deck growing battery.

Birds in the first treatment received a vitamin B_6 deficient purified diet. Feed and water were provided *ad libitum*. Birds in the second treatment received a vitamin B_6 adequate purified diet and were "pair-fed" with the vitamin B_6 deficient group. In order to equalize the feed consumption of this group with that of the vitamin B_6 deficient, a record of daily feed intake of the latter group was kept by weighing their feed every morning, thus calculating their feed consumption of the previous day. This amount of feed was then fed to the former group. Birds in the third treatment also received a vitamin B_6 adequate purified diet, but were "pair-weighed" with the vitamin B_6 deficient group. In order to equalize their rate of growth with that of the vitamin B_6 deficient group, only two thirds of the amount of feed consumed by the deficient birds was fed to them. This was purely an empirical method of regulating the growth, and as can be seen from the growth curves, worked out satisfactorily.

Birds receiving treatments two and three finished their daily feed within one hour and therefore remained off feed till the next morning. Birds receiving the vitamin

B_6 deficient diet engaged in severe cannibalism resulting in a total loss of four birds.

The birds in all the treatments were weighed weekly and blood samples for cholesterol determination were drawn from five birds in each treatment. Thus every bird was bled once every two weeks. This period gave them a chance to recover from the stress of bleeding. The experiment lasted a total of twelve weeks including the initial four weeks period.

Results

Growth: Data on body weight are presented in Table 16 and Figure 2. As can be observed from the graph, the vitamin B_6 deficient and the pair-weighed group were kept nearly equal in body weight throughout the experiment. The pair fed birds, however, grew to a greater extent even though their growth was much below the normal rate. The objectives of the experiment, i.e., simulating growth rate and feed consumption of vitamin B_6 deficient birds in vitamin B_6 adequate ones were thus accomplished.

Deficiency symptoms: Learning from the second experiment that vitamin B_6 deficiency symptoms develop very rapidly in very young chicks, the birds in this experiment were grown for a period of four weeks on a vitamin B_6 adequate diet before being put on a deficient diet. As was

Table 16 - Weekly body weights in grams - Experiment IV.

Weeks	Treatments		
	Vitamin B_6-deficient	Pair-fed	Pair-weighed
5	444*	443	445
6	554	615	554
7	590 (9)	660	581
8	600 (8)	684	584
9	580 (7)	698	606
10	598	717	613
11	611	738	625
12	598 (6)	698	599

* All values are averages of ten birds except those in which numbers are indicated between brackets.

