The effect of varying levels of dietary calcium upon lipid excretion and deposition in swine
by Arthur Leo Hecker

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Abstract:
One trial involving 24 pigs was conducted to investigate the effect of varying levels of dietary calcium upon lipid excretion and deposition in swine. Phosphorus and calcium were maintained in a 1:1 ratio in all diets. Calcium levels, 0.4, 0.8 and 1.2 percent, and calcium sources, limestone and defluorninated rock phosphate, appeared to be satisfactory for normal growth. Although the effect was nonsignificant, the 0.8 percent dietary calcium level appeared to induce the highest average daily gain. Treatment had no effect on feed efficiency. Average daily gain did not demonstrate any significant difference in the,efficacy of the two calcium sources. The serum calcium level was significantly (P< .05) affected by the calcium source and 0.8 percent calcium intake level; Increased dietary calcium intake had no effect on fecal lipid levels. The diets used in the present investigation contained no added fats.

These diets contained only low melting point fats which are not subject to interference by calcium as are the high melting point fats. The lack of significance could be due to this reason. Data revealed that increased calcium ingestion caused a significant (P< .01) drop in' serum cholesterol but not in total serum lipids. Liver and heart total lipid levels showed -no effect due to treatment. The limestone calcium source when compared to defluorinated rock phosphate appeared to cause a significant (P< 0.5) decrease in heart cholesterol content. The limestone calcium source supplied the calcium in the form of calcium carbonate compared to tricalcium phosphate from the defluorinated rock phosphate. This difference in anion ,form may explain the difference in effect due to source here , and elsewhere in the study. Fecal, tissue and serum lipid phosphorus remained unaffected by treatment.

The investigation indicated that gilts had a lower (P<.05) serum cholesterol level than did the barrows. The sex hormones may be involved in this difference. At the 0.8 percent dietary calcium level, sex again appeared to introduce some effect (P<.05) causing an opposite flucuation in the heart cholesterol content between barrows and gilts.

There was a significant (P<.05) three-way interaction found in analyzing the effect of calcium level, calcium source and sex on serum lipid phosphorus.. As there were no significant,effects due to main treatments on this parameter, it is suspected that this interaction may have been due to random variation.

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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ABSTRACT

One trial involving 24 pigs was conducted to investigate the effect of varying levels of dietary calcium upon lipid excretion and deposition in swine. Phosphorus and calcium were maintained in a 1:1 ratio in all diets. Calcium levels, 0.4, 0.8 and 1.2 percent, and calcium sources, limestone and defluorninated rock phosphate, appeared to be satisfactory for normal growth. Although the effect was nonsignificant, the 0.8 percent dietary calcium level appeared to induce the highest average daily gain. Treatment had no effect on feed efficiency. Average daily gain did not demonstrate any significant difference in the efficacy of the two calcium sources. The serum calcium level was significantly ($P < .05$) affected by the calcium source and 0.8 percent calcium intake level.

Increased dietary calcium intake had no effect on fecal lipid levels. The diets used in the present investigation contained no added fats. These diets contained only low melting point fats which are not subject to interference by calcium as are the high melting point fats. The lack of significance could be due to this reason. Data revealed that increased calcium ingestion caused a significant ($P < .01$) drop in serum cholesterol but not in total serum lipids. Liver and heart total lipid levels showed no effect due to treatment. The limestone calcium source when compared to defluorinated rock phosphate appeared to cause a significant ($P < 0.5$) decrease in heart cholesterol content. The limestone calcium source supplied the calcium in the form of calcium carbonate compared to tricalcium phosphate from the defluorinated rock phosphate. This difference in anion form may explain the difference in effect due to source here and elsewhere in the study. Fecal, tissue and serum lipid phosphorus remained unaffected by treatment.

The investigation indicated that gilts had a lower ($P < .05$) serum cholesterol level than did the barrows. The sex hormones may be involved in this difference. At the 0.8 percent dietary calcium level, sex again appeared to introduce some effect ($P < .05$) causing an opposite flucuation in the heart cholesterol content between barrows and gilts.

There was a significant ($P < .05$) three-way interaction found in analyzing the effect of calcium level, calcium source and sex on serum lipid phosphorus. As there were no significant effects due to main treatments on this parameter, it is suspected that this interaction may have been due to random variation.

INTRODUCTION

Experimental animal nutrition has transformed from an age of new discovery into one of interrelationship exploration. While much of our current knowledge has resulted from concentrated research on specific nutritional problems of animals and man, equally important discoveries have evolved from basic studies of the physiological and biochemical changes in the animal organism as affected by various dietary variables.

The nutritional and physiological importance of certain minerals has been recognized since the early 1800's. It has only been in the last fifty years, however, that a relationship has been demonstrated between minerals and other nutrients. It is known that proper calcium utilization depends upon not only the presence of phosphorus and vitamin D but also upon the availability of adequate amounts of them. With the advent of added fats in animal diets, an interrelationship involving calcium and lipids has evolved. Extended research has disclosed evidence supporting the theory that increased dietary calcium levels have an inhibitory effect upon lipid utilization.

Recent developments have served to stress the interrelationships between human and animal nutrition. Nutritional data from swine investigations may be extrapolated to human nutrition, since the nutritional requirements and digestive systems of man and swine are quite similar. High energy diets, as are now being fed to swine, approach the caloric value and origin of those consumed by man.

An effective reduction of fat absorption might be desirable in reference to human nutrition. In a human population accustomed to a

high caloric and relatively high fat intake, it would seem difficult to change dietary habits; therefore, if a significant portion of ingested fat could be rendered nonabsorbable, it would probably constitute an aid in effecting weight loss or preventing weight gain. This calcium-lipid relationship takes on additional importance when you consider that certain lipids are suspected to be the crucial substances responsible for the development of arteriosclerosis in man. An effective control of serum lipid types and levels would be very advantageous.

This investigation was designed to study the effects of varying levels of dietary calcium upon lipid excretion and deposition in swine.

REVIEW OF LITERATURE

Calcium-Phosphorus Requirements of Swine

Dunlop (1935) stated that the true requirement of an animal for calcium and phosphorus is dependent on growth rate and efficiency of feed conversion. His investigations indicated that a swine diet with calcium and phosphorus levels of 0.45 percent of the dry matter was optimum for average daily gains of 1.0 to 1.4 pounds per day and efficiency of 3 to 4 pounds of dry matter per pound of gain. Low calcium intakes did not change blood calcium levels.

Aubel et al. (1936) established the minimum phosphorus requirements of swine at 0.27 to 0.30 percent of the diet. Aubel and Hughes (1937) found that pigs receiving rations of 0.51 percent phosphorus and 0.79 percent calcium had the best appetites, gains, bone formation and efficiency of gain. Blood levels of serum inorganic phosphorus dropped on low phosphorus diets from an initial 5.5 milligram percent to 2.6 milligram percent, while serum calcium level was maintained or increased. There was no effect on blood phosphorus at 0.23 percent dietary phosphorus or above. It was observed that carcasses of pigs fed low levels of phosphorus carried more fat than pigs receiving higher levels, though there was no consistent effect on dressing percent.

Theiler et al. (1937) conducted experiments with swine on the effect of rations deficient in calcium or phosphorus or both. The rations were considered deficient at 0.1 percent calcium and adequate at 0.2 percent. Theiler stated that serum calcium was not lowered by low levels of calcium in the diet.

Swine rations containing 0.1 percent calcium were considered deficient by Aubel et al. (1941). Pigs exhibited poor appetites, diarrhea, became coarse haired and nervous. One half of the pigs recovered after the 10th week and were fairly normal at the end of twenty-four weeks except in weight. The remainder showed continual disturbances and developed paralysis of the hindquarters. A small percentage of pigs receiving 0.25 percent dietary calcium showed deficiency symptoms and those appearing normal had soft bones when sacrificed. Blood calcium dropped intially and then rose to normal on the low calcium diets. Pigs were able to overcome the deficiency as they matured, indicating requirements to be greater in young pigs and decreasing with maturity.

Miller et al. (1960) reported that 0.6 percent calcium in the presence of 0.5 percent phosphorus supported maximum growth and feed response in baby pigs. Analysis of humerus ash, calcium, phosphorus, specific gravity and the breaking strength of femurs, however, showed that the minimum calcium level for maximum skeletal formation was 0.8 percent of the ration.

Rutledge et al. (1961) fed early weaned pigs practical corn-soybean meal diets containing 0.4, 0.6, 0.8 or 1.0 percent calcium and 0.6 percent phosphorus. Highly significant linear trends toward increased ash and calcium content of femur samples and of humeri breaking strength resulted from increases in dietary calcium. Rate of gain, feed efficiency and serum calcium and phosphorus levels were not affected significantly by calcium content of the diet. Radiographs of femurs and humeri indicated marked increases in degree of calcification and bone density with increases

in dietary calcium. The minimum calcium level suggested was 0.8 percent of the ration.

Calcium and phosphorus requirements of growing-finishing swine fed rations fortified with vitamins, trace minerals, and an antibiotic were investigated by Chapman et al. (1962). Dietary levels of calcium and phosphorus ranged from 0.2 through 0.8 and 0.2 through 0.7 percent, respectively. Rate of gain decreased as calcium content increased when the diet contained 0.2 or 0.3 percent phosphorus, thus demonstrating the importance of a narrow calcium-phosphorus ratio when the dietary level of phosphorus is low. As the dietary phosphorus level increased, there was a significant increase in both femur weight and femur fat content. Increasing phosphorus to 0.6 percent of the diet resulted in increased gains to 100 pounds for all levels of calcium fed. There was a highly significant increase in femur weight with increases in dietary calcium or phosphorus, though there were no differences in calcium or phosphorus content of femurs due to treatment. There was a significant correlation between increasing calcium and phosphorus levels and femur breaking strength. Dietary requirements, to assure maximum rate of gain and optimum skeletal development, appeared to be 0.8 percent calcium and 0.6 percent phosphorus for the pig from 25 to 100 pounds, and 0.7 percent calcium and 0.5 percent phosphorus from 100 to 200 pounds.

Calcium-Phosphorus Interrelationship

Forbes (1914) was one of the first to demonstrate that a phosphorus deficiency was rendered more acute by adding calcium carbonate to diets

low in phosphorus.

A close relationship between calcium and phosphorus intake and calcium retention was observed by Haag and Palmer (1927). These workers reported 70 to 80 percent retention by pigs on diets containing 0.34 percent calcium and 0.65 percent phosphorus. Their data indicated that this level of calcium was below the point where maximum retention occurred. Results were quite variable with phosphorus, and it was postulated that low retention was due to the low calcium content of the diet.

Bethke et al. (1932) emphasized the importance of calcium-phosphorus ratios in rat diets for optimum bone development. Increasing the calcium-phosphorus ration from 1 to 5 caused progressive decreases in growth, bone ash and serum inorganic phosphorus. Changing the ration from 1 to 0.25 depressed growth and ash content of femurs and serum calcium was depressed, whereas serum inorganic phosphorus was increased. The most favorable ratio was two parts calcium to one part phosphorus.

Haldt et al. (1939) observed that when large amounts of calcium and phosphorus were added to rat diets, practically all excess calcium was recovered from the feces, whereas phosphorus in the feces was considerably less than the intake. Excess phosphorus and little more than normal amounts of calcium were excreted through the kidneys. The large amount of calcium and phosphorus interfered to a slight extent with the utilization of nutrients.

Data presented by Vermeulen (1959) showed that rats fed extra high calcium levels excreted greater amounts through the urine. Formation of

calcium phosphate stones was inhibited at high levels of calcium, and a profound drop in urine phosphorus was observed. Addition of phosphorus to high-calcium diets produced an increase in urine phosphate and a drop in urine calcium.

Clark and Cordero (1964) reported that with rats, in the absence of dietary phosphorus, urinary calcium was constant regardless of calcium intake and unless phosphorus intake was high, increasing calcium intake did not increase calcium retention. On a constant and adequate calcium diet, however, increasing dietary phosphorus increased calcium retention. When rats were fed calcium-free diets, urinary phosphorus accounted for as much as 75 percent of ingested phosphorus. Data indicated that level of phosphorus in the diet was as important as that of calcium for promoting calcium retention.

Zimmerman et al. (1963) conducted a series of experiments to determine the optimum calcium and phosphorus levels in high-milk-product rations for maximum body weight gain, efficiency of feed utilization and metatarsal calcification of swine 2 to 7 weeks of age. Low phosphorus levels and high calcium levels in the rations slowed the rate of body weight gain. In general, calcium-phosphorus ratios of 1.6:1.0 or wider adversely influenced gains. Calcium and phosphorus appeared to independently influence the efficiency of feed utilization. Calcium levels above 0.8 percent of the diet reduced feed efficiency, whereas phosphorus up to approximately 0.6 percent of the ration improved efficiency of feed utilization. The highest levels of calcium (1.0 percent) and phosphorus

(0.7 percent) in the ration produced maximum responses in metatarsal calcification; however, the largest increments of response come between the lower levels, especially with regard to phosphorus. Although high calcium levels in the ration lowered serum chlortetracycline values, it was demonstrated that this was not the cause of the adverse effect of high calcium intakes on body weight gains.

Effect of Source on Utilization of Calcium and Phosphorus

Thomas et al. (1933) conducted an investigation comparing the relative efficacy of ground limestone, steamed bone meal, and Dicapho (a commercial dicalcium phosphate supplement) as calcium supplements to rations for growing and fattening pigs. Data were collected in three metabolism experiments designed to determine the efficacy of the three calcium sources fed at equivalent low and high levels of calcium intake as supplements to basal rations deficient in calcium. Vitamin D was left out in an attempt to augment the possibility of detecting significant differences in the efficiency with which these compounds met the calcium requirements of the pig. The metabolism study showed that for equivalent quantities of calcium from each source, fed at either low or high levels of intake, calcium and phosphorus retention was essentially the same. A feedlot experiment comparing the three calcium sources was conducted concurrently with the metabolism trials. The data collected in this part of the study did not demonstrate any significant differences in the efficacy of the calcium supplements compared.

McCampbell and Aubel (1934) conducted several trials in an attempt

to make a comparison between calcium carbonate and calcium sulfate (gypsum) as a source of calcium in swine rations. All pigs were fed the same basal ration: linseed oil meal, wheat shorts, and corn, to equal the amount consumed by the pig eating the least. There were no significant differences in gains, and there appeared to be no effect on the appetite or physical condition of the pigs. This study indicated that calcium sulfate could be used as an effective substitute for calcium carbonate as a source of calcium for swine rations.

The effect of pulverized limestone and dicalcium phosphate supplements on the nutritive value of dairy cattle feed were studied by Colovos et al. (1958). Results of these experiments indicated that pulverized limestone, when added to both all roughage and mixed dairy rations, decreased the digestibility of both protein and energy in dairy heifers. Similar amounts of dicalcium phosphate did not have this effect. The feeding of dicalcium phosphate along with the pulverized limestone decreased the depressing effects of the limestone. These results indicated that the feeding of calcium in excess of requirements, particularly in wide calcium:phosphorus ratios, may reduce the digestibilities of protein and energy. There was nothing in this data to suggest any involvement of the carbonate radical as the causative agent in the decreased digestibilities of protein and energy.

Data collected by Combs and Wallace (1962) showed little evidence of a dietary calcium source effect on average daily gain, feed consumption or feed efficiency in growing pigs. The source of calcium did not significant-

ly affect the apparent digestibility of ether extract. In three experiments where calcium levels of 0.40 and 0.88 percent were compared, the higher level significantly depressed growth rate. Similar differences in growth rate were also obtained with rations containing either 0.52 or 0.72 percent calcium. When ration calcium was increased by increments of 0.10 from 0.40 to 0.80 percent, daily gains tended to decrease linearly with increasing calcium. In all experiments, the most efficient utilization of feed occurred with low calcium rations. Neither rate nor efficiency of gain were significantly influenced by the sources of supplementary calcium used in this study or by varying the diet concentration of chlortetracycline. Contrasting results were obtained with apparent digestibility determinations. In one experiment, the digestion coefficients for dry matter, ether extract and crude protein were comparable with pigs that received either 0.40 or 0.88 percent dietary calcium. The 0.88 percent calcium in another experiment significantly reduced digestibility of ether extract and crude protein. Dry matter and crude protein digestibilities were significantly lowered when gypsum rather than ground limestone or oyster shell flour supplied the supplemental calcium.

Fleishmann et al. (1967) compared the effect of several different sources of calcium on serum and fecal lipid levels. He found that irregardless of calcium sources; calcium carbonate, dibasic phosphate, calcium gluconate or Beta-glycerophosphate, an increase in intake caused a significant lowering of serum cholesterol level. Triglycerides

were lowered only by carbonate and gluconate. The Beta-glycerophosphate yielded unexpected results in that it caused a significant decrease in serum cholesterol at the 0.2 percent level but an increase at the 0.7 percent level. Fecal lipid excretion was significantly elevated at the 0.7 percent calcium level with no difference shown between sources. All salts except Beta-glycerophosphate significantly increased fecal bile acid excretion at the 0.2 percent level. The authors postulated that the hypolipemic action of dietary calcium may be partially anion dependent.

Studies conducted by Chapman et al. (1955) concluded that there was some effect due to different sources of phosphorus. Eight phosphorus treatments were fed with and without chlortetracycline. Diets were formulated so that each had the same calcium and phosphorus level and ratio. Percentages of calcium and phosphorus used in the experiment were 0.7 and 0.5, respectively. Steamed bonemeal, dicalcium phosphate, colloidal clay and plant phosphorus represented the different phosphorus sources. The experiment was conducted in two phases, one extending through the first eight weeks and the second running from the eighth week weight to 200 pounds live animal weight. Data from the first period showed no significant difference in rate of gain, average daily feed intake or feed efficiency. The source of phosphorus had no significant effect on either average daily feed intake or feed efficiency; however, average daily gains were significantly reduced in those pigs receiving collodial clay compared with those fed steamed bonemeal or dicalcium phosphate.

Pigs receiving the higher levels of phosphorus from the inorganic phosphorus supplements ate significantly less feed per day and required significantly less feed per pound of gain than those receiving more of the phosphorus from plant sources. In all cases there was no significance due to the presence of antibiotics in the ration. In direct contrast to the initial eight week period, the second phase of the experiment demonstrated a small but significant increase in average daily gain due to the presence of the antibiotic. Once again those pigs receiving colloidal clay gained significantly slower than those receiving either steamed bonemeal or dicalcium phosphate, but there was little difference in the rates of gain between pigs receiving the latter two supplements. The daily feed intake of pigs fed colloidal clay was significantly less than for pigs fed dicalcium phosphate. The difference between colloidal clay and steamed bonemeal was just short of significance. The feed efficiency of the pigs receiving colloidal clay was significantly greater than that of pigs fed dicalcium phosphate. There was no effect on rate of gain when the phosphorus level was increased; however, the amount of feed required per pound of gain and the average daily feed intake were significantly decreased by increased phosphorus consumption. Considering both phases of the trial, the performance of pigs by the all-plant phosphorus rations was progressively improved as the content of phosphorus from plant sources was decreased and replaced by an equivalent amount of phosphorus from dicalcium phosphate. These studies indicated a significant decrease in the rate of gain and feed efficiency when phosphorus was supplied

entirely by plant sources.

After comparing dicalcium phosphate, steamed bonemeal and defluorinated rock phosphate as sources of dietary phosphorus for pigs, Van Zante et al. (1967) reported no difference in bone ash between the three sources. However, at low levels deficiency symptoms developed in the pigs on steamed bonemeal and defluorinated rock phosphate.

Interrelationship Between Calcium, Protein and Carbohydrates

Woodman et al. (1937) found no evidence to warrant the conclusion that mineral metabolism was affected by higher levels of protein. On the other hand, McCance et al. (1942) showed that in healthy, adult human subjects, increasing protein intake increased calcium and magnesium absorption. Lehman and Pollak (1942) observed that alpha-amino acids increased the solubility of calcium salts and proposed that their presence aided in mineral absorption. Frandsen et al. (1954) reported calcium utilization and bone mineralization were greatly reduced in rats fed low protein or low quality protein rations. Lowery et al. (1962) demonstrated that the apparent protein digestibility of practical-type swine rations was not influenced by the addition of fat.

Lactose feeding has been shown to bring about changes in the pH of the contents of the whole intestinal tract below the duodenum (Robinson and Duncan, 1931) and to favor the development of an acidophilic flora (Rettger and Cheplin, 1923). The bacteriological studies of Cannon and McNease (1923) and Cruickshank (1928) might be cited as favoring the view

that any effect of lactose on calcium metabolism is a specific one and not the result merely of an increased solubility of calcium salts in the more acid medium.

The influence of lactose in the diet on the utilization of calcium and phosphorus was studied by French and Cowgill (1937). The data obtained from young animals indicated that the presence of lactose in the diet favorably influenced the utilization of calcium and phosphorus; although this was not observed with mature animals. They were unable to determine whether this favorable action of lactose was due to increased absorption from or diminished excretion into the intestine. Even though they were unable to prove the exact mode of action, it did appear that a diminished excretion of calcium into the intestine was the most plausable. They very definitely confirmed with dogs that immaturity played a role in the effect of lactose on the utilization of calcium.

Kline et al. (1932) reported lactose to have a favorable effect on calcium absorption and skeleton building in chicks fed a rachitic ration. These workers postulated that the acid effect produced in the intestine by lactose was responsible for improved calcium utilization. Neither maltose nor citrate were of value in aiding calcium absorption or increasing intestinal acidity. Recent work of Charley and Saltman (1963) has strengthened these findings. In experiments with rats, they proposed evidence for the chelation of calcium ions by sugars. Lactose and other polyols prevented or delayed the precipitation of calcium ions in the presence of bicarbonate buffer. A charged calcium-lactose complex was

demonstrated by electrophoresis and calcium-45. Greenwald et al. (1963) published somewhat conflicting data. They reported lactose had little effect on absorption and excretion of calcium in humans. Radioisotope studies carried out in a single patient indicated that absorption of calcium-45 was only slightly improved by lactose.

Lengemann et al. (1959) found that lactose enhanced the absorption of oral doses of calcium whether administered as chloride, lactate, gluconate or acetate. Even though direct derivatives of lactose were not able to increase calcium absorption, many other sugars were. Lactose increased calcium absorption both in the presence and absence of vitamin D. The greatest absorption took place in the duodenum and jejunum. Antibiotics had no effect on the rate of absorption, thus indicating that bacterial fermentation is not involved in the absorption mechanism.

Lloyd et al. (1961) found that by increasing the calcium level from 1.2 percent to 4.0 percent in the diets of young pigs, average daily gains and apparent digestibility of total carbohydrate were significantly decreased, but no other adverse effects were observed.

Interrelationship Between Calcium and Fat

A relationship between calcium and fat was shown in the work of Givens (1917). This author reported that poor utilization of dietary fats or fatty acids increased the excretion of calcium. Bosworth et al. (1918) pointed out the importance of calcium in the diet of human infants because of the elimination of calcium soaps when cows' milk was fed in place of breast feeding. Elimination of calcium soaps in the stools increased directly in proportion to the degree of solubility of diet calcium.

Because the infant cannot absorb the excess calcium from cow's milk, insoluble soaps are formed with the nonvolatile free fatty acids.

Telfer (1930) indicated that the higher calcium content of cows' milk as compared with human milk is the factor that leads to the greater loss of calcium soaps in the stools of bottle fed infants.

Holt et al. (1919_a, 1919_b) concluded from a study using infants, that a high calcium intake did not necessarily cause a large loss of fat in the feces. In a later study, Holt et al. (1920) found that the excretion of calcium in the feces was not related to the excretion of total fat, but some relationship existed to excretion of fat as a soap.

Telfer (1921) made the following postulation: if fatty acids are in excess, the calcium is excreted as soap; if alkalinity predominates, calcium phosphate is formed; if both fats and phosphate are low in the diet, calcium may be excreted as carbonate. These facts tend to indicate that the lower fatty acids readily dissolve calcium carbonate and calcium phosphate. In a later study, Telfer (1923) found that in the presence of higher fatty acids and their soluble soaps, a secondary reaction may then result in the formation of insoluble calcium soaps and the liberation of carbonic and phosphoric acids.

Boyd et al. (1932) stated that loss of calcium through insoluble calcium soap complexes depended upon other factors other than the amounts and nature of calcium compounds and fats in the diet. One such factor discussed was intestinal acidity. The addition of fat to a diet containing calcium ion as calcium chloride increased intestinal acidity

and increased absorption of calcium and phosphorus thus decreasing the loss of soaps in the feces. These authors reported that young rats capable of storing 30 to 40 milligrams of calcium daily, utilized 25, 38, and 90 percent of calcium soaps of stearic, palmitic and oleic acids, respectively. When calcium intake was lowered, greater utilization of calcium stearate and calcium palmitate occurred.

The previous references all support the theory that there is an insoluble calcium soap complex formed where dietary calcium and fat levels are relatively high. In direct contrast to this theory is an early report by Klinke (1928). He reported that calcium-fatty acid-soap complexes were found to form soluble complexes with bile salts. He demonstrated this by excluding bile from the intestine of a dog which resulted in greater fecal calcium. Beznak (1931) supported Klinke when he found that by adding sodium taurocholate to diets high in fat, calcium absorption was increased. A possible explantion was postulated by Verzar (1936). He suggested that through the hydrotropic action of bile, bile acids are able to complex with calcium and fatty acids, thus making them water soluble.

Haldt (1939) demonstrated that rats fed high calcium and phosphorus diets had lower dry body weights than those fed normal calcium and phosphorus levels. A lowered body fat content was assumed to account for the weight loss.

Bassett et al. (1939) reported that a reduction in calcium intake facilitated the absorption of fatty acids. Increasing levels of dietary

fat, increased calcium loss at the expense of stored calcium. Ingestion of alkaline phosphatase, a calcium salt or both caused an increase in total fecal fat.

In a study of the effects of calcium, manganese and other mineral elements upon the metabolism of lipids in dairy cattle, