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CHARACTERISTICS OF THE RACHITOGENIC ACTIVITY IN ISOLATED SOYBEAN PROTEIN

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IN ISOLATED SOYBEAN PROTEIN

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

CHARACTERISTICS OF THE RACHITOGENIC ACTIVITY IN ISOLATED SOYBEAN PROTEIN

Abstract

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Under the supervision of Drs. C. W. Carlson and I. S. Palmer

The characteristics of the rachitogenic activity in isolated soybean protein were investigated. Day-old Broad Breasted White and/or Wrolstad Small White turkey poults were used. A purified glucose-isolated soybean protein diet was used. Levels of calcium, phosphorus and vitamin D_3 were equal to or greater than National Research Council recommendations. Criteria used for evaluation were body weight gains, tibia ash, serum alkaline phosphatase, serum inorganic phosphate and serum calcium.

Trays containing dry isolated soybean protein spread to a uniform depth of two and one-half centimeters were placed in an autoclave and brought to 100°C. by steaming and then to 120°C. for a designated period of time.

The poults were kept in electrically heated wire-floor batteries. Water and food were supplied ad libitum for the 4 week experimental periods.

When serum values were studied, blood was taken by heart puncture from a representative sample of birds (five when possible) from each group. The serum was removed, placed in vials and stored at -20°C.

Under the conditions of this investigation the following observations were made:

1. In general, poults fed untreated isolated soybean protein consistently exhibited rachitic symptoms, namely low tibia ash values and concomitant elevated serum alkaline phosphatase levels. The effects of the isolated soybean protein as measured by the other criteria were less consistent.

2. Subjecting isolated soybean protein to autoclaving destroyed its rachitogenic activity. Autoclaving proved to be the most effective way of reducing the rachitogenic property of isolated soybean protein.

3. Sixty minutes autoclaving time at 120°C. proved optimum in destroying the rachitogenic activity of isolated soybean protein.

4. Substituting dibasic calcium phosphate, hydrous, U.S.P., for dicalcium phosphate, feed grade, markedly reduced the rachitogenic activity of the isolated soybean protein.

5. The inclusion of vitamin D_3 levels four times the National Research Council recommendations was less effective than autoclaving in reducing the rachitogenic activity of isolated soybean protein.

6. It appears that the Broad Breasted White strain of poults is more susceptible to the onset of rickets as induced by untreated isolated soybean protein compared to the Wrolstad Small White strain.

7. Tibia ash gains should be studied when investigating the effect of ration treatments on tibia ash in order to take into consideration any initial tibia ash differences that might be present due to strain or nutritional effects.

8. The dilution of isolated soybean protein with autoclaved isolated soybean protein in the ratio of 2:2 was effective in reducing

the rachitogenic activity of isolated soybean protein, however, it required one part of untreated isolated soybean protein to three parts of autoclaved isolated soybean protein to overcome the growth depressing action of isolated soybean protein.

9. The rachitogenic effect of isolated soybean protein exerts its greatest effect on the birds between 2 and 4 weeks of age.

10. Two-way interrelationships existed between autoclaving × vitamin D_3, autoclaving × source of calcium and phosphate, autoclaving × strain and vitamin D_3 × strain; also there was a three-way interrelationship between autoclaving × source of calcium and phosphate × vitamin D_3 level.

11. The variability in the Ca^{45} experiment was great and consequently raises a question as to the reliability of the data. Nevertheless, the data suggest that feeding autoclaved isolated soybean protein like increasing vitamin D_3 does play a role in increasing calcium deposition by increasing calcium absorption and/or decreasing calcium secretion into the intestine.

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INTRODUCTION

Today, when the shortage of food is a serious problem in many of the densely populated areas of the world, increasing attention is being directed toward research involved in finding good sources of nutrients which are economically feasible to produce. For mainly economic reasons, animals as a possible source of protein do not seem to have as much appeal as plant sources. In this connection much attention has already been focused on soybean protein as a very valuable source of protein to supply human and animal needs.

However, Osborne and Mendel (1917) reported impaired growth in rats fed raw soybean meal and observed that dry heat had no significant effect on the nutritional value of the meal. Cooking in a steam bath for three hours, however, yielded a meal which promoted normal growth of rats. They concluded that the raw meal was not toxic since none of the rats on this dietary regimé died. They suggested that cooking improved palatability of the meal, thereby making it more acceptable to the rats. Similar growth depressing effects have been observed in other classes of animals fed soybean protein.

In spite of the extensive experimental investigations that have taken place with soybean protein, the complete nature of all the nutritional and antinutritional factors is not known. However, soybean hemagglutinins, soybean saponins and soybean isoflavones have been considered as factors which affect the nutritional value of soybean protein. Research has demonstrated that the activity of soybean trypsin inhibitors and soybean hemagglutinins can be destroyed by

moist heat, whereas the soybean saponins and soybean isoflavones are heat stable.

Several studies have demonstrated that isolated soybean protein contains an antinutritional property, namely, rachitogenic activity. In contrast to the antinutritive activity observed in isolated soybean protein, antirachitic activity has been reported in commercial soybean meal and extracts therefrom.

Definitions of isolated soybean protein, soybean meal and whey proteins may be helpful to show the relationships of these by-products of soybeans. Soybean meal is made from soybeans (plain or dehulled) after the oil has been extracted with hexane. The beans are then toasted, flaked and ground to yield soybean meal. Isolated soybean protein is obtained by acid precipitation of the protein from the water extract of the soybean meal. The liquid phase which results after the separation of the soybean protein contains the whey proteins.

At the outset of my program, this study was oriented toward the isolation and possible identification of the antirachitic factor(s) in soybean meal. The results of several studies were inconsistent. While trying to rectify the inconsistencies, it became evident that autoclaving isolated soybean protein beyond 30 minutes further destroyed its rachitogenic activity. Consequently, the prime interest in this study was directed from the antirachitic activity in soybean meal to the rachitogenic activity in isolated soybean protein.

Objectives of this study were to determine the optimum autoclaving time required to destroy the rachitogenic activity in

isolated soybean protein; to ascertain the relative effectiveness of vitamin D_3 and sources of calcium and phosphate in overcoming the rachitogenic activity in isolated soybean protein; to determine the effect of a wider range of vitamin D_3 levels than used previously; to evaluate the resistance of strains of experimental animals to the rachitogenic activity of isolated soybean protein; to ascertain whether there is a direct relationship between the rachitic condition of poults as measured by tibia ash with that as measured by serum alkaline phosphatase activity; and to ascertain by the use of Ca^{45} how autoclaved isolated soybean protein affects calcium metabolism.

Hopefully, the characterization of some of the rachitogenic properties of isolated soybean protein would provide beneficial information to nutritionists, which will assist them in making recommendations concerning its usefulness as a source of protein.

LITERATURE REVIEW

Antinutritional Factors in Soybeans

Soybean Trypsin Inhibitors. Bowman (1944) was one of the first to discover soybean trypsin inhibitor and recognized it as one of the antinutritional factors in soybeans. Kunitz (1945) reported the crystallization and characterization of soybean trypsin inhibitor. Although not soluble in water at its isoelectric point, trypsin inhibitor is soluble in soybean whey at pH 4.2 to 4.6. He reported that the crystalline inhibitor contains no sulfhydryl groups, but it is crosslinked by two cystine disulfide bonds. Steiner (1965) observed that the reduction of the two disulfide bonds inactivates the inhibitor, but activity is regained upon oxidation of the sulfhydryl groups. In addition, Kunitz (1947) showed that if the trypsin inhibitor is heated at 90°C. for 2 minutes it is completely inactivated. The activity gradually returns on cooling. The extent of reversibility decreases as the time of heating is increased.

Although crystalline soybean trypsin inhibitor has been widely studied and assumed to be homogeneous, recent studies of Wu and Scheraga (1962) and Eldridge et al. (1966) have indicated that is is a mixture of proteins. Eldridge et al. (1966) reported that polyacrylamide gel electrophoresis shows large variations among commercial preparations of the inhibitor; some preparations consist of a major protein component, plus several minor compounds, whereas other preparations approach the complexity of soybean whey protein. An

additional complication in elucidation of the role of trypsin inhibitor activity in soybeans is the occurrence of multiple forms of inhibitors. Bowman (1948) obtained, by fractional precipitation with organic solvents, three inhibitors, one of which was believed identical to the inhibitor isolated by Kunitz (1945). Rackis and Anderson (1964) reported four inhibitor fractions, while Stead et al. (1966) reported seven fractions separated from soybean whey proteins by diethylaminoethyl cellulose chromatography; Rackis and Anderson observed that one fraction had properties similar to the inhibitor of Kunitz (1945).

Some of the biological activities attributed to trypsin inhibitor are inhibition of trypsin in vitro, inhibition of trypsin in vivo, growth inhibition, pancreatic hypertrophy and pancreatic stimulation. However, the literature on this subject is voluminous and often controversial. Although in vivo inhibition of trypsin may occur, Westfall et al. (1948) suggested that it was doubtful that this factor is significant in the inhibition of the growth of animals. Nevertheless, preparations of crystalline trypsin inhibitor will inhibit growth of rats (Khayambashi and Lyman, 1966) and chicks (Garlich and Nesheim, 1966).

Soybean Hemagglutinins. Following the discovery of the soybean trypsin inhibitor by Bowman (1948), Liener and Pallansch (1952), and Pallansch and Liener (1953) isolated and characterized soybean hemagglutinin, a protein distinctly different from soybean trypsin inhibitor. This discovery exemplifies the complexity of the composition of soybean whey proteins. Investigators feel that further careful

fractionation and biological testing of purified whey fractions may reveal other proteins that affect the nutritive value of raw soybeans.

In contrast to crystalline trypsin inhibitor which is a mixture of proteins, hemagglutinin is an albumin. It also has a higher isoelectric point than the inhibitor. Birk and Gertler (1961) observed that because of these two properties, hemagglutinin occurs primarily in whey after soybean meal is fractionated. Other major differences between the two proteins are the higher molecular weight of the hemagglutinin, its multichain structure as indicated by the number of amino- and carboxyl-terminal residues and its content of mannose and glucosamine.

Rackis et al. (1959) and Stead et al. (1966) reported that multiforms of hemagglutinins were also isolated. Neither the significance nor origin of these multiple forms is as yet understood. In partial explanation of the multiple forms, Weil et al. (1966) demonstrated protease activity in soybeans and suggested that proteolysis of parent trypsin inhibitor and hemagglutinin molecules during islation procedures may be responsible for the multiple forms of the two proteins.

Liener (1951, 1953 and 1955) contributed most of the present knowledge about the biological properties of the hemagglutinins. Soybean hemagglutinins agglutinated red blood cells in vitro, were toxic when injected intraperitoneally and inhibited the growth of rats.

Soybean Saponins. Considerations of the antinutritional factors in soybeans are often extended to include saponins, but supporting evidence to substantiate these claims is rather sparse. Gestetner

et al. (1966b) reported that defatted soybean meal contains 0.6% saponins. These saponins are glycosides of five triterpenoid alcohols. Gestetner et al. (1966a) also observed that the sugars present in soybean saponins are galactose, glucose, rhamanose, xylose, arabinose and glucuronic acid.

Ishaaya and Birk (1965) obtained from defatted soybean meal a 0.4% yield of crude saponin mixture, and subsequently Nash et al. (1967) reported that the carbohydrate content of soybean saponins ranged from 24 to 27%. The carbohydrate content gives the saponins their polar character. Birk et al. (1963) studied the chemical properties of the soybean saponins and reported that they are soluble in water, soluble in 80% alcohol, surface active and heat stable. They also found that in contrast to the trypsin inhibitors and hemagglutinins, the saponins in soybean meal are stable to autoclaving. The earlier suggestion made by Potter and Kummerow in 1954 that cooking of meal caused hydrolysis of saponins appears incorrect.

The biological properties of the soybean saponins have been determined by Birk et al. (1963) and Ishaaya and Birk (1965). They found that the saponins hemolyzed red blood cells in vitro, inhibited the activity of trypsin, chymotrypsin, papain, cholinesterase and a protease enzyme of Tribolium costaneum. Although the hemolysis of red blood cells could serve as a useful tool for detecting soybean saponins, Birk et al. (1963) reported that apparently this phenomenon does not occur when soybean meal is ingested. Wolf (1966) mentioned other investigators' work with rats, chicks and mice which demonstrated

that the soybean saponins pass through the stomach and the small intestine without being hydrolyzed or absorbed. They are hydrolyzed by bacterial enzymes in the lower digestive tract, but again are not absorbed. Neither saponins nor sapogenins were detectable in blood of the test animals. It is conceivable that absorption of sapogenins in the upper digestive tract needs to be investigated since hydrolysis of saponins may occur during processing of fermented foods. High concentrations of soybean saponins will inhibit a number of enzymes, but this interaction appears to be nonspecific and similar to that observed between proteins and other surface-active materials, such as detergents. Ishaaya and Birk (1965) observed that the enzyme inhibitory effect can be overcome by casein and soybean proteins.

At present the stability of soybean saponins to autoclaving raises the question of their possible role in growth inhibition by soybean diets. In this connection, Wolf (1966), in a compiled report, mentioned that there was no growth impairment of chicks, rats or mice when soybean saponins were added to the diet. Many authorities seem to think the saponins may be able to be removed from the list of antinutritional factors in soybeans, since much evidence shows that the saponins do not inhibit growth.

Soybean Isoflavones. The isoflavones are another group of compounds in soybeans which must be considered in assessing the nutritive value of soybean products. Walz (1931) stated that only two isoflavones are believed to occur in soybeans, genistein and diadzein. A third isoflavone, 6,7,4'-trihydroxyisoflavone, was recently isolated

from tempeh, a fermented soybean food, but its occurrence in raw soybeans is still undetermined (Gyorgy et al., 1964). Walter (1941) reported the physical and chemical properties of the isoflavones. The isoflavones are glucosides, yellow in color, phenolic in character, insoluble in water and soluble in alcohol. The stability of the isoflavones to heat has not been completely resolved, as Walter isolated genistin from commercial defatted soybean meal which presumably had been heated; hence, it is assumed that genistin is stable to autoclaving. On the contrary, Alexander and Watson (1951) found that drying subterranean clover decreased its estrogenic activity caused by genistein and other isoflavones. The biological properties of genistein and genistin are estrogenic and growth inhibitory. These substances were found to induce increased uterine weight, vaginal cornification and infertility in rats.

Interest in the biological properties of isoflavones stems from the work of Bradbury and White (1951) who discovered that genistein is estrogenic and that it causes infertility in sheep ingesting it by grazing on pastures of subterranean clover. Although estrogenic, genistein has a low activity and it is reported to be only one-hundred-thousandth as active as the synthetic estrogen, diethylstilbestrol. In addition, Wong and Flux (1962) reported that diadzein, the other isoflavone in soybeans, is only one-fourth as active as genistein. In spite of their low estrogenic activities, soybean isoflavones may have physiological effects because of their concentrations in soybean meal. Walz (1931) reported 0.15% genistin and 0.007%

diadzein in defatted meal, whereas Walter (1941) found 0.1% genistin in similar defatted meal. Both estimates are based on yields of isolated isoflavones and are therefore likely to be low. Because of the higher concentration and estrogenic activity of genistein compared to diadzein, genistein is the isoflavone of greatest interest in soybeans.

Much has been reported concerning the physiological effects of genistein and genistin isolated from soybean meal, but little information is available about their effects when ingested at the levels occurring in meal and other soybean fractions. Experimental evidence presented by Carter et al. (1953) showed that commercial meal was estrogenic when fed to mice; nearly a threefold increase in uterine weight occurred. Meal from which genistin had been removed was inactive. No details were given about the composition of the diet or the amount of diet consumed. However, results appear consistent with data of Wong and Flux (1962) who found that mice receiving 5.9 mg. of genistein in 15 g. of diet over a period of 6 days showed a nearly twofold increase in uterine weight.

Pieterse and Andrews (1956) extracted soybean and cottonseed meals with 95% ethanol and incorporated the meal extractables into a ration at a level equivalent to feeding the meals as the sole source of the diet. Both meals gave positive estrogenic responses as measured by increase in uterine weights of mice. Data for feeding the alcoholic extracts at lower levels, however, were not reported.

Although no adverse effects concerning genistin and diadzein in

soybeans under practical conditions are reported in the literature, more research is needed before deciding whether they are completely innocuous at the levels commonly ingested.

Soybean Phytin. Phytic acid is another constituent that has been associated with antinutritional properties of soybeans. However, there does not seem to be sufficient evidence to demonstrate that phytic acid is deleterious at practical levels, except as a chelator of trace minerals.

As early as 1948, McCance and Walsham reported that large amounts of phytic acid included in human diets resulted in negative calcium balance. In these studies it was found that the phytic acid forms insoluble salts with calcium (phytin) in the intestine and thus the calcium was unavailable for absorption. Further work of Bonner et al. (1956) with adolescent boys demonstrated that Ca^{45} absorption was not affected by phytates in the diet when the test meal contained 80 mg. of phytic phosphorus and 239 mg. of calcium, an amount typical of that in the breakfast of many children in the United States. From these and other studies it was agreed that food phytic acid becomes a nutritional problem only under special conditions of very low calcium intake or excessive phytic acid intake or both.

In considering the effect of phytic acid at a practical level, O'Dell and Savage (1960) suggested that since soybean protein contains about 0.5% phytic acid phosphorus, perhaps it was exerting some influence in binding trace minerals. They also showed the zinc requirement for chicks to be greater with an isolated soybean protein

diet than with a casein-gelatin diet. But in addition, they suggested that phytic acid could be complexed with casein to increase the zinc requirement. A subsequent report by Likuski and Forbes (1964) has shown that phytic acid added to either an amino acid or casein diet greatly reduced growth by tying up the zinc. Ethylenediaminetetra-acetic acid (EDTA) at 223 p.p.m. largely overcame the growth retardation but only brought the zinc content of tibia ash part-way back to normal.

Effects of Additives on the Growth Depressing and/or Rachitogenic Activity of Isolated Soybean Protein

Many researchers have made several attempts to ascertain the cause and nature of the growth depressing and/or rachitogenic activity of C-1 protein. Various soybean preparations, chelating agents, high levels of vitamin D, minerals, other additives and autoclaving have been tried with C-1 protein in an attempt to reduce its deleterious effect. To date some treatments have been effective, however neither the mode of action nor the exact nature of this antinutritional factor(s) is known.

Various Soybean Preparations. Kratzer et al. (1964) working with a corn starch-C-1 protein diet studied the growth promoting activity in various fractions of soybean meal, namely, crude methanol extracts, ether insoluble fractions, water-acetic acid soluble and insoluble fractions, benzene soluble and insoluble fractions and some acidified fractions of soybean meal. These fractions were fed to turkey poults

in various quantities in order to determine their efficacy in overcoming the growth inhibitory nature of the C-1 protein. A portion of the growth promoting activity of the methanol extract of the soybean oil meal was found in the benzene soluble fraction of the material after acidification with acetic acid. This growth response was not altered by the addition of excessive vitamin D_3.

Griffith and Young (1966) also conducted similar studies. They reported that a water extract of raw soybean meal increased the growth of turkey poults when added to purified diets based on isolated soybean protein or casein-gelatin. The growth promoting factor could be extracted from heated soybean meal with methanol. This factor appeared to be partially dialyzable and insoluble in phenol. Extraction of the water extract with methanol brought about a concentration of the growth factor in the methanol-soluble portion. The factor appeared to be organic in nature, although a small response to the ash was obtained. Extraction of the methanol-soluble fraction with chloroform-methanol inactivated or destroyed the factor. It was also reported that the growth factor could be made unavailable to the poult by reacting it with acetic anhydride, however, it was reactivated by KOH hydrolysis.

Likewise, Wilcox et al. (1961a) presented evidence for a water-soluble growth promoting factor(s) in soybean oil meal. The increased growth appeared to be due to both organic and inorganic components. This active material was not extracted from soybean oil meal by 100% acetone or 100% ethanol. Wilcox et al. (1961b) furnished further

evidence to support the fact that water extract of soybean oil meal overcomes the growth inhibitory property of C-1 protein.

Chelating Agents. Much speculation has been made concerning the growth inhibitory action of C-1 protein. Several researchers have tried to determine which metabolic system is most seriously affected by the antinutritional property of the C-1 protein. In this regard, O'Dell and Savage (1957) first demonstrated a chelating-like action of the C-1 protein by showing that it interfered with trace mineral utilization, since the zinc requirement of chicks fed a C-1 protein semi-purified diet was greater than for chicks fed a diet containing casein and gelatin. Davis et al. (1962) using a similar basal diet showed that C-1 protein fed to chicks also interfered with the utilization of manganese and copper, as well as zinc. Also, Fitch et al. (1964) observed that absorption of Fe^{59} in monkeys was significantly lower in the presence of C-1 protein than in the presence of casein. Reid et al. (1956) demonstrated that C-1 protein interfered with molybdenum absorption in chicks and turkey poults.

Kratzer et al. (1959) investigated methods of overcoming the rachitogenic activity of C-1 protein. They reported that the zinc requirement of turkey poults fed a diet containing C-1 protein was reduced by adding ethylenediaminetetraacetic acid (EDTA) or by autoclaving the protein. Further extensive investigations on the effect of chelating agents on mineral metabolism in the presence of C-1 protein was conducted by Vohra and Kratzer (1964) with turkey poults. Their results show that several chelating agents were of no value.

These included aminonaphtholsulfonic acid, diaminocyclohexanetetra-acetic acid, dihydroxyethylglycine, dihydroxybenzenedisulfonic acid, ethylenediaminetetrapropionic acid, ethylenediaminetetraethanol, glutamic acid, ethylenediaminebitartrate, iminodiacetic acid, hydroxy-quinolinesulfonic acid, mercaptosuccinic acid, proline, thalidomide, triethylenetetraamine (TETA), terephthalic acid and thiodipropionic acid. They gave growth responses either poorer than the zinc-deficient basal diet or of the same order.

Compounds such as nitrilotriacetic acid, hydroxyethylethylene-diaminetriacetic acid, propylenediaminetetraacetic acid, dihydroxy-ethylethylenediaminediacetic acid, ethylenediamine-N,N'-diacetic acid-N,N'-dipropionic acid and ethylenediaminediacetic acid gave definite improvement in the growth of the poults. They observed that the relative growth for some of these compounds was of the same order as that observed for EDTA. A definite growth response but less than that for EDTA was obtained for ethylenebis (oxyethylenenitrilo) tetra-acetic acid (EBONTA), hydroxyethyliminodiacetic acid and diethylene-triaminepentaacetic acid. A high degree of mortality was observed for ethylenediamine (dihydroxyphenyl) acetic acid in a single experiment in which it was used and it is difficult to determine whether or not that response was meaningful.

In general, the growth response to the chelating agents appeared to be satisfactory over the stability constant range of 13 to 17 with an optimum at 14.5. Compounds like EBONTA and TETA were less effective for improving zinc availability than could be predicted on the

basis of their stability constants. The reason for this is not known, but may be attributed to a definite requirement for a certain size of the molecule, steric configuration or toxicity of these compounds.

Vohra and Kratzer (1964) explained that one mechanism by which a chelating agent might improve mineral availability depends upon the chelating agent having a stronger stability constant for the metal than the metal binding substance in the feed so that the metal is complexed with the chelating agent in the gastrointestinal tract. The metal chelate can then be absorbed if it is a relatively small molecule. Tracer studies by Darwish and Kratzer (1963) and Koike et al. (1963) indicated significant absorption of EDTA, Zn-EDTA and Ca-EDTA complexes from the gastrointestinal tract of chickens and hens. After absorption the metal might be available for specific body functions if it can be removed from the chelating agent. This means that the various systems in which the metal is required for proper functioning (e.g., enzyme) should have higher stability constants for the metal than the chelating agent with which it is absorbed.

Carlson et al. (1964b) reported that EDTA fed together with C-1 protein had no effect in alleviating the rachitogenic property of C-1 protein in one study. In another study, EDTA actually caused a reduction in calcification.

Vitamin D. The rachitogenic activity of C-1 protein can be partially overcome by high levels of vitamin D_3. The National Research Council (N.R.C.) (1960) reports that vitamin D_3 fed at the level of 900 I.C.U./kg. of diet should be adequate for 4-week old

turkey poults receiving a practical diet. Carlson et al. (1964a, 1964b) have reported that turkey poults fed a glucose-C-1 protein diet containing 900 I.C.U./kg. of diet exhibited symptoms of severe rickets, namely poor growth and low bone ash values. These investigations also revealed that poults which were fed diets containing levels of vitamin D_3 in excess of the N.R.C. recommendations showed a significant reduction in the incidence of rickets.

Jensen and Mraz (1966) reported that chicks fed a C-1 protein diet containing excessively high levels of vitamin D_3, 2000 I.C.U./kg. of diet, displayed evidence of reduced incidence of rickets. These chicks gained more weight and tibia ash and concomitantly showed higher levels of tibia Ca^{45} and P^{32} uptake compared with the control fed diets containing 200 I.C.U. of vitamin D_3/kg. of diet. In this connection Jensen and Mraz (1966) also stated that the rachitogenic effect of isolated soybean protein is probably a general phenomenon among monogastric animals as Miller et al. (1965) reported that the vitamin D_2 requirement of swine fed soy protein was greatly increased over that of swine receiving a casein diet.

The mechanism responsible for the role of vitamin D_3 in calcification under normal conditions is not known. Whether the mode of action of high levels of vitamin D_3 under rachitic conditions is merely due to mass action, is purely conjecture at this time. However, several scientists have put forward a number of theories in attempts to elucidate certain aspects of the vitamin D_3-calcification interrelationship.

Nicolaysen and Eeg-Larsen (1953) reported that the vitamin elevates serum calcium and phosphate by causing increased absorption of these ions from the intestine, and at the same time mineral mobilization from the bone takes place (Carlsson, 1952). Dowdle et al. (1960) and Schachter et al. (1961) attributed the former phenomenon, at least, to stimulated active transport of calcium, and Harrison and Harrison (1961) showed that calcium transport activates phosphate transport. Zull et al. (1966) mentioned in a report that vitamin D apparently prevents rickets by maintaining the serum (Ca^{++}) ($HPO_4^{=}$) product at a level which is supersaturated with respect to bone mineral.

Zull et al. (1966) investigated the relationship between vitamin D action and actinomycin-sensitive processes. They observed that the kinetic patterns of vitamin D-actinomycin interactions correspond with the concept that the action of the vitamin is to initiate events which lead to DNA-directed production of RNA. They thought that the significance of the mitochondrial and intestinal permeability responses to vitamin D was of some concern. Although the mitochondrial system is poorly understood (Engstrom and DeLuca, 1964), it seems likely that it reflects altered membrane permeability to calcium, thus completing the analogy to the intestinal mucosal permeability response. Zull et al. suggested earlier in 1965, that such permeability effects of the vitamin may be secondary, seen only when excessive or large amounts of vitamin D interact nonspecifically with membranes. However, the interaction is rather specific both for vitamin D and for calcium. Furthermore, it would be a striking coincidence to find a system which

relates vitamin D with membrane permeability to calcium, but which is completely unrelated to the fact that the vitamin is an essential agent in normal calcium transport across biological membranes.

On the strength of the foregoing evidence, Zull et al. (1966) attempted to integrate these permeability effects into a working hypothesis, although there is no direct evidence that vitamin D_3 does in fact interact with the nuclear membrane in the manner proposed. Taking into account the fact that the nucleus is already implicated in the action of vitamin D because of the actinomycin inhibition, it may be that the action of vitamin D is to interact with the nuclear membrane to change its permeability to calcium. According to this view, calcium itself then becomes the controlling agent for the synthesis of the parathyroid hormone-stimulated calcium transport enzyme proposed in their earlier paper (Zull et al., 1965).

This hypothesis allows us to consider the known permeability effects as models of vitamin D action on the membrane. The requirement of large amounts of vitamin D in these model systems might then be explained as due to lack of vitamin specific sites.

A further advantage of this new hypothesis is that it provides for feed-back control and metabolic economy, since synthesis of the transport enzyme is proposed to be sensitive to the calcium environment, rather than to the presence of vitamin D, per se. In fact, Kimberg et al. (1961) claimed that the transport of calcium by the intestine is known to be an adaptive function, being reduced in activity under conditions of high dietary calcium, and increased when

the supply of calcium is limited. Furthermore, this adaptive nature is lost in vitamin D deficiency.

Minerals. The influence of the dietary calcium and phosphate source has been observed to greatly affect the extent of rickets induced by C-1 protein.

Griffith and Young (1967) studied the effect of anhydrous dicalcium phosphate versus the hydrated form in turkey poults. The hydrated dicalcium phosphate induced more weight gains and bone ash, and simultaneously lowered mortality compared to those birds fed the anhydrous form. These observations are consistent with those of Gillis et al. (1962) who reported that phosphorus in anhydrous dicalcium phosphate is much less available to the turkey poult than that in the hydrated form.

Gillis et al. (1962) indicated that there are other factors which affect the utilization of the calcium and phosphate source. They report that for chicks and turkey poults, in general, the primary calcium phosphate salt is the most available, followed by the secondary, with the tertiary salt being the least available. Scott et al. (1956, 1962) also observed that the phosphorus in anhydrous dicalcium phosphate (reagent grade) was poorly utilized by the poult.

Factors to consider in assessing the value of possible calcium and phosphorus sources for turkey poults were investigated by Wilcox et al. (1954). They found that the phosphorus in monocalcium phosphate was well utilized by the poult and that the phosphorus from dicalcium phosphate (U.S.P. grade) was utilized to a lesser extent,

while the phosphorus in tricalcium phosphate (N.F. grade) and β-tricalcium phosphate was poorly utilized by turkey poults as compared with the other calcium orthophosphates.

Other Additives. Increasing the bulk in the diet and substitution for part of the C-1 protein have been effective in reducing the rachitogenic activity of C-1 protein. Griffith and Young (1967) studied the influence of fiber on the availability of phosphorus to turkey poults and reported that soybean hulls, oat hulls or alkali-treated soybean hulls were highly effective in improving the phosphorus availability when added to a purified C-1 protein diet. The responses were considerably larger than that from 0.15% additional phosphorus. The soybean hulls used in this experiment contained 0.12% phosphorus and when fed at the rate of 8% of the diet, supplied only 0.01% phosphorus.

Purified cellulose (Solka-Floc) was also fed to turkey poults at the rate of 8% of the diet. However, it was much less effective than the other sources of fiber in increasing bone ash of poults, but caused a significant improvement as compared with the bone ash of poults receiving the 1.6% calcium basal diet containing 3% cellulose. It appeared that all the ability of soybean hulls to improve phosphorus availability could not be accounted for by their cellulose content.

Jensen and Mraz (1966) studied the effect of a 40% C-1 protein diet fed to chicks. In these investigations they substituted 10, 20 and 30% of the C-1 protein with soybean meal and observed that all levels of substitution improved bone ash but only the 30% level also

improved growth. This evidence also suggests that soybean meal contains two factors, namely, a growth factor and an antirachitic factor. This observation seems consistent with that of Griffith and Young (1964) who have also presented evidence that soybean meal contains two factors for the turkey poults. One is a water-soluble factor which improves growth. The second factor remains with the insoluble residue and improves the biological value of phosphorus from anhydrous dicalcium phosphate. In this regard, Kratzer et al. (1964) reported that large amounts of vitamin D_3 did not alter the growth response of turkey poults obtained with a methanol extract of soybean meal, also indicating that factors in soybeans affecting growth and calcification were different. It also appears that simply reducing the level of isolated soybean protein in the diet markedly improved bone ash. This suggests that the antirachitic effect of soybean meal may be due in part to a reduction in the level of the isolated soybean protein. Likewise, Griffith et al. (1966) found that when 30 or 60% soybean meal was substituted isonitrogenously for isolated soy protein and starch, a growth response and increased bone ash were elicited.

Autoclaving. The subjection of C-1 protein to autoclaving has proven effective in destroying the rachitogenic property of C-1 protein. However, the mechanism involved in this heat treatment is not understood.

Carlson et al. (1964b) made an interesting observation concerning autoclaved soybean meal included in a diet for turkey poults. Autoclaving raw soybean meal or C-1 protein for 30 minutes at 120°C.

overcame the growth-depressing and rachitogenic effects of both the raw soybean meal and C-1 protein, whereas, increasing the vitamin D_3 supplementation tenfold only partially overcame these deleterious effects of the protein. These results demonstrated that the basal diet was rachitogenic, largely due to the high level of raw soybean protein.

Although it was shown that raw soybean meal possesses rachitogenic properties, C-1 protein appeared to be a much more potent source of the activity. This could be explained on the basis that the raw soybean meal contained both antirachitic and rachitogenic factors that to some extent masked each other, whereas the C-1 protein contained only the rachitogenic factor. Consequently, the rachitogenic factor appears to be labile to heat whereas the antirachitic factor is heat stable.

Some Characteristics of Rickets

Nicolaysen and Eeg-Larsen (1953) referred to rickets as a bone disease, which is characterized by lameness, swelling of the joints and bone deformities. Such deformities are caused by a disturbance of the utilization and retention of calcium and phosphorus salts (principally calcium phosphate) in the body, whereby these salts are either not deposited or they are deposited in insufficient amounts in the growing bone. Even with an abundance of calcium and phosphorus in the diet, the bird cannot make use of them unless vitamin D or ultraviolet radiation is also provided. A mineral deficiency obviously will

produce rickets. Unless minerals are present (and preferably in correct calcium-phosphorus balance), no minerals can be deposited; but even a large excess of calcium and phosphorus cannot be utilized by the bird without the involvement of vitamin D.

According to Ewing (1963), rickets may be a result of insufficient vitamin D, calcium or phosphorus or excess calcium. Tendency toward rickets is enhanced by coccidiosis infection, presumably by impairing the intestinal absorption of calcium.

Reddy and Srikantia (1967) reported that rachitic children exhibited high levels of serum alkaline phosphatase, decreased serum calcium and serum inorganic phosphate levels. Guyton (1966), reporting on calcium and phosphate in the blood of patients with rickets, stated that ordinarily the level of calcium in the blood of patients with rickets is only slightly depressed, but the level of phosphate is greatly depressed. The parathyroid glands prevent the calcium level from falling by promoting bone resorption every time the calcium level begins to fall. On the other hand, there is no good regulatory system for controlling a falling level of phosphate, and increased parathyroid activity enhances the excretion of phosphates in the urine.

Serum alkaline phosphatase may be used in conjunction with other criteria as a diagnostic means of determining the onset of rickets. This is advisable, as Guyton points out that although serum alkaline phosphatase activity is always elevated in rachitic conditions, this phenomenon is also associated with several other metabolic disorders. Such conditions exist following major bone fractures, in

any bone disease that causes bone destruction where repair is by osteoblastic activity similar to rickets, in osteomalacia and in osteitis fibrosa cystica.

EXPERIMENTAL METHODS

Originally the objective of this investigation was to isolate and possibly identify the antirachitic factor(s) or property of soybean meal. As a result, water extracts were obtained from soybean meal (50% protein) which was defatted by the Cargill industrial hexane solvent extraction method. Water extracts were prepared from 225 kg. of soybean meal in the modified method employed by Wilcox et al. (1961a). A 15.52% yield of dried water extract was obtained by this method. However, the inconsistent results from the initial studies with the water extract demonstrated the need to try and control results between experiments. Because of the experimental inconsistencies, it was deemed important to formulate a diet which could be used as a control and still produce 65 to 75% livability and also be marginal enough to produce significant responses to rather slight nutritional improvements, for example, the factors reported present in small percentages of the water extract of soybean meal. While attempting to effect this dietary improvement, the C-1 protein was inadvertently allowed to be autoclaved for 40 minutes, instead of 30 minutes. The results indicated that 40 minutes autoclaving appeared more effective in reducing the rachitogenic activity of the C-1 protein compared to 30 minutes autoclaving.

Consequently, the main interest in this investigation was diverted to further studies concerning aspects of autoclaving and other treatments in reducing the rachitogenic activity of C-1 protein. A complete analysis of the C-1 protein (Skidmore) is shown in

Appendix A.

In the subsequent investigations with turkey poults, the diets contained 44% C-1 protein as shown in Table 1. However, in one experiment a practical turkey starter of the formulation shown in Table 2 was also used. The levels of calcium, phosphorus and vitamin D_3 were higher than the National Research Council recommendations (1960). Dry C-1 protein was autoclaved by placing 5-kg. batches in enamel trays and spreading them to a uniform depth of 2.6 cm. The trays were then placed in an autoclave and brought to 100°C. by steaming and then to 120°C. for the designated period of time.

Male day-old Wrolstad Small White (W.S.W.) and /or Broad Breasted White (B.B.W.) poults were kept in electrically heated wire-floor batteries. Water and feed were supplied ad libitum for the 4-week experimental periods; during these periods it was desirable to collect data at 2 weeks of age, in some experiments. Each pen contained 9, 10 or 11 poults and 3 pens were allotted to each ration treatment. The only exception to this rule was experiment 6b, where effects on individual birds were studied and 4 replicates were used to test the effect of the oral administration of Ca^{45}, while 5 replicates were used to test the effect of the intravenous administration of Ca^{45}.

Experiments 1, 2, 3, 5 and 6b were conducted as random complete block designs with factorial arrangements, while experiments 4 and 6a were conducted as random complete block design studies.

The criteria used in this study were body weight gains, tibia ash, serum alkaline phosphatase activity, serum inorganic phosphate,

TABLE 1. COMPOSITION OF BASAL DIET

Ingredient	Amount %
Isolated Soybean Protein[1]	44.0
Glucose Monohydrate[2]	42.0
Cellulose[3]	3.0
Corn Oil	2.0
Source of Calcium and Phosphate[4]	4.0
Limestone	1.5
dl-Methionine	0.7
Glycine	0.5
Minerals[5], Vitamins[6], Antibiotic[7] and Antioxidant[8]	2.3

[1]Assay Protein C-1 (90% protein), Skidmore Enterprises, Cincinnati, Ohio.

[2]Cerelose, Corn Products Company, Argo, Illinois.

[3]Solka Floc, Brown Company, Berlin, New Hampshire.

[4]Dicalcium phosphate, feed grade, Central States Phosphate, Inc., Weeping Water, Nebraska, used in experiments 1-3 and 5, while dibasic calcium phosphate, hydrous, U.S.P., was used in experiments 4-6b.

[5]Added minerals in mg./kg. of diet were: NaCl, 4994.0; KCl, 4994.0; $MgSO_4 \cdot 7H_2O$, 3014.0; $MnSO_4 \cdot H_2O$, 198.0; $FeSO_4 \cdot 7H_2O$, 308.0; $CuSO_4 \cdot 5H_2O$, 8.8; $ZnSO_4 \cdot H_2O$, 308.0; KI, 11.0; $Na_2MoO_4 \cdot 2H_2O$, 2.2; Na_2SeO_3, 4.4; H_3BO_3, 11.0; $AlK(SO_4)_2 \cdot 12H_2O$, 123.2; Na_2SiO_3, 50.6; NaBr, 2.2; $CoCl_2 \cdot 6H_2O$, 22.0.

[6]Added vitamins in mg./kg. of diet (except where stated otherwise) were: folic acid, 8.8; pyridoxine, 22.0; riboflavin, 22.0; thiamine, 22.0; calcium pantothenate, 44.0; niacin, 99.0; choline (25% in wheat middling carrier), 14,080.0; p-aminobenzoic acid, 110.0; ascorbic acid, 22.0; biotin, 440.0 µg./kg.; vitamin B_{12} (1 mg./gm.), 33.0; vitamin E (275,000 I.C.U. dl-α-tocopheryl acetate/kg.), 35.0; vitamin A (325,000 I.C.U./gm.), 51.2; menadione sodium bisulfite (11.44 mg./kg.), 22.0.

[7]Oxytetracycline, 22.0 mg./kg.

[8]Ethoxyquin, 110.0 mg./kg.

TABLE 2. COMPOSITION OF A PRACTICAL TURKEY STARTER DIET

Ingredient	Amount
	%
Ground Yellow Corn	40.2
Soybean Meal (50% protein)	42.0
Alfalfa Meal (17% protein)	2.0
Dried Whey	2.0
Fish Meal	2.0
Dicalcium Phosphate	2.0
Limestone	2.0
Dried Brewer's Yeast	1.0
dl-Methionine	0.1
Lysine (50% concentrate)	0.2
Yellow Grease	5.0
Salt[1]	0.5
Vitamins, antioxidant and antibiotic[2]	1.0

[1]Salt contained in gm./kg. of salt mix: Mn, 2.25; Zn, 2.50; I, 0.049; Co, 0.049; Cu, 0.249; Fe, 0.849; S, 1.49; NaCl, 484.4.

[2]Vitamins, antioxidant and antibiotic were added in the following amounts/kg. of diet: vitamin A, 10,560 I.C.U.; vitamin D_3, 2,750 I.C.U.; vitamin E, 44 I.C.U.; menadione (sodium bisulfite), 2.2 mg.; riboflavin, 8.8 mg.; pantothenic acid, 17.6 mg.; niacin, 88.0 mg.; choline, 880 mg.; cobalamine (B_{12}), 17.6 µg.; folic acid, 2,200 µg.; biotin, 220 µg.; ethoxyquin, 220 mg.; Terramycin, 22 mg.

serum calcium (total Ca and Ca^{45}) and fecal Ca^{45}. Body weights were determined and averaged initially for each group of birds, similar measurements were taken for each succeeding week to the termination of each experiment. Weight gains were then computed from these data.

Tibia ash was determined by a modified A.O.A.C. (1960) method. This method suggests that the bone be crushed during extraction, but instead they were subjected to the extraction procedure as complete tibias.

All serum determinations were made from blood taken at the termination of each study. Blood was taken by heart puncture from a representative sample of birds from each group. Five birds were used whenever this was possible. The blood was pooled and kept on ice until centrifuged. After centrifugation the serum was removed, placed in vials and stored at $-20^{\circ}C$. Serum alkaline phosphatase was determined by the method of Bodansky (1932, 1933). Serum inorganic phosphate was determined by the Fiske and Subbarow (1925) method, while serum calcium was measured by the use of a Perkin-Elmer 303 atomic absorption spectrophotometer.

In the study, Ca^{45} was administered orally and intravenously on the basis of 0.1 ml./100 gm. and 0.05 ml./100 gm. body weight, respectively. The stock solutions of Ca^{45} for oral administration contained 250 μc./1.0 ml., whereas the stock solution of Ca^{45} for intravenous administration contained 500 μc./1.0 ml. These solutions were prepared from 0.256 ml. of 0.5 N hydrochloride solution containing 7.69 mc./mg. Ca. The above dilutions were made to reduce the

volume of the intravenous administration to one half that of the oral administration and still maintain the same dosage of Ca^{45}/100 gm. body weight. Blood samples were taken from the brachial veins of each bird at the following periods after administration: 20, 40, 60 and 1860 minutes. At the termination of this experiment total fecal collections were made from each bird. Fecal calcium was then taken up into solution according to the procedure outlined in the A.O.A.C. (1960) method. Also tibia ash determinations were made as mentioned above, then the ash was dissolved in hydrochloric acid in accordance with the above method prescribed for calcium determination. All Ca^{45} activity was determined with the aid of a Packard Model 3002 Tri-Carb Liquid Scintillation Spectrometer. Samples were counted in a hyamine-toluene system similar to that mentioned by Herberg (1960).

All data were subjected to analysis of variance, F, L.S.D. (experiments 1-3) and Dunnett's (experiments 4 and 6a) tests using the procedures cited by Snedecor (1956).

RESULTS

Experiment 1

The first investigation, experiment 1, was designed to ascertain the effect of autoclaving and vitamin D_3 levels with respect to reducing the rachitogenic activity of C-1 protein. Consequently, four levels of vitamin D_3, namely 880, 1760, 3520 and 7040 I.C.U./kg. of diet were fed with untreated C-1 protein or C-1 protein autoclaved for 40 minutes.

The results of experiment 1 are presented in Tables 3 and 4. They show that body weight gains, serum calcium and serum inorganic phosphate levels were not affected by either the level of vitamin D_3 or by use of autoclaved C-1 protein in the diet.

Increasing the vitamin D_3 level did not significantly alter tibia ash, whereas autoclaving the C-1 protein for 40 minutes significantly ($P < 0.05$) increased tibia ash. There was a significant ($P < 0.01$) interaction between vitamin D_3 and autoclaving. The data suggest that the interaction was apparently due to the greater magnitude of response by poults fed autoclaved C-1 protein to the higher vitamin D_3 levels. Changes in vitamin D_3 levels for poults on untreated C-1 protein gave inconsistent results in tibia ash.

Serum alkaline phosphatase activity was significantly ($P < 0.01$) reduced by increasing the vitamin D_3 level from 880 to 1760 I.C.U./kg. of diet. Increasing the vitamin D_3 level above 1760 I.C.U./kg. of diet did not cause any further change in alkaline phosphatase activity.

TABLE 3. THE INFLUENCE OF AUTOCLAVING (40 MIN.) AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

Vitamin D_3 Level	Body Weight Gain			Tibia Ash			Serum Alkaline Phosphatase Activity			Serum Inorganic Phosphate			Serum Calcium		
	Autoclaving[1] Time			Autoclaving Time			Autoclaving Time			Autoclaving Time			Autoclaving Time		
	0 min.	40 min.	Mean[2]	0 min.	40 min.	Mean	0 min.	40 min.	Mean	0 min.	40 min.	Mean	0 min.	40 min.	Mean
I.C.U./kg. of diet	gm.	gm.		%	%		Bodansky unit[3]			mg./100 ml.			mg./100 ml.		
880	262[4]	230	246	34.5	35.4	35.0	190	208	199[a]	5.6	6.0	5.8	10.2	10.0	10.1
1760	258	250	254	32.7	40.6	36.7	156	100	128[b]	4.9	5.6	5.3	9.9	9.3	9.6
3520	210	214	212	31.5	40.5	36.0	153	70	111[b]	5.5	6.5	6.0	10.6	9.5	10.1
7040	236	233	235	35.2	41.3	38.3	125	108	117[b]	6.4	7.8	7.1	9.7	10.2	10.0
Mean[5]	242	232		33.5	39.5*		156	122*		5.6	6.5		10.1	9.7	

[1]Autoclaved at 120°C.

[2]Means in the same column bearing different superscript letters are significantly different at $P < 0.01$.

[3]One Bodansky unit = 1 mg. inorganic phosphate released/100 ml. serum/1 hr. incubation at 37°C.

[4]Mean of 3 pens/treatment.

[5]Means under each criterion bearing 1 asterisk are significantly different at $P < 0.05$.

TABLE 4. ANALYSIS OF VARIANCE FOR THE INFLUENCE OF AUTOCLAVING (40 MIN.) AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

		Mean Squares				
Source of Variation	Degrees of Freedom	Body Wt. Gains	Tibia Ash	Serum Alkaline Phosphatase Activity	Inorganic Phosphate	Serum Calcium
Blocks	2	602.32	6.16	1,331.88	1.29	0.25
Autoclaving[1]	1	541.50	212.95*	7,336.01*	4.84	0.48
Autoclaving × Replicate	2	3,552.14	3.46	102.03	1.38	0.01
Vitamin D_3 Level	3	2,037.57	11.03	10,022.94*	3.42	0.20
Vitamin D_3 Level × Replicate	6	1,078.45	2.92	1,082.88	1.08	0.22
Vitamin D_3 Level × Autoclaving	3	360.88	19.16**	2,935.17*	0.26	0.46
Residual	6	1,354.73	1.31	357.29	0.87	0.49
Total	23					

[1]Autoclaving refers to untreated or autoclaved isolated soybean protein.

*Mean square significant at $P < 0.05$.

**Mean square significant at $P < 0.01$.

The data in Tables 3 and 4 also show that including autoclaved C-1 protein in the diet significantly ($P < 0.05$) lowered serum alkaline phosphatase activity. As with tibia ash values there was a significant ($P < 0.05$) interaction between autoclaving and vitamin D_3 level. This also was apparently due to the greater response to changes in vitamin D_3 level exhibited by the poults on autoclaved C-1 protein.

Experiment 2

In experiment 2, autoclaving time was increased to 60 minutes to determine whether further destruction of the rachitogenic properties of C-1 protein could be obtained, since 40 minutes autoclaving time proved effective in destroying rachitogenic properties of the protein. This experiment was similar to the first one except that only three vitamin D_3 levels were used, namely 880, 1760 and 3520 I.C.U./kg. of diet and the autoclaving time was increased to 60 minutes.

The data for experiment 2 are presented in Tables 5 and 6. As in the first experiment, serum inorganic phosphate was not significantly altered by autoclaving C-1 protein nor by increasing the vitamin D_3 level. In contrast with the first experiment, serum calcium was significantly ($P < 0.05$) increased as a result of autoclaving the C-1 protein, although increasing vitamin D_3 levels did not markedly alter serum calcium levels. Body weight gains were significantly increased by vitamin D_3 and by autoclaving at the $P < 0.01$ level. Serum alkaline phosphatase was significantly lowered by each increment of vitamin D_3 and by autoclaving, at $P < 0.05$ and $P < 0.01$ levels, respectively. Tibia ash values were also significantly ($P < 0.05$)

TABLE 5. THE INFLUENCE OF AUTOCLAVING (60 MIN.) AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

Vitamin D_3 Level	Body Weight Gain			Tibia Ash			Serum Alkaline Phosphatase Activity			Serum Inorganic Phosphate			Serum Calcium		
	Autoclaving[1] Time			Autoclaving Time			Autoclaving Time			Autoclaving Time			Autoclaving Time		
	0 min.	60 min.	Mean[2]	0 min.	60 min.	Mean	0 min.	60 min.	Mean	0 min.	60 min.	Mean	0 min.	60 min.	Mean
I.C.U./kg. of diet	gm.	gm.		%	%		Bodansky unit[3]			mg./100 ml.			mg./100 ml.		
880	149[4]	354	252[a]	27.8	44.5	36.2[a]	237	73	155[a]	3.8	5.5	4.7	9.7	11.2	10.5
1760	257	401	329[b]	34.8	47.5	41.2[b]	157	39	98[b]	5.4	5.9	5.7	9.5	11.6	10.6
3520	328	433	381[c]	39.2	47.6	43.4[c]	101	62	81[c]	5.2	6.5	5.9	10.2	12.0	11.1
Mean[5,6]	254	396**		33.9	46.5*		165	58**		4.8	5.9		9.8	11.6*	

[1]Autoclaved at 120°C.

[2]Means in the same column bearing superscript letters b and c are significantly different from the control, a, at $P < 0.05$ and $P < 0.01$, respectively.

[3]One Bodansky unit = 1 mg. inorganic phosphate released/100 ml. serum/1 hr. incubation at 37°C.

[4]Mean of 3 pens/treatment.

[5]Means under each criterion bearing 1 asterisk are significantly different at $P < 0.05$.

[6]Means under each criterion bearing 2 asterisks are significantly different at $P < 0.01$.

TABLE 6. ANALYSIS OF VARIANCE FOR THE INFLUENCE OF AUTOCLAVING (60 MIN.) AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

		Mean Squares				
Source of Variation	Degrees of Freedom	Body Wt. Gains	Tibia Ash	Serum Alkaline Phosphatase Activity	Inorganic Phosphate	Serum Calcium
Blocks	2	3,666.57	4.29	123.58	2.42	0.58
Vitamin D_3 Level	2	25,074.64**	82.06*	8,948.87*	2.61	0.73
Vitamin D_3 Level × Replicate	4	800.58	4.84	110.12	1.32	0.21
Autoclaving[1]	1	102,212.27**	712.28*	51,541.90**	5.88	15.35*
Autoclaving × Replicate	2	756.33	8.53	421.47	1.12	0.55
Vitamin D_3 Level × Autoclaving	2	3,863.94	25.88*	6,010.44*	0.53	0.11
Residual	4	1,852.45	2.25	390.79	0.96	1.18
Total	17					

[1]Autoclaving refers to untreated or autoclaved isolated soybean protein.

*Mean square significant at $P < 0.05$.

**Mean square significant at $P < 0.01$.

increased by each increment of vitamin D_3 and by autoclaving the C-1 protein. Statistical analysis of the data revealed that for tibia ash and serum alkaline phosphatase activity there was a significant ($P < 0.05$) interaction between autoclaving and level of vitamin D_3. For both criteria little additional response with poults receiving the autoclaved protein was obtained from the highest level of vitamin D_3, namely 3520 I.C.U./kg. of diet, whereas all vitamin additions produced considerable response in poults fed the untreated protein.

Experiment 3

Experiment 3 was undertaken mainly to test the effects of increasing autoclaving time by 20 minute intervals up to 60 minutes. In this investigation, two levels of vitamin D_3, namely 1760 and 3520 I.C.U./kg. of diet were fed with untreated C-1 protein or C-1 protein autoclaved for 20, 40 or 60 minutes. Figures 1, 2, 3, and 4 illustrate the beneficial effects gained from autoclaving the C-1 protein and increasing the level of vitamin D_3 in the diet.

Tables 7 and 8 show the data for this experiment. Again, serum inorganic phosphate was not affected by either autoclaving time or increasing vitamin D_3 levels. Increasing vitamin D_3 levels did not affect the overall serum calcium means, but there was a significant ($P < 0.05$) interaction between autoclaving and vitamin D_3. The poults on the lower vitamin level, namely 1760 I.C.U./kg. of diet, exhibited the greater response to autoclaving.

Although weight gains, tibia ash and serum calcium values

1760-D_3
AUTOCLAVED-0 MINS.

3520-D_3
AUTOCLAVED-0 MINS.

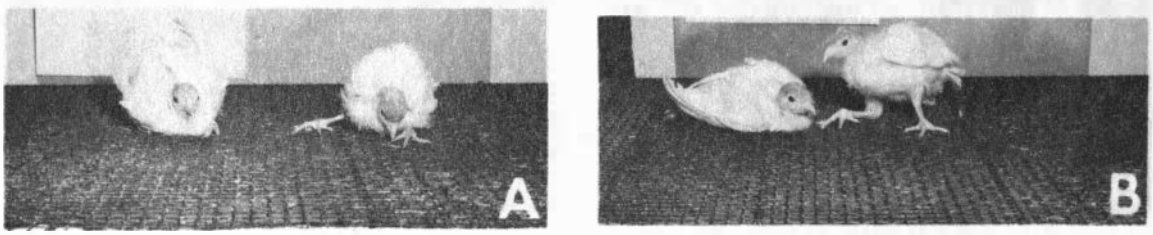

Figure 1. Poults showing the effect of vitamin D_3 levels and unautoclaved isolated soybean protein. A. 1760 I.C.U. of vitamin D_3/kg. of diet - small body size, poor feather condition and severe leg weakness. B. 3520 I.C.U. of vitamin D_3/kg. of diet - slightly larger body size, better feather condition and indication of leg strength.

1760-D_3
AUTOCLAVED-20 MINS.

3520-D_3
AUTOCLAVED-20 MINS.

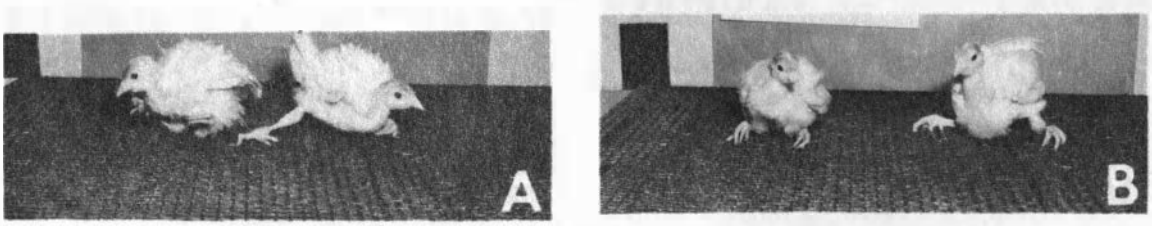

Figure 2. Poults showing the influence of vitamin D_3 levels and isolated soybean protein autoclaved for 20 minutes. A. 1760 I.C.U. of vitamin D_3/kg. of diet - small body size, poor feather condition and weak legs. B. 3520 I.C.U. of vitamin D_3/kg. of diet - slightly improved feather condition and body size.

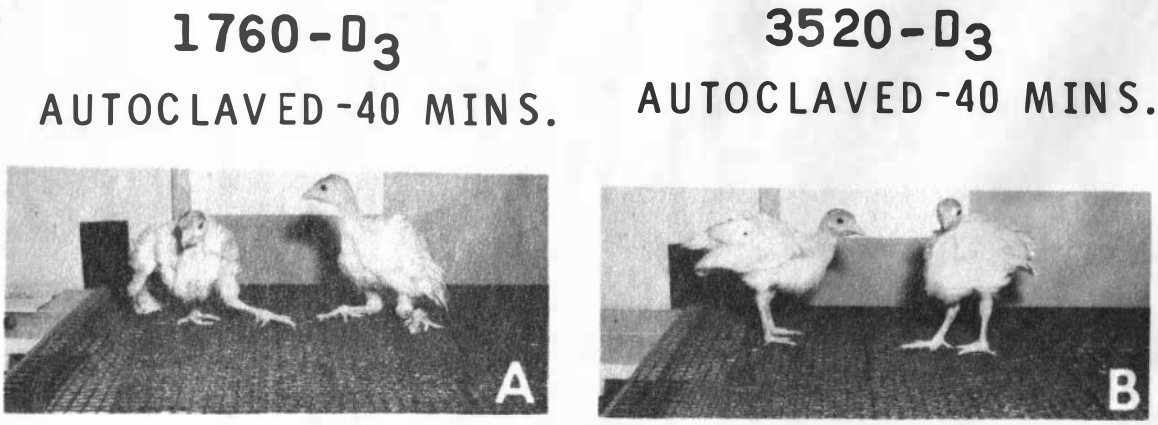

Figure 3. Poults showing the effect of vitamin D_3 levels and isolated soybean protein autoclaved for 40 minutes. A. 1760 I.C.U. of vitamin D_3/kg. of diet - signs of leg weakness but improved body size compared to birds in Figures 1 and 2. B. 3520 I.C.U. of vitamin D_3/kg. of diet - no signs of leg weakness and larger body size compared to birds in A.

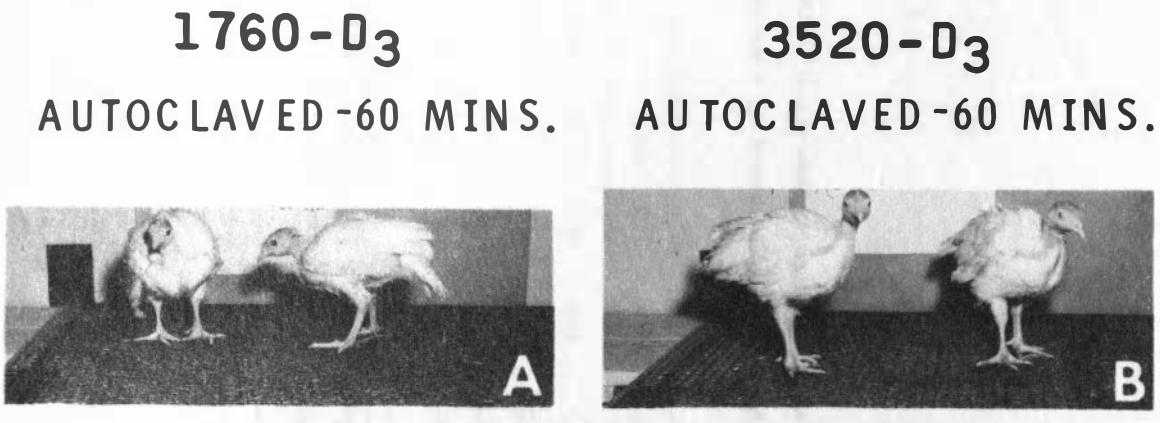

Figure 4. Poults showing the influence of vitamin D_3 levels and isolated soybean protein autoclaved for 60 minutes. A. 1760 I.C.U. of vitamin D_3/kg. of diet - no visual signs of leg weakness, body size greater than that of poults represented in Figures 1, 2 or 3. B. 3520 I.C.U. of vitamin D_3/kg. of diet - strong legs and larger body size compared to birds in A.

TABLE 7. THE EFFECT OF VARYING AUTOCLAVING TIME AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

Autoclaving[1] Time	Body Weight Gain			Tibia Ash			Serum Alkaline Phosphatase Activity			Serum Inorganic Phosphate			Serum Calcium		
	Vitamin D_3, I.C.U./kg.			Vitamin D_3, I.C.U./kg.			Vitamin D_3, I.C.U./kg.			Vitamin D_3, I.C.U./kg.			Vitamin D_3, I.C.U./kg.		
	1760	3520	Mean[2]	1760	3520	Mean	1760	3520	Mean	1760	3520	Mean	1760	3520	Mean
min.	gm.	gm.		%	%		Bodansky unit[3]			mg./100 ml.			mg./100 ml.		
0	181[4]	209	195[a]	29.6	30.7	30.2[a]	163	159	161[a]	7.2	5.0	6.0	8.0	9.1	8.6[a]
20	281	218	250	32.0	32.3	32.2	153	123	135	4.2	4.7	4.5	9.6	9.1	9.4
40	318	312	315[b]	31.1	35.0	33.1[b]	131	113	122	4.9	4.9	4.9	10.1	10.5	10.3[c]
60	309	412	361[b]	39.4	40.0	40.0[c]	96	99	98[c]	4.6	4.5	4.5	10.8	10.6	10.7[c]
Mean	272	287		33.0	35.0		136	124		5.2	4.8		9.6	9.8	

[1]Autoclaved at 120°C.

[2]Means in the same column bearing superscript letters b and c are significantly different from the control, a, at $P < 0.05$ and $P < 0.01$, respectively.

[3]One Bodansky unit = 1 mg. inorganic phosphate released/100 ml. serum/1 hr. incubation at 37°C.

[4]Mean of 3 pens/treatment.

TABLE 8. ANALYSIS OF VARIANCE FOR THE EFFECT OF VARYING TIME AND VITAMIN D_3 LEVELS ON THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

Source of Variation	Degrees of Freedom	Mean Squares				
		Body Wt. Gains	Tibia Ash	Serum Alkaline Phosphatase Activity	Inorganic Phosphate	Serum Calcium
Blocks	2	4,866.79	8.32	381.71	0.44	0.21
Vitamin D_3 Level	1	1,577.88	14.21	955.08	1.20	0.21
Vitamin D_3 Level × Replicate	2	887.49	11.40	225.89	0.70	0.45
Autoclaving[1]	3	33,310.59*	103.83**	4,350.20*	3.36	5.89**
Autoclaving × Replicate	6	6,943.77	3.42	795.80	1.07	0.25
Vitamin D_3 Level × Autoclaving	3	6,001.96	3.34	369.47	1.94	0.73*
Residual	6	4,484.47	8.45	508.27	0.52	0.12
Total	23					

[1]Autoclaving refers to untreated or autoclaved isolated soybean protein.

*Mean square significant at $P < 0.05$.

**Mean square significant at $P < 0.01$.

appeared to be affected by feeding C-1 protein which had been autoclaved for 20 minutes, the first statistically significant ($P < 0.05$) response was produced by feeding C-1 protein which had been autoclaved for 40 minutes. Increasing the autoclaving time to 60 minutes increased the response in each of the criteria although the further changes were not statistically significant in all cases. There was a consistent decrease in alkaline phosphatase activity as the time of autoclaving the C-1 protein was increased; however, 60 minutes of autoclaving was required to give the first statistically significant ($P < 0.01$) response.

Experiment 4

Since previous studies indicated a progressive beneficial effect from increasing autoclaving time, it was deemed necessary to ascertain the optimum autoclaving time with respect to reducing the rachitogenic activity of C-1 protein. In experiment 4, 1760 I.C.U. of vitamin D_3/kg. of diet was fed with untreated C-1 protein or C-1 protein autoclaved for 60, 80, 120, 180 or 240 minutes.

The 2 and 4 week data for this experiment are shown in Table 9. In general, at 2 and 4 weeks of age, body weights were not affected by autoclaving the C-1 protein for periods of 80 minutes or less. At 2 weeks of age, autoclaving the protein for 180 and 240 minutes significantly ($P < 0.01$) lowered body weight gains compared with the control (60 minutes). This same trend was evident at 4 weeks.

At both 2 and 4 weeks, poults fed untreated C-1 protein exhibited

TABLE 9. DETERMINATION OF THE OPTIMUM AUTOCLAVING TIME REQUIRED TO DESTROY THE RACHITOGENIC PROPERTY OF C-1 PROTEIN

Autoclaving[1] Time	Mean[2] Body Weight Gain		Tibia Ash		Serum Alkaline Phosphatase Activity		Serum Inorganic Phosphate		Serum Calcium	
	2 week	4 week	2 week	4 week	2 week	4 week	2 week	4 week	2 week	4 week
min.	gm.		%		Bodansky unit[3]		mg./100 ml.		mg./100 ml.	
0	123[4]	542	41.0[b]	44.2[c]	79.1[c]	90.4[c]	5.4[b]	4.4[c]	11.9	11.5
60	112[a]	535[a]	43.1[a]	48.4[a]	58.5[a]	32.3[a]	8.2[a]	9.1[a]	12.5	12.3
80	117	538	43.9	49.4	55.2	47.0	8.1	10.7	11.5	12.3
120	97	483	44.3	49.0	51.8	44.3	8.1	8.7	12.1	12.5
180	60[c]	355[c]	43.5	48.9	46.0	50.7	7.0	8.9	11.4	11.5
240	55[c]	306[c]	43.5	49.2	42.6[b]	48.9	7.2	9.4	11.5	11.2

[1]Autoclaved at 120°C.

[2]Means in the same column bearing superscript letters b and c are significantly different from the control, a, at $P < 0.05$ and $P < 0.01$, respectively.

[3]One Bodansky unit = 1 mg. inorganic phosphate released/100 ml. serum/1 hr. incubation at 37°C.

[4]Mean of 3 pens/treatment.

significantly lower tibia ash than the controls. The level of significance increased with age, that is, from $P < 0.05$ at 2 weeks to $P < 0.01$ at 4 weeks. At 2 and 4 weeks, autoclaving, per se, did little to alter the tibia ash values between birds fed these treatments.

At 2 weeks, serum alkaline phosphatase activity decreased progressively as autoclaving time increased. Poults receiving C-1 protein autoclaved for 240 minutes exhibited a significantly ($P < 0.05$) lower serum alkaline activity, while those fed untreated C-1 protein exhibited the highest ($P < 0.01$) activity compared to the control. However, at 4 weeks only the untreated group was significantly different from the control group. For the first time in these investigations serum inorganic phosphate was significantly altered by ration treatments. At 2 weeks, poults receiving the untreated C-1 protein showed a significantly ($P < 0.05$) lower serum inorganic phosphate level compared to the control, while at 4 weeks, the same trend was evident, but at a higher level of significance ($P < 0.01$). Serum calcium was not significantly affected by autoclaving.

Experiment 5

The main objective of experiment 5 was to establish whether or not there was an interrelationship between the sources of calcium and phosphate, autoclaving, vitamin D_3 and the strains of poults in relation to the reduction of the rachitogenic property of the protein. This experiment was conducted as a $2 \times 2 \times 2 \times 2$ factorial design involving two sources of calcium and phosphate (dibasic calcium

phosphate, hydrous, U.S.P. and commercial dicalcium phosphate, feed grade), two vitamin D_3 levels (1760 and 3520 I.C.U./kg. of diet), two strains of turkey poults (B.B.W. and W.S.W.) fed with two types of protein (untreated C-1 protein and C-1 protein autoclaved for 80 minutes).

The data presented in Tables 10 and 11 show the influence of the various treatments on body weight gains at 2 and 4 week periods. Results of the statistical analysis of data are shown in Table 12. For easy comparison, derived Tables 1, 2 and 3 in Appendix B each show the effects of two factors, only, on body weight gains of the poults at both 2 and 4 weeks of age. The data show that at 2 weeks of age there was a slight improvement in body weight of the poults receiving the dibasic calcium phosphate, hydrous, U.S.P., as compared to those fed the commercial dicalcium phosphate, feed grade. This difference was not statistically significant. However, at 4 weeks of age this difference between the two calcium and phosphate sources increased to the $P < 0.01$ level of significance. A similar trend was also observed with respect to the efficacy of autoclaving. At 2 weeks, there was little difference in body weight gains when either the untreated C-1 protein or the autoclaved C-1 protein was fed. However, at 4 weeks, autoclaving the C-1 protein significantly increased ($P < 0.05$) body weight gains.

Although the main effects of source of calcium and phosphate and autoclaving did not significantly alter body weight gains at 2 weeks, a significant interaction ($P < 0.05$) was observed between source of

TABLE 10. THE EFFECT OF SOURCE OF CALCIUM AND PHOSPHATE, AUTOCLAVING, STRAIN AND VITAMIN D_3 LEVEL ON BODY WEIGHT GAINS AT 2 WEEKS

Variable Constituent	Strain				
	Broad Breasted White		Wrolstad Small White		
	Untreated C-1 Protein	Autoclaved[1] C-1 Protein	Untreated C-1 Protein	Autoclaved C-1 Protein	Mean
	gm.	gm.	gm.	gm.	
880 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	123[2]	151	133	131	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	148	169	154	121	
Mean	136[3]	160	144	128	142
1760 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	142	187	127	128	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	178	179	157	122	
Mean	160	183	142	125	153*
Mean	160*		135		

[1]Autoclaved for 80 min. at 120°C.

[2]Mean of 3 pens/treatment.

[3]Mean of 6 pens/treatment.

*Mean square significant at $P < 0.05$.

TABLE 11. THE EFFECT OF SOURCE OF CALCIUM AND PHOSPHATE, AUTOCLAVING, STRAIN AND VITAMIN D_3 LEVEL ON BODY WEIGHT GAINS AT 4 WEEKS

Variable Constituent	Strain				
	Broad Breasted White		Wrolstad Small White		
	Untreated C-1 Protein	Autoclaved[1] C-1 Protein	Untreated C-1 Protein	Autoclaved C-1 Protein	Mean
	gm.	gm.	gm.	gm.	
880 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	170[2]	307	251	324	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	197	415	349	334	
Mean	184[3]	361	300	329	294
1760 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	211	494	280	394	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	463	497	429	353	
Mean	337	495	354	374	390*
Mean	344		339		

[1]Autoclaved for 80 min. at 120°C.

[2]Mean of 3 pens/treatment.

[3]Mean of 6 pens/treatment.

*Mean square significant at $P < 0.05$.

TABLE 12. ANALYSIS OF VARIANCE FOR THE INFLUENCE OF SOURCE OF CALCIUM AND PHOSPHATE, AUTOCLAVING, STRAIN AND VITAMIN D_3 LEVEL ON BODY WEIGHT GAINS AND TIBIA ASH

		Mean Squares		
		2 Week	4 Week	
Source of Variation	df	Body Wt. Gains	Body Wt. Gains	Tibia Ash
Blocks	2	78.65	1,292.65	4.29
V[1]	1	1,409.42*	111,988.38*	330.49*
R[2] × V	2	36.12	2,385.05	4.36
Ca & PO_4[3]	1	2,067.49	68,818.88**	424.06**
R × Ca & PO_4	2	134.43	668.38	1.58
V × Ca & PO_4	1	0.73	2,734.61	1.33
R × V × Ca & PO_4	2	44.08	1,913.96	6.69
A[4]	1	212.94	110,716.84*	1,070.40**
R × A	2	40.37	2,997.94	2.97
V × A	1	2.21	621.36	0.004
R × V × A	2	650.05	1,854.21	2.72
Ca & PO_4 × A	1	2,795.33*	37,280.03	37.83*
R × Ca & PO_4 × A	2	71.11	4,152.58	1.55
V × Ca & PO_4 × A	1	329.18*	35,278.78*	19.62
R × V × Ca & PO_4 × A	2	6.68	494.91	11.75
S[5]	1	6,167.60*	313.65	177.06*
R × S	2	82.97	291.13	3.30
V × S	1	2,042.33	26,616.21*	3.66
R × V × S	2	196.71	1,144.66	5.01
Ca & PO_4 × S	1	274.09	5,652.85	0.66
R × Ca & PO_4 × S	2	89.73	2,497.84	0.49

TABLE 12 CONTINUED

Source of Variation	df	Mean Squares 2 Week Body Wt. Gains	4 Week Body Wt. Gains	4 Week Tibia Ash
V × Ca & PO_4 × S	1	134.33	2,707.50	1.42
R × V × Ca & PO_4 × S	2	440.45	3,115.85	10.29
A × S	1	5,110.88*	61,970.63*	0.38
R × A × S	2	171.37	3,209.99	7.84
V × A × S	1	6.83	63.71	0.03
R × V × A × S	2	89.99	2,205.24	1.00
Ca & PO_4 × A × S	1	74.75	2,286.66	3.05
R × Ca & PO_4 × A × S	2	302.42	2,939.70	10.03
V × Ca & PO_4 × A × S	1	246.16	9,744.15	9.32
R × V × Ca & PO_4 × A × S	2	484.51	1,520.40	1.03
Total	47			

[1]Vitamin D_3 levels were 880 and 1760 I.C.U./kg. of diet.

[2]Replicates were 3 pens/ration treatment.

[3]Calcium and phosphate sources were commercial dicalcium phosphate, feed grade, and $CaHPO_4 \cdot 2H_2O$, U.S.P.

[4]Autoclaving refers to untreated C-1 protein and C-1 protein autoclaved for 80 min. at 120°C.

[5]Strain refers to Broad Breasted White and Wrolstad Small White.

*Mean square significant at $P < 0.05$.

**Mean square significant at $P < 0.01$.

calcium and phosphate and autoclaving. This interaction was attributable to the fact that the commercial dicalcium phosphate in combination with autoclaved C-1 protein induced greater weight gains when it was fed together with the untreated C-1 protein, whereas the situation was reversed when dibasic calcium phosphate was substituted for commercial dicalcium phosphate. At 2 weeks, the main effects show that B.B.W. strain of poults gained significantly more ($P < 0.05$) body weight than the W.S.W. strain. However, at 4 weeks, this difference was not apparent. A significant interaction ($P < 0.05$) was observed between autoclaving and strain at 2 and 4 weeks. At 2 weeks the interaction was attributable to the difference in magnitude of response between the strains at the 0 and 80 minute levels of autoclaving, while at 4 weeks the interaction was due to the difference in direction of response with the 0 and 80 minute levels of autoclaving.

Increasing the vitamin D_3 level exerted much influence on the poults. At 2 and 4 weeks, doubling the vitamin D_3 level significantly increased ($P < 0.05$) body weight gains. At 4 weeks there was a significant interaction ($P < 0.05$) between vitamin D_3 level and strain. This interaction was due to a difference in direction of response of the strains at vitamin D_3 levels of 880 and 1760 I.C.U./kg. of diet.

Tables 12 and 13 show the statistical analysis and the complete effects, respectively, of the tibia ash data. In addition, derived Tables 4 and 5 in Appendix B each illustrates the effects of two factors only on tibia ash values of the poults at both 2 and 4 weeks of age. All single treatment effects significantly altered tibia ash

TABLE 13. THE INFLUENCE OF SOURCE OF CALCIUM AND PHOSPHATE, AUTOCLAVING, STRAIN AND VITAMIN D_3 LEVEL ON TIBIA ASH

Variable Constituent	Strain				
	Broad Breasted White		Wrolstad Small White		
	Untreated C-1 Protein	Autoclaved[1] C-1 Protein	Untreated C-1 Protein	Autoclaved C-1 Protein	Mean
	%	%	%	%	
880 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	26.1[2]	34.8	29.2	40.3	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	30.7	41.2	36.8	44.1	
Mean	28.4[3]	38.0	33.0	42.2	35.4
1760 I.C.U. Vitamin D_3/kg. of diet					
4% Commercial Dicalcium Phosphate, feed grade	29.1	42.1	33.8	45.4	
4% $CaHPO_4 \cdot 2H_2O$, U.S.P.	39.4	45.5	41.8	48.5	
Mean	34.3	43.8	37.8	47.0	40.7
Mean	36.2		40.0*		

[1]Autoclaved for 80 min. at 120°C.

[2]Mean of 3 pens/treatment.

[3]Mean of 6 pens/treatment.

*Mean square significant at $P < 0.05$.

values. The substitution of dibasic calcium phosphate for commercial dicalcium phosphate, as well as autoclaving the C-1 protein, significantly increased ($P < 0.01$) tibia ash values. A significant interaction ($P < 0.05$) resulted due to the difference in magnitude of response to autoclaving with two sources of calcium and phosphate. The substitution of W.S.W. for the B.B.W. strain, as well as increasing the vitamin D_3 level, significantly increased ($P < 0.05$) tibia ash values. It is of interest to point out that the pooled initial tibia ash values of the B.B.W. and W.S.W. poults were 32.6 and 36.6%, respectively. This initial strain relationship regarding calcification was similar to that observed at 4 weeks, namely 36.2 and 40.0%, respectively.

Experiment 6a

Experiment 6a was designed to determine the diluting effect of autoclaved C-1 protein when fed with the untreated protein. Various proportions of the C-1 protein autoclaved for 60 minutes were used: 4:0, 3:1, 2:2, 1:3 and 0:4. A practical turkey starter of the formulation shown in Table 2 was also fed separately in this study.

The data shown in Table 14 indicate the effect of varying ratios of untreated C-1 protein to autoclaved C-1 protein on body weight gains, tibia ash and serum calcium of the turkey poults. Data at 2 weeks of age indicated that poults fed autoclaved C-1 protein as their main source of dietary protein, gained significantly more ($P < 0.01$) body weight than those poults fed untreated C-1 protein. Poults fed

TABLE 14. THE DILUTING EFFECT OF AUTOCLAVED ISOLATED SOYBEAN (C-1) PROTEIN ON THE RACHITOGENIC ACTIVITY OF UNTREATED C-1 PROTEIN

Ratio of Untreated C-1 Protein : Autoclaved[1] C-1 Protein	2 Week Data[2]	4 Week Data		
	Body Wt. Gains	Body Wt. Gains	Tibia Ash	Serum Calcium
	gm.	gm.	%	mg./100 ml.
4 : 0	107[c3]	241[c]	28.6[c]	9.8
3 : 1	128	289[b]	32.6[b]	10.9
2 : 2	132	295[b]	35.3	11.1
1 : 3	125	348	37.9	10.7
0 : 4	147[a]	405[a]	38.3[a]	11.0[a]
Practical Turkey Starter	146	464	36.6	13.0[b]

[1]Autoclaved for 80 min. at 120°C.

[2]Data in the same column bearing superscript letters b and c are significantly different from the control, a, at $P < 0.05$ and $P < 0.01$, respectively.

[3]Mean of 3 pens/treatment.

diets containing a mixture of untreated C-1 protein and autoclaved C-1 protein exhibited differences in body weight gains, however, these differences were not significant. The efficacy of the practical turkey starter with respect to body weight gains proved equal to that of the autoclaved C-1 protein.

At 4 weeks, both body weight gains and tibia ash were influenced similarly by the ration treatments. A progressive reduction in both criteria was observed as the ratio of untreated C-1 protein to autoclaved C-1 protein increased from 0:4 to 4:0. However, poults fed protein ratios of 2:2 and 3:1 grew significantly less ($P < 0.05$) than the control group which was fed a diet containing protein in the ratio of 0:4, and the magnitude of difference was further increased by feeding the protein ratio of 4:0. Unlike body weight gains, the tibia ash of poults fed protein in the ratio of 2:2 were not significantly different from that of the control. Like body weight gains, tibia ash of poults fed proteins in the ratios of 3:1 and 4:0 were less than the control at the $P < 0.05$ and $P < 0.01$ level of significance, respectively.

Serum calcium was not significantly altered by varying the proportions of untreated C-1 protein in the diet.

At 4 weeks, poults fed the practical turkey starter did not grow more rapidly nor show higher tibia ash compared to the control. However, poults fed the starter diet did exhibit significantly higher ($P < 0.05$) serum calcium compared to the control.

Experiment 6b

The last study, experiment 6b, was an attempt to learn more about the effect of autoclaving on the absorption and secretion of Ca^{45} in turkey poults. Ca^{45} was administered orally to study the absorption of Ca^{45} while the isotope was given intravenously to study the secretion of Ca^{45} into the gut. It was conducted as a 2 × 2 factorial with 4-week old birds obtained from experiment 6a. The two factors were: (1) method of administration (oral and intravenous) and (2) treatment of protein (unautoclaved C-1 protein and C-1 protein autoclaved for 60 minutes).

Table 15 shows the effect of the route of Ca^{45} administration and dietary C-1 protein (untreated or autoclaved) on the Ca^{45} activity in ash, serum and excreta of turkey poults. There was wide variation between replicates, consequently, the data were not subjected to statistical analysis.

Tibia ash values of poults fed autoclaved C-1 protein were markedly greater than those of poults fed untreated C-1 protein. When Ca^{45} was administered orally, the poults fed untreated C-1 protein showed a slightly lower Ca^{45} specific activity in tibia as compared to those fed the autoclaved C-1 protein. However, when turkey poults were given Ca^{45} intravenously, the mean specific activity values as influenced by the untreated protein and autoclaved protein were reversed. As might be expected tibia ash of birds that received Ca^{45} intravenously had higher mean Ca^{45} specific activity compared to that of birds given Ca^{45} orally.

TABLE 15. EFFECT OF Ca^{45} ADMINISTRATION AND AUTOCLAVED[1] ISOLATED SOYBEAN PROTEIN ON Ca^{45} METABOLISM IN TURKEY POULTS

Method of Ca^{45} Administration and Diet	Bird No.	Dose[2,3]	Body Weight	Tibia Data		Serum Ca^{45} at Different Intervals					Ca^{45} Excreted
			Individual	Ash	Ca^{45} in Ash	20 min.	40 min.	60 min.	1860 min.	Total	Total
		ml.	gm.	%	c.p.m./gm.			c.p.m./ml.			c.p.m.
Oral											
Untreated C-1 Protein	I	0.65	650	40.52	5,733,399	4,688	28,770	6,113	4,610	44,181	28,514,649
	III	0.52	520	34.17	7,719,727	37,403	28,223	27,364	1,661	94,751	37,108,395
	V	0.37	365	29.48	4,213,868	703	3,047	3,574	3,340	10,664	24,393,555
	VII	0.31	305	31.78	2,952,149	11,856	12,500	11,699	1,387	37,442	22,694,336
	IX	0.32	315	29.30	3,856,589	349	469	446	3,876	5,140	32,634,690
Mean		0.43	431	33.05	4,895,146	10,999	14,602	9,839	2,975	38,436	29,069,121
Autoclaved C-1 Protein	II	0.70	695	43.57	3,747,071	606	742	1,309	1,739	4,396	29,903,320
	IV	0.77	770	47.76	5,441,407	489	274	274	3,750	4,787	23,366,211
	VI	0.66	655	47.88	5,128,907	2,168	2,559	6,113	2,031	12,871	21,812,500
	VIII	0.59	590	48.12	6,433,594	88,126	44,961	43,086	1,661	177,834	35,148,105
	X	0.56	555	44.50	7,015,503	126,318	72,218	42,500	2,326	243,362	23,156,008
Mean		0.66	653	46.37	5,553,296	43,541	24,151	18,656	2,301	88,650	26,677,221
Intravenous											
Untreated C-1 Protein	I	0.31	610	33.86	11,321,289	77,832	76,035	39,454	4,600	197,921	3,502,540
	III	0.20	398	30.71	6,598,633	70,625	134,551	83,613	5,000	293,789	4,164,063
	V	0.22	431	33.03	8,764,649	44,004	35,735	37,500	4,922	122,161	3,148,438
	VII	0.15	295	37.51	6,848,632	112,930	62,793	47,168	4,219	227,110	4,679,688
Mean		0.22	434	34.03	8,383,308	76,348	77,279	76,934	4,703	210,245	3,873,682
Autoclaved C-1 Protein	II	0.43	850	43.57	6,464,844	316,543	63,203	91,133	2,227	473,106	2,463,907
	IV	0.33	664	43.00	8,091,797	64,688	35,860	32,578	1,934	135,060	2,095,704
	VI	0.31	611	47.11	5,857,422	95,293	----	105,957	4,199	205,449	19,849,610
	VIII	0.38	760	48.09	5,800,781	122,520	91,308	36,074	2,989	252,891	3,515,625
Mean		0.36	721	45.44	6,553,711	149,761	63,457	66,436	2,837	266,626	6,981,211

[1]Autoclaved for 60 min. at 120°C.

[2]Oral dose administered on the basis of 250 μc./1.0 ml.; specific activity of Ca^{45} was 7.69 mc./mg. Ca.

[3]Intravenous dose administered on the basis of 250 μc./0.05 ml.; specific activity of Ca^{45} was 7.69 mc./mg. Ca.

The specific activity of serum Ca^{45} (c.p.m./ml.) collected proved to be quite variable in this experiment. Hematocrit values were determined at each interval and correction was made for differences in serum volumes between intervals and between birds. Birds that received Ca^{45} orally, exhibited 260 fold variation in serum Ca^{45} between replicates. Irrespective of the method in which Ca^{45} was administered, the mean total serum Ca^{45} specific activity was higher in the birds fed autoclaved C-1 protein.

Mean total fecal Ca^{45} was highest in birds that received Ca^{45} orally. However, in this group, poults fed untreated C-1 protein excreted slightly more total Ca^{45} than those that received autoclaved C-1 protein. This situation was reversed when Ca^{45} was administered intravenously, as poults fed autoclaved C-1 protein excreted more total Ca^{45} than poults fed the untreated C-1 protein.

DISCUSSION

These investigations indicate that the rachitogenic activity of C-1 protein was markedly reduced by autoclaving, increased levels of vitamin D_3 and the apparent increased availability of the calcium and phosphorus source. The B.B.W. was more susceptible to the onset of rickets as compared to the W.S.W. in this study.

In general, autoclaving proved to be the most effective means of overcoming the rachitogenic property of the protein. Sixty minutes of autoclaving at 120°C. was the optimum time required to produce the greatest reduction in the rachitogenic property of C-1 protein, although shorter treatment periods had some effect. Increasing the vitamin D_3 level usually decreased the rachitogenic activity of the protein as measured by tibia ash and serum alkaline phosphatase. The extent of reduction was not significant in all instances. Also, dibasic calcium phosphate proved more effective than commercial dicalcium phosphate in overcoming the rachitogenic property of the C-1 protein. It is regretable that a Ca^{45} study designed to provide more information concerning the mode of action of autoclaving yielded inconclusive results.

Feeding autoclaved C-1 protein caused varied responses in weight gains, serum calcium and serum inorganic phosphate between different experiments. However, tibia ash values and serum alkaline phosphatase activity were affected similarly in each of the experiments. Inclusion of autoclaved protein in the diet always caused a significant increase in tibia ash and a concomitant decrease in serum alkaline phosphatase

activity. Conversely, feeding the untreated protein in the diet produced low tibia ash and high serum alkaline phosphatase activity. These symptoms are probably related to the induced rachitic conditions. In this regard, Reddy and Srikantia (1967) and Guyton (1966) reported high serum alkaline phosphatase activity in patients suffering from rickets. Therefore, it appears that the level of serum alkaline phosphatase may in fact be related to the extent of rickets in poults and the measurement of phosphatase activity may be useful in detecting the onset or alleviation of rickets within a group of poults.

The use of alkaline phosphatase levels as a criterion for rickets seems especially valid in controlled studies where conditions are primarily designed to produce rickets. For example, in experiment 4 when it was desired to ascertain the optimum autoclaving time required to destroy the rachitogenic property of C-1 protein, the induced differences in body weight gains, tibia ash, serum inorganic phosphate and serum calcium did not provide conclusive evidence. It was the difference in serum alkaline phosphatase levels together with the other criteria that proved to be the most sensitive index in evaluating the extent of rickets in the birds. As a result of this evaluation, 60 minutes autoclaving proved most effective in reducing the rachitogenic property of the protein.

It probably would be unwise to use this criteria alone as an absolute measure of rickets, since serum alkaline phosphatase levels are influenced by several other physiological disorders and little is known concerning the normal phosphatase levels in turkeys. In this

connection, Guyton (1966) mentioned that elevated serum alkaline phosphatase levels are associated with conditions that exist following major bone fractures or with any bone disease that causes bone destruction where repair is by osteoblastic activity similar to rickets, for example, osteomalacia and osteitis fibrosa cystica. Consequently, when serum alkaline phosphatase is used as a diagnostic tool to evaluate rickets, it should be used in conjunction with other criteria.

The significant increases in tibia ash that resulted when autoclaved C-1 protein was fed to poults is similar to the observations of Carlson et al. (1964b) and Jensen and Mraz (1966). Feeding protein which had been autoclaved for at least 60 minutes at 120°C. always produced tibia ash values in excess of 39%. From these experiments it appears that tibia values of 39% or greater are equivalent to a serum alkaline phosphatase activity of 100 or less Bodansky units.

It is remarkable to note that in experiment 4 when the C-1 protein was autoclaved for various times ranging from 60 to 240 minutes, the differences between tibia ash values at the 2 and 4 week periods were very similar in spite of the fact that this range of times produced a decline in weight gains. Throughout this study, irrespective of vitamin D_3 level used, autoclaving always enhanced tibia ash values; and also a comparison of the 2 and 4 week tibia ash data clearly shows that the most beneficial effects of autoclaving are realized at the period of greatest growth during the experiment (4 weeks). When the birds attained 4 weeks of age, attempts were made to determine what effects autoclaving exerted on the metabolism of Ca^{45}

in the tibias of the birds. Due to the great variability between replicates, the reliability of these results is somewhat doubtful. As expected, the poults that received Ca^{45} intravenously exhibited higher specific activities in the ash than those poults that received Ca^{45} orally. Under these conditions, autoclaving appeared to increase Ca^{45} deposition when the isotope was administered orally, but depressed Ca^{45} metabolism when the isotope was administered intravenously. As a result of the variation associated with this experiment, it may not seem meaningful to attempt to explain the role of autoclaving in calcium metabolism until further studies are conducted.

Under the conditions of the experiments reported herein, the effect of feeding autoclaved C-1 protein on weight gains varied. In experiments 2, 3, 5 and 6, increased weight gains were obtained while in experiments 1 and 4 feeding autoclaved C-1 protein did not increase weight gains. The reasons for this variability are not fully understood. However, differences in the initial nutritional status of the groups of poults may have contributed to the variation observed between the experiments. This is conceivable as the poults were obtained from different commercial hatcheries in different states at different times of the year and the dams were probably not fed the same dietary regimé.

Extended periods of autoclaving had a deleterious effect on weight gains. Subjecting the C-1 protein to periods of autoclaving beyond 80 minutes caused a progressive decline in body weight gains at 2 and 4 weeks of age. This indicates that above 80 minutes of

autoclaving there is probably a denaturation of the protein which reduces amino acid availability and thereby inhibits growth. The data suggest that within this range of autoclaving, namely 120 to 240 minutes, protein metabolism is impaired, while the metabolism of the minerals observed is not appreciably altered. The results are in agreement with the fact that heat treatment frequently causes the destruction of certain amino acids in protein thereby impairing its availability for growth.

Serum calcium was increased in two out of five experiments by feeding autoclaved C-1 protein. This variation was most likely due to the initial nutritional status of the birds that certainly influenced their susceptibility to rickets. The mechanism by which feeding autoclaved protein increased serum calcium could not be ascertained from this study.

Tibia ash values of poults fed untreated protein were altered markedly by increasing the vitamin D_3 levels without any effect being reflected in serum calcium levels (experiment 3). In this connection, Reddy and Srikantia (1967) reported that there is usually a very marked reduction in serum calcium level of patients showing severe symptoms of rickets.

However, the Ca^{45} data suggest certain trends concerning the role of autoclaving in calcium metabolism. Although the variation in specific activity of serum Ca^{45} between replicates raises the question as to the reliability of the data, it appeared that feeding autoclaved C-1 protein enhanced the mean serum Ca^{45} values approximately two-fold

up to 60 minutes after administration. Nevertheless, 1860 minutes after administration, these values approached equality in terms of specific activity. This is an apparent indication that feeding autoclaved protein like increasing vitamin D_3 levels reduces the incidence of rickets by increasing calcium absorption. Whether this is a direct effect of autoclaving at the intestinal mucosa level or an indirect effect mediated via a vitamin D_3 system could not be determined in this study. Other data in this investigation suggest that the net effect of autoclaving is similar to that of vitamin D_3 relative to calcium metabolism. This is supported by further evidence concerning the excretion of orally administered Ca^{45} which seems to support the observation that autoclaving could be improving calcium absorption. The specific activity of excreta from the birds fed untreated C-1 protein was higher than that in the excreta from birds fed autoclaved C-1 protein. Thus, the data suggest that for some unknown reason(s), less dietary calcium is absorbed from untreated C-1 protein diets than from diets containing autoclaved C-1 protein.

Ca^{45} was administered intravenously, hopefully to ascertain whether autoclaving exerts any effect on the secretion of calcium into the gut. At the end of 1860 minutes after Ca^{45} administration, poults that received untreated C-1 protein appeared to retain more Ca^{45} in their serum than those poults that received autoclaved C-1 protein. It is likely that the birds with the higher serum Ca^{45} levels have a greater calcium requirement and consequently retained higher serum Ca^{45} levels for subsequent deposition. This is compatible with the fact

that the birds fed untreated C-1 protein secreted less Ca^{45} compared to the birds fed autoclaved C-1 protein.

There are several modes of action by which untreated C-1 protein exerts its deleterious effects. As previously mentioned, calcium absorption seems to be impaired at the membrane level; whether the ability of the system to deposit calcium is affected could not be assessed in this study, but it is conceivable that this phenomenon could be a contributing factor.

It appears that the reliability of this data would be greatly enhanced if initial individual controls were established. For example, knowledge concerning individual blood volumes and serum calcium would be of great value in reducing variation between replicates.

Serum inorganic phosphate appeared to be least affected by rickets under the conditions of these experiments. In only one study was there a significant decline in serum inorganic phosphate levels as a result of feeding untreated C-1 protein. This decline was accentuated as the birds approached maturity. According to Guyton (1966), the level of phosphate in the blood of rachitic patients is greatly depressed, in contrast to calcium levels which are ordinarily only slightly depressed. However, the data in the present study indicate that serum inorganic phosphate levels were the least sensitive criteria used to evaluate the incidence of rickets in poults.

In an attempt to gain further information as to the mechanism by which autoclaving reduces the rachitogenic property of C-1 protein, various proportions of autoclaved protein and untreated C-1 protein

were mixed and fed to poults. In general, the progressive dilution of C-1 protein with the autoclaved protein produced corresponding reduction in its rachitogenic activity. It appears that the reduction of rachitogenic activity may be due to simple dilution of the rachitogenic factor(s). Similar conclusions have been stated by Jensen and Mraz (1966) who effected a partial reduction in rachitogenic activity of C-1 protein by substituting soybean meal. They interpreted their results as indicating a dilution of the rachitogenic activity. Of course the possibility still remains that autoclaving may actually produce an antirachitic substance which has an overriding effect on the rachitogenic activity of C-1 protein.

The effects obtained by varying vitamin D_3 levels were similar in all four experiments although the changes were not significant in experiment 3. In experiment 3, however, the vitamin D_3 levels were both high. Less response might be expected with the addition of more vitamin D_3 to an already high level. Increasing vitamin D_3 levels increased tibia ash values and decreased alkaline phosphatase activity. These responses were similar to those obtained by feeding autoclaved C-1 protein. Whether the mode of action of these two treatments is the same is conjecture. In any event, autoclaving C-1 protein for 60 minutes was more effective in reducing the rachitogenic properties of C-1 protein than the addition of vitamin D_3 at the rate of four times N.R.C. recommendations (experiment 2). It is conceivable that vitamin D_3 exerts an overriding effect on the rachitogenic activity of C-1 protein, whereas, autoclaving involves the actual destruction of the

activity. As stated before, serum calcium levels were increased in two out of five experiments by feeding autoclaved C-1 protein; increasing vitamin D_3 levels did not affect serum calcium markedly. The possibility exists, therefore, that the effect of feeding autoclaved protein is mediated by a pathway different from that involved in the control of rickets by vitamin D_3 additions.

In three out of four experiments, there was an interrelationship between autoclaving and vitamin D_3. Two out of three experiments show that interrelationships were associated with tibia ash and serum alkaline phosphatase, while the third affected serum calcium levels. In all instances the interaction resulted from the larger responses to vitamin D_3 increases by poults fed untreated C-1 protein as compared to the poults fed autoclaved C-1 protein. This vitamin D_3 × autoclaving interaction is further supporting evidence that autoclaving is more effective in reducing the rachitogenic effect of C-1 protein than vitamin D_3.

Dibasic calcium phosphate was more effective than dicalcium phosphate in overcoming the rachitogenic effect exhibited by C-1 protein, since turkey poults fed dibasic calcium phosphate gained body weight more rapidly and exhibited higher tibia ash values than those birds which received dicalcium phosphate. In the case of weight gains, this difference was accentuated with time. It appears that the dibasic calcium phosphate was more available than the dicalcium phosphate, and thus contributed more toward the promotion of growth. Probably, improved calcification could also be attributable to the

above suggested phenomenon. These findings are similar to those of Wilcox et al. (1955) who reported that dibasic calcium phosphate was superior to other sources of calcium and phosphate when included in practical-type diets (corn-soybean oil meal) for turkey poults.

Just as with autoclaving, the effect of source of calcium and phosphate was more marked at the 4 week growing period when the birds attained their maximum growth. These results suggest that the effect of the rachitogenic activity of C-1 protein becomes increasingly noticeable as stress becomes greater due to increased bone calcification needs. However, the growth suppressing trends were already evident when the poults attained 2 weeks of age. Other investigations (unpublished data) in this laboratory have shown that the rachitogenic activity of C-1 protein exerted significant inhibitory effects on poults at 2 weeks of age.

The relationship that appeared to exist between the source of calcium and phosphate, and autoclaving also seemed to be dependent on age. The two week data indicated that feeding either dibasic calcium phosphate together with the untreated C-1 protein or the dicalcium phosphate with autoclaved C-1 protein was sufficient to overcome the growth inhibitory activity of the C-1 protein. However, at 2 weeks, when autoclaved C-1 protein was fed with dibasic calcium phosphate, little response was obtained while this same ingredient combination elicited the type gains seen in the later stages of growth. At 4 weeks, when the birds nutritional requirements for growth were more critical, the dibasic calcium phosphate and the autoclaved C-1 protein were both

utilized for maximum growth of the birds.

A somewhat similar relationship seemed to exist between source of calcium and phosphate, and autoclaving with respect to bone calcification. Autoclaving was more effective in reducing the rachitogenic effect of C-1 protein than dibasic calcium phosphate, and thus, produced higher tibia ash values. Dibasic calcium phosphate in combination with autoclaving was most effective in enhancing calcification. This effect probably was mediated by increased availability of calcium and phosphorus, thus overcoming the rachitogenic activity of the C-1 protein.

The difference between main effects of response to the two strains appeared to be more marked during the early stages of growth. The B.B.W. gain significantly more weight than the W.S.W. in the early stages of growth. Toward the later stages of growth when the demands for growth were greater, the effect of the rachitogenic activity of the C-1 protein on the faster growing B.B.W. was more marked, consequently weight gains were lower than those of the W.S.W.

A definite relationship appears to exist between autoclaving × strain with respect to growth as the B.B.W. gained more weight than the W.S.W. when both strains were fed autoclaved C-1 protein. This fact was indicated by the significant interaction that existed between autoclaving and strain.

Although there were absolute differences between the final tibia ash values of the two strains, this was probably not attributable to the ration treatment effects, as there was virtually no difference

between the amounts of ash gained by the two strains. This demonstrates the need to determine initial tibia ash values in studies of this nature where it is neither feasible nor desirable to completely randomize the animals at the outset of the study.

A definite relationship between the following factors were indicated in experiment 5. An interaction between vitamin D_3 and strain was shown, in that doubling the vitamin D_3 level produced a greater response in the B.B.W. compared to the W.S.W. strain. There was also an interrelationship between source of calcium and phosphate, vitamin D_3 and autoclaving.

SUMMARY AND CONCLUSIONS

The characteristics of the rachitogenic activity in isolated soybean protein were investigated in seven studies involving day-old Broad Breasted White and/or Wrolstad Small White turkey poults. A purified-glucose C-1 protein diet was used. Levels of calcium, phosphorus and vitamin D_3 were equal to or greater than National Research Council recommendations. Criteria used for evaluation were body weight gains, tibia ash, serum alkaline phosphatase, serum inorganic phosphate and serum calcium.

Under the conditions of this investigation the following observations were made:

1. In general, poults fed untreated C-1 protein, consistently exhibited rachitic symptoms, namely, low tibia ash values and concomitant elevated serum alkaline phosphatase levels. The effects of the C-1 protein as measured by the other criteria were less consistent.

2. Subjecting C-1 protein to autoclaving destroyed its rachitogenic activity. Autoclaving proved to be the most effective way of reducing the rachitogenic property of C-1 protein.

3. Sixty minutes autoclaving time at 120°C. proved optimum in destroying the rachitogenic activity of C-1 protein.

4. Substituting dibasic-calcium phosphate, hydrous, U. S. P. for dicalcium phosphate, feed grade, markedly reduced the rachitogenic activity of the C-1 protein.

5. The inclusion of vitamin D_3 levels 4 times the N. R. C. recommendations was less effective than autoclaving in reducing the rachitogenic activity of C-1 protein.

6. It appears that the B. B. W. strain of poults is more susceptible to the onset of rickets as induced by untreated C-1 protein compared to the W. S. W. strain.

7. Tibia ash gains should be studied when investigating the effect of ration treatments on tibia ash, in order to take into consideration any initial tibia ash differences that might be present, due to strain or nutritional effects.

8. The dilution of C-1 protein with autoclaved C-1 protein in the ratio of 2:2 was effective in reducing the rachitogenic activity of C-1 protein, however, it required one part of untreated C-1 to three parts of autoclaved C-1 protein to overcome the growth depressing action of C-1 protein.

9. The rachitogenic effect of isolated soybean protein exerts its greatest effect on the birds between two and four weeks of age.

10. Two-way interrelationships existed between autoclaving × vitamin D_3, autoclaving × source of calcium and phosphate, autoclaving × strain and vitamin D_3 × strain; also there was a three-way interrelationship between autoclaving × source of calcium and phosphate × vitamin D_3 level.

11. The variability in the Ca^{45} experiment was great and consequently raises a question as to the reliability of the data.

Nevertheless, the data suggests that feeding autoclaved C-1 protein like increasing vitamin D_3 does play a role in increasing calcium deposition by increasing calcium absorption and/or decreasing calcium secretion into the intestine.

In conclusion, the rachitogenic activity of isolated soybean protein may be reduced by improving the source of calcium and phosphate, by autoclaving the isolated soybean protein and by increasing the level of vitamin D_3. The B. B. W. strain of turkey poults are more susceptible to the rachitogenic effect of untreated isolated soybean protein than the W. S. W. strain.

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APPENDIX A

COMPLETE ANALYSIS OF ASSAY PROTEIN C-1 TOGETHER WITH SPECIFICATIONS AND USES COMPILED BY SKIDMORE ENTERPRISES, CINCINNATI

ASSAY PROTEIN C-1[1]

Specifications and Uses

Assay Protein C-1 is a high purity, chemically isolated soy protein, widely used in nutritional research. It is a complete protein requiring no supplementation with gelatin.

Because Assay Protein C-1 is low in vitamins and minerals, it is a splendid material for evaluating the vitamin interrelationships and nutrient requirements for various animals. Its economy has enabled nutritionists to expand research with experimental animals. It is used in diverse studies utilizing growing chicks, laying hens, turkey poults, weanling and growing pigs, sheep, calves, rabbits, guinea pigs and rats.

The following characteristics recommend Assay Protein C-1 as the finest protein for nutritional research studies:

High Purity - it is essentially depleted of vitamins and minerals.

High Biological Availability - has superior nitrogen and amino acid utilization -- readily assimilated.

Palatability - readily eaten by animals and poultry.

Regular Supply - A Standard item, always available.

Economy - finest quality, economically priced.

Uniformity - produced to meet the following specifications under rigid quality control procedures.

Protein - - - - 90% or more (moisture free, N × 6.25)
Moisture - - - 9.0% ± 1.5%
Ash - - - - - - Less than 2.5% (moisture free)

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[1]Skidmore Enterprises, 3217 Buell Avenue, Cincinnati, Ohio 45211.

Fat - - - - - - - Less than 0.5% (moisture free)
SO_2 - - - - - - - Less than 0.05% (moisture free)

The remainder, about 6%, is composed of carbohydrates and phosphorus and sulfur compounds. A major portion of these is held in close association with the protein. The phosphorus compounds are largely organic, amounting to about 2% of the balance and include phytin and sugar phosphates. The sulfur portion approximating 1% consists largely of inorganic sulfate. Carbohydrates amounting to about 2.5% include simple sugars, hemicellulose and cellulose.

Vitamin, Amino Acid and Element Analysis

The vitamin and amino acid composition of Assay Protein C-1 has been determined by microbiological assay, with the exception of thiamine for which the thiochrome method was used. The accuracy of microbiological assays is approximately ± 5%.

A large portion of the water soluble vitamins are removed during the isolation of Assay Protein C-1.

Vitamins - values are expressed as micrograms per gram of moisture-free protein.

Amino Acids - the first 10 in the listing, arginine through valine, are the familiar "essential" amino acids. Glycine also is essential for chicks. All values are expressed as grams of amino acid per 100 grams of moisture-free protein.

Rations for experimental animals may require supplementation with methionine and/or glycine depending upon the animal's requirements and upon the level of C-1 used. Appropriate literature sources should be consulted.

VITAMIN ANALYSIS

	Micro grams
Biotin	0.3
Choline	2.09
Citrovorum Factor	0.92
Folic Acid	2.5
Inositol	300.0
Niacin	6.0
Panthenol	None
Pantothenic Acid	4.2
ρ-Aminobenzoic Acid	0.7
Pyridoxine	5.4
Riboflavin	1.2
Thiamine	0.25
Vitamin B_{12}	<0.0005

AMINO ACID ANALYSIS

	Grams
Arginine	8.3
Histidine	2.6
Isoleucine	6.5
Leucine	7.5
Lysine	6.8
Methionine	1.0
Phenylalanine	5.0
Threonine	3.9
Tryptophan	1.0
Valine	5.5
Glycine	4.1
Alanine	3.6
Aspartic Acid	6.2
Cystine	0.6
Glutamic Acid	19.5
Proline	2.5
Serine	6.9
Tyrosine	3.4

ELEMENT ANALYSIS

	Percent
Aluminum	0.0031
Arsenic	0.00001
Calcium	0.0185
Chlorine	0.0019
Cobalt	0.00001
Copper	0.00077
Fluorine	0.00007
Iodine	0.00001
Iron	0.0131
Lead	0.00014
Magnesium	0.0132
Manganese	0.0049
Phosphorus	0.7638
Potassium	0.1825
Selenium	<0.00002
Silicon	0.0021
Sodium	0.0668
Sulfur	0.7140
Zinc	0.0023

APPENDIX B

DERIVED TABLES, EACH SHOWING THE EFFECTS OF TWO FACTORS, ONLY, ON BODY WEIGHT GAINS AND TIBIA ASH OF POULTS AT 2 AND 4 WEEKS OF AGE

TABLE 1. THE EFFECT OF AUTOCLAVING AND SOURCE OF CALCIUM AND PHOSPHATE ON BODY WEIGHT GAINS AT 2 AND 4 WEEKS

Calcium and Phosphate Source	2 Week Data			4 Week Data		
	Autoclaving[1] Time			Autoclaving Time		
	0 min.	80 min.	Mean	0 min.	80 min.	Mean
4% of diet	gm.	gm.		gm.	gm.	
Commercial Dicalcium Phosphate, feed grade	131[2]	150	141	228	380	304
Dibasic Calcium Phosphate, hydrous, U.S.P.	159	148	153	357	400	379**
Mean	145	149		293	390*	

[1]Autoclaved for 80 min. at 120°C.

[2]Mean of 12 pens/treatment

*Mean square significant at $P < 0.05$.

**Mean square significant at $P < 0.01$.

TABLE 2. THE EFFECT OF AUTOCLAVING AND STRAIN ON BODY WEIGHT GAINS AT 2 AND 4 WEEKS

Autoclaving[1] Time	2 Week Data			4 Week Data		
	Strain			Strain		
	Broad Breasted White	Wrolstad Small White	Mean	Broad Breasted White	Wrolstad Small White	Mean
min.	gm.	gm.		gm.	gm.	
0	148[2]	143	146	260	327	294
80	173	127	150	428	351	390*
Mean	160*	135		344	339	

[1]Autoclaved at 120°C.

[2]Mean of 12 pens/treatment.

*Mean square significant at $P < 0.05$.

TABLE 3. THE INFLUENCE OF VITAMIN D_3 LEVEL AND STRAIN ON BODY WEIGHT GAINS AT 2 AND 4 WEEKS

	2 Week Data			4 Week Data		
	Strain			Strain		
Vitamin D3 Level	Broad Breasted White	Wrolstad Small White	Mean	Broad Breasted White	Wrolstad Small White	Mean
I.C.U./kg. of diet	gm.	gm.		gm.	gm.	
880	148[1]	136	142	273	315	294
1760	172	134	153*	416	364	390*
Mean	160*	135		345	339	

[1]Mean of 12 pens/treatment.

*Mean square significant at $P < 0.05$.

TABLE 4. THE INFLUENCE OF AUTOCLAVING AND SOURCE OF CALCIUM AND PHOSPHATE ON TIBIA ASH

Calcium and Phosphate Source	Autoclaving Time		Mean
	0 minutes	80 minutes	
4% of diet	%	%	
Commercial Dicalcium Phosphate, feed grade	29.5[1]	40.7	35.1
Dibasic Calcium Phosphate, hydrous, U.S.P.	37.2	44.8	41.0**
Mean	33.4	42.8**	

[1]Mean of 12 pens/treatment.

**Mean square significant at $P < 0.01$.

TABLE 5. THE INFLUENCE OF VITAMIN D_3 LEVEL AND STRAIN ON TIBIA ASH

Vitamin D3 Level	Strain		
	Broad Breasted White	Wrolstad Small White	Mean
I.C.U./kg. of diet	%	%	
880	33.2[1]	37.6	35.4
1760	39.1	42.4	40.8*
Mean	36.2	40.0*	

[1]Mean of 12 pens/treatment.

*Mean square significant at $P < 0.05$.