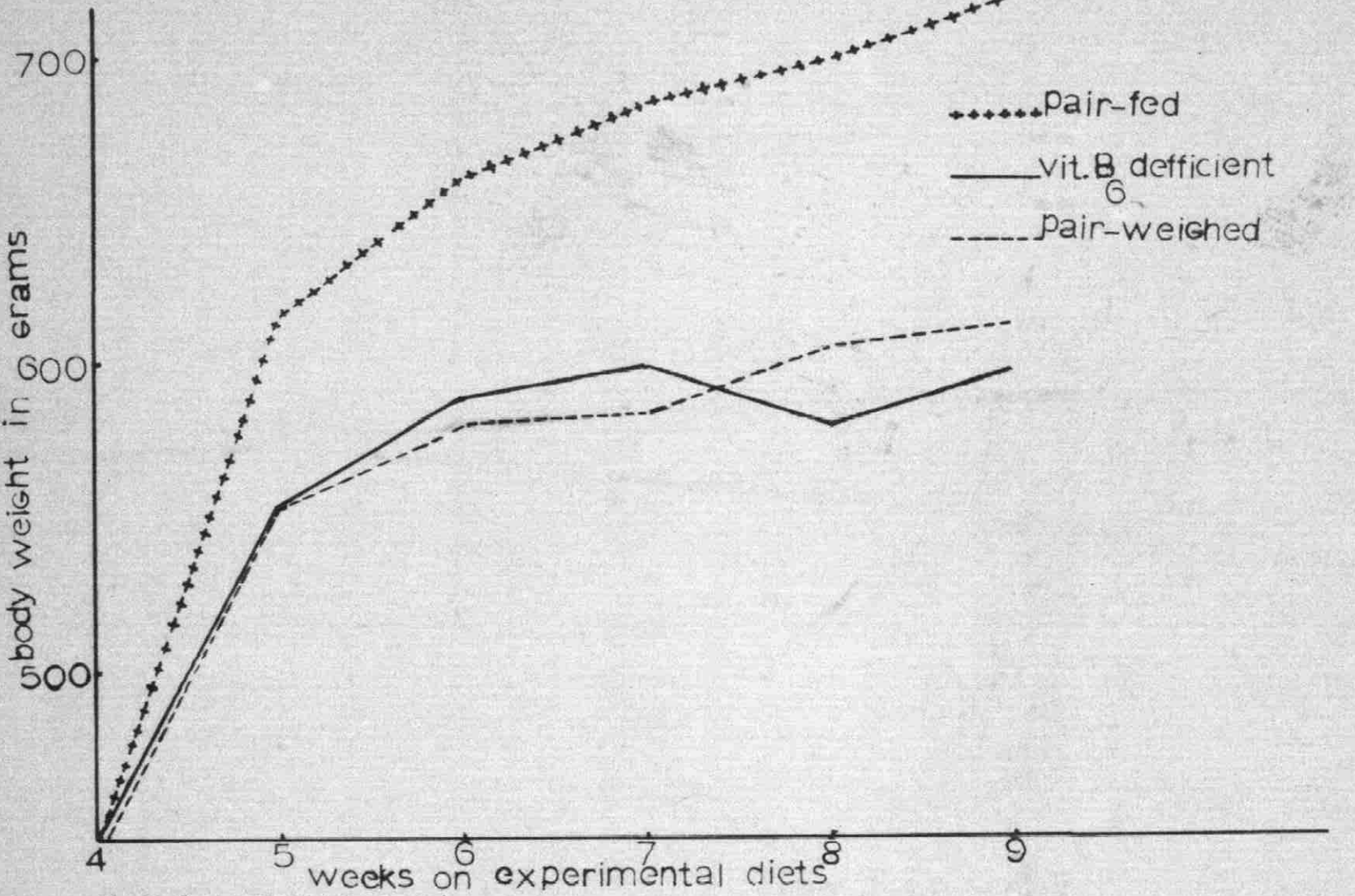

FIG. 2 WEEKLY BODY WEIGHTS OF THE CHICKS USED IN EXPERIMENT IV

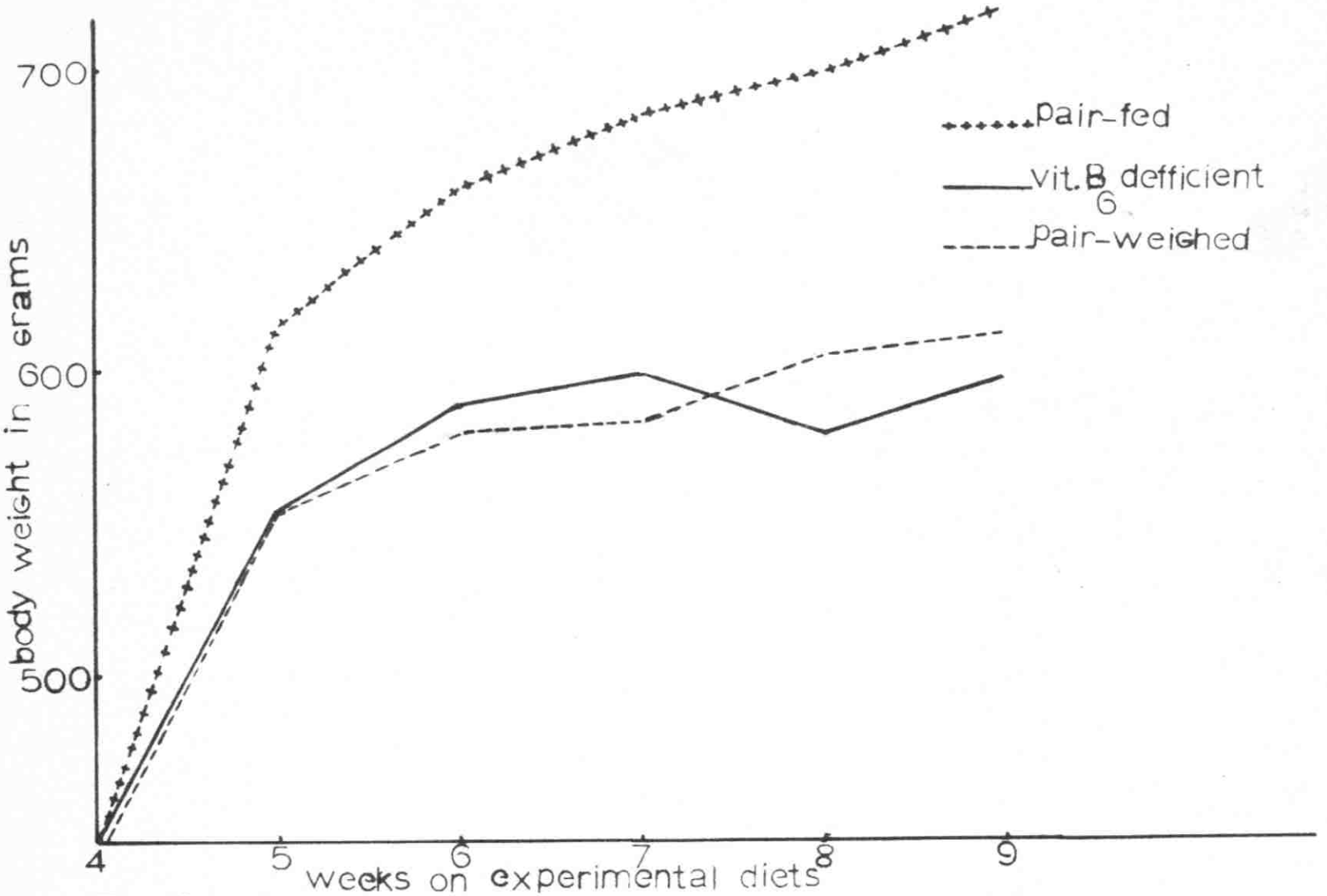

FIG.2 WEEKLY BODY WEIGHTS OF THE CHICKS USED IN EXPERIMENT IV

expected, deficiency symptoms became milder and were limited to a loss of appetite and unthriftiness with occasional convulsions. None of the deficient birds actually died from the vitamin B_6 deficiency. Loss of appetite occurred in a short time, almost within one week.

Serum cholesterol: Data on serum cholesterol are presented in Table 17 and Figure 3. The highest serum cholesterol values belong to the "pair weighed" vitamin B_6 adequate group and the lowest serum values belong to the vitamin B_6 deficient birds. The pair-fed group had serum values between the two. This indicates that it is not the vitamin B_6 deficiency *per se* that causes elevated serum cholesterol, but rather conditions caused by the deficiency, namely reduced feed consumption and growth. Statistical analysis showed no significant difference between the three treatments but it is the author's opinion that with larger numbers of birds and a more extensive study period the above trends could assume statistical significance.

Table 17 - Weekly serum cholesterol (mg %) - Experiment IV.

Weeks	Treatments		
	Vitamin B_6-deficient	Pair-fed	Pair-weighed
5	197 ± 6*	238 ± 9	218 ± 11
6	251 ± 12	238 ± 16	272 ± 12
7	285 ± 17	294 ± 6	311 ± 22
8	249 ± 17	271 ± 7	327 ± 12
9	286 ± 31	349 ± 27	302 ± 22
10	289 ± 18	295 ± 29	333 ± 34
11	280 ± 29	309 ± 5	302 ± 23
Average	262 ± 18	285 ± 14	295 ± 19

* Values are averages of 5 determinations ± standard error.

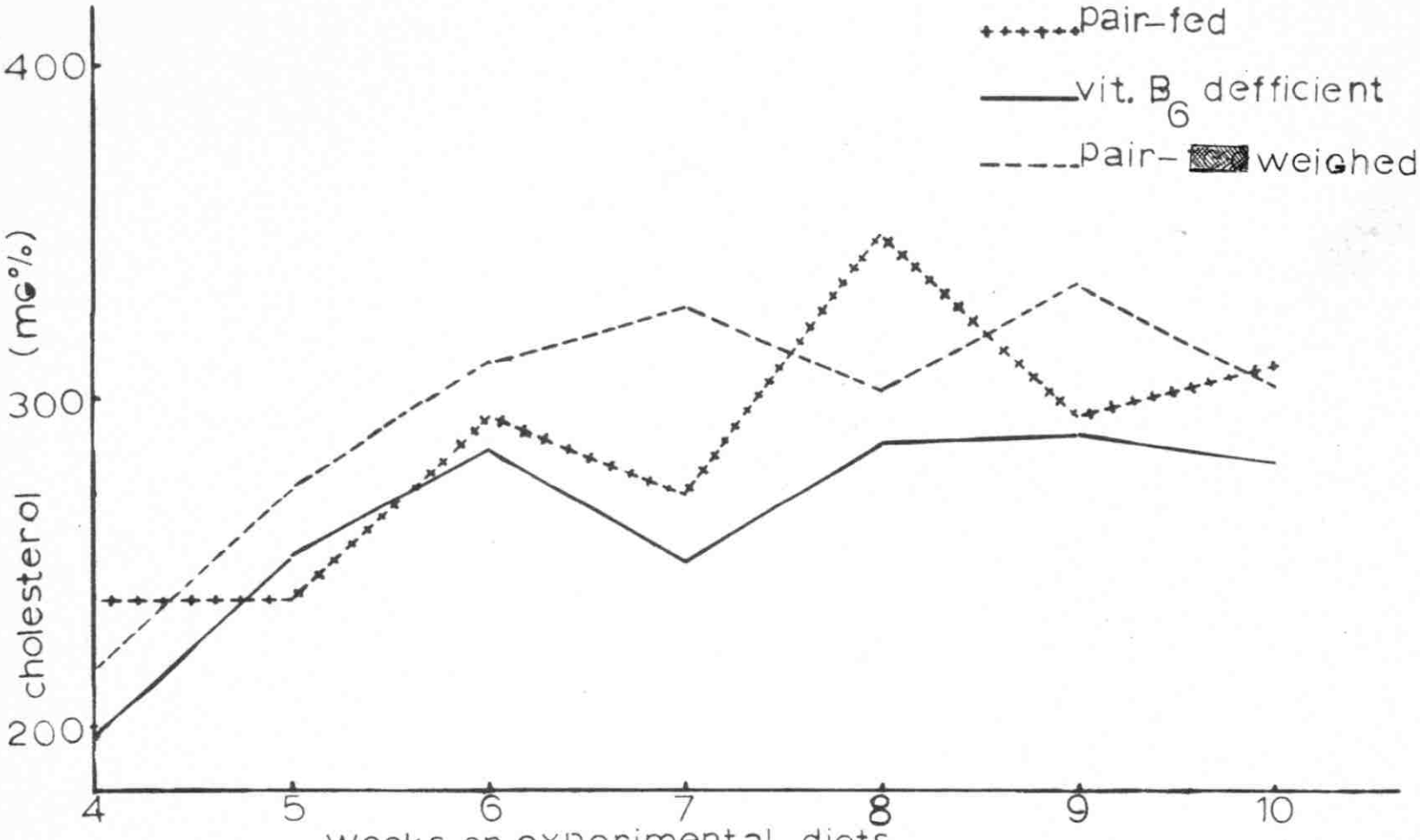

FIG. 3 WEEKLY SERUM CHOLESTEROL VALUES OF TREATMENTS USED IN EXPERIMENT IV

GENERAL DISCUSSION

Results of the first experiment indicate that, in the chick, cholesterol is adequately absorbed from a purified fat-free diet. Comparing serum cholesterol values from the two treatments in this experiment, it is observed that cholesterol when fed in either a fat-free or a fat adequate diet resulted in elevation of serum cholesterol. Normal serum cholesterol values in the chick range between 100-200 mg percent. The two treatments in this experiment resulted in cholesterol values between 200-300 mg percent, which are reasonably hypercholesterolemic.

That fat supplementation improves cholesterol absorption is both plausible and evidential. The work of March et al. (1964) in cockerels, indicating that cholesterol absorption is related to the efficiency with which dietary fat is absorbed is in line with the results of this experiment. Comparing the feces cholesterol values of the two treatments, it is observed that there is considerably less cholesterol absorbed from a fat-free diet than from a fat adequate one. In a purified fat-free diet, supplemented with 1 percent cholesterol, however, there is enough cholesterol absorption by the chick to induce hypercholesterolemia.

The results of the second experiment indicate that the growth rate of vitamin B_6 deficient chicks was significantly

lower than the vitamin B_6 adequate ones. Neither fat nor cholesterol supplementation had any significant effect on growth rate of the chicks. As was indicated in the first experiment, fat supplementation in general increased serum cholesterol. Partial answer to the question as to whether fat supplementation improves cholesterol absorption or itself causes increased serum cholesterol or both is provided by the results of this experiment. The fat supplemented diets resulted in higher serum cholesterol values when compared to diets which were fat-free, but otherwise identical. Diets adequate in both fat and cholesterol resulted in even higher serum cholesterol values than when each was fed separately. It is, therefore, apparent that fat supplementation both improves cholesterol absorption and itself is hypercholesterolemic. Adamson (1961) and March et al. (1964) have reported similar findings.

Vitamin B_6 deficiency slightly increased serum cholesterol. This effect, however, was not observed in the presence of fat. Dam et al. (1958) and Daghir and Balloun (1962) have both reported significant increases in the plasma and aorta cholesterol levels of vitamin B_6 deficient chicks. Whether vitamin B_6 deficiency significantly increases serum cholesterol has not been well established yet. Dietary cholesterol was the most significant agent in increasing serum cholesterol, among those studied. The increase, however, was not great enough to be considered severely hypercholes-

terolemic. Young chicks with a vigorous metabolism do not seem to be very favorable for the induction of hypercholesterolemia. Liver cholesterol values followed similar trends, but the effects were more pronounced. Dietary fat and cholesterol fed separately or in combination all caused a statistically significant increase in liver cholesterol.

Fatty acid composition of the abdominal fats from the different treatments in the second experiment indicate that no linoleic acid was recovered from the abdominal fats of the chicks receiving the fat-free diets. Under the conditions of the present experiment, conversion of linoleic acid into arachidonic acid apparently did not occur whether in the presence or absence of vitamin B_6. This is contrary to some reported literature. Witten and Holman (1952) reported that vitamin B_6 deficiency impairs the mechanism involving conversion of linoleic acid to a more unsaturated fatty acid and that this impairment can be corrected by the addition of pyridoxine to the diet.

The proportion of fatty acids in the fats remained fairly constant regardless of the dietary treatment, indicating that vitamin B_6 did not seem to play a role in conversion of linoleic acid into arachidonic acid. These findings are in conflict with

many reports. Dam et al. (1958) reported that pyridoxine deprived chicks, on a fat-free diet had more heart and liver arachidonate than pyridoxine supplemented ones. The fact that the chick does not synthesize linoleic acid precludes the conversion of this fatty acid into arachidonic acids, thus arachidonic acid seems to be independent of linoleic acid, but itself may be related to pyridoxine metabolism.

In the third experiment, egg production was lowered by feeding a diet containing 8 percent beef tallow, while birds on a 1 percent cholesterol supplemented diet maintained egg production and even produced more than the control group. Their serum cholesterol was also the highest. A theory may be advanced that egg production may stimulate cholesterol synthesis or conversely the supplementation of the diet with cholesterol may enhance egg production. Since serum cholesterol is only a reflection of "transport" of cholesterol from the site of synthesis (or absorption) to the ovaries and other organs one would expect higher serum cholesterol values in actively laying hens than in non-producing ones.

The graph in Figure 1 clearly shows that only dietary cholesterol resulted in hypercholesterolemia. Beef tallow when compared to the control did not cause any hypercholesterolemia. The fact that vitamin B_6 injections at fifty times the daily requirement were entirely ineffective in altering serum cholesterol is further reason to doubt any direct relationship between cholesterol and this vitamin. A more likely

possibility would be the indirect influence of vitamin B_6 "deficiency" on cholesterol metabolism.

In the fourth experiment, having respectively equalized growth and feed consumption of two groups of birds on a pyridoxine adequate diet with those of a pyridoxine deficient group, it was observed that it is the lower feed consumption and reduced body growth secondary to pyridoxine deficiency that are responsible for elevation of serum cholesterol. According to the results of this experiment, it is not the vitamin B_6 deficiency in itself, but rather conditions created by it, that lead to such elevated serum cholesterol values. This is probably due to an increased catabolic rate resulting from lack of growth and lowered feed consumption leading to excessive formation of 2 carbon units which may subsequently be transformed into cholesterol.

SUMMARY AND CONCLUSIONS

Four experiments were conducted to investigate possible relationships between vitamin B_6, cholesterol and fat. In the first experiment, to determine whether supplementary cholesterol is adequately absorbed from a fat-free diet, two groups of 10 day-old chicks were fed a purified diet containing 1 percent cholesterol and no fat and one containing 1 percent cholesterol and 4 percent corn oil. The chicks remained on their respective diets for a period of 2 weeks, at the end of which serum and feces cholesterol contents were determined.

In the second experiment, the three variables fat, pyridoxine and cholesterol were incorporated into eight different treatments, thus including all possible combinations. Ten, day-old male chicks were used per treatment and were kept on their respective diets for a period of 3 weeks. Criteria studied were body weight, serum cholesterol, liver cholesterol, liver lipids, and fatty acid composition of abdominal fat.

In the third experiment, the effect of feeding cholesterol at 1 percent of the diet or beef tallow at 8 percent of the diet on the development of hypercholesterolemia in the laying chicken, and the subsequent effect of vitamin B_6 injections on this hypercholesterolemia were investigated.

Ten layers were used per treatment and the experiment lasted for ten weeks. Changes in serum cholesterol were followed by weekly determinations and vitamin B_6 injections at the rate of fifty times the daily requirement were administered after the first five weeks.

The fourth experiment was designed to investigate whether vitamin B_6 deficiency directly influences serum cholesterol or rather conditions of this deficiency, namely, reduced feed intake and lower growth are responsible for changes in serum cholesterol. Three groups of 10 day-old chicks were first grown to four weeks of age on a nutritionally complete purified diet. At the end of four weeks the first group received a purified pyridoxine deficient diet and feed was provided ad libitum, the second group received a purified, pyridoxine adequate diet with feed consumption equalized to that of the first group, while the third group received a purified, pyridoxine adequate diet and feed consumption was so controlled as to equalize growth of this group with that of the first. Weekly serum cholesterol samples were collected for a period of 6 weeks.

In the growing chick, cholesterol was adequately absorbed from a purified fat-free diet. Fat improved cholesterol absorption. Dietary cholesterol caused a significant increase in serum and liver cholesterol regardless of of the presence or absence of vitamin B_6 in the diet. Vitamin B_6 deficiency however, irrespective of the presence

or absence of dietary cholesterol, mildly increased serum cholesterol, but its effect on liver cholesterol was variable. Chicks fed a fat-free diet did not synthesize linoleic acid in either the presence or absence of vitamin B_6. Conversion of linoleic acid into arachidonic acid was not observed in any of the treatments.

In the laying chicken, dietary cholesterol caused an immediate elevation of serum cholesterol, which was unaffected by vitamin B_6 injections. Beef tallow did not, in the course of 10 weeks, cause a significant increase in serum cholesterol when compared with the control. Here again, cholesterol level remained unaffected by vitamin B_6 injections.

The increased serum cholesterol in vitamin B_6 deficient chicks was not apparently due to the vitamin itself but rather as a result of conditions created by this deficiency. Vitamin B_6-adequate birds feeding or growing to the extent of vitamin B_6-deficient ones, developed even slightly higher serum cholesterol values than the deficient group.

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A P P E N D I X

Table 18 - Analysis of variance of body weights, serum cholesterol, liver cholesterol and fat - Experiment II.

Source of variation	d.f.	M.S.			
		4-week weights	Serum cholesterol	Liver cholesterol	Liver fat
Replication	1	1040	2575	0.7	2.6
Vitamin B_6	1	36960**	1008	47.0	76.5
Cholesterol	1	1040	35815*	942.5**	323.9*
Fat	1	3164*	25201	416.2**	327.5*
Vitamin B_6 x cholesterol	1	2115	28	26.5	70.6
Vitamin B_6 x fat	1	0	11006	30.7	59.4
Cholesterol x fat	1	2142	17227	388.1**	73.2
Vitamin B_6 x fat x cholesterol	1	730	1205	126.6	78.2
Error	7	474	5479	42.6	38.1

* Significant at 5% level of probability.

** Significant at 1% level of probability.

Table 19 - Analysis of variance of serum cholesterol values before and after vitamin B_6 injections - Experiment III.

Source of variation	d.f.	M.S.	
		Before injections	After injections
Treatments	2	37267**	40850**
Individuals	27	4829	5181

** Significant at 1% level of probability.

Table 20 - Separation of the means by Duncan's Multiple Range Test - Experiment III.

	Treatment means*		
	1% cholesterol	8% beef tallow	Control
Before B_6 injections	342	240	235
After B_6 injections	353	233	249

* Means not underlined by same continuous line are significantly different.

Table 21 - Analysis of variance of serum cholesterol values - Experiment IV.

Source of variation	d.f.	M.S.
Replications	4	234
Treatments	2	1232
Error	8	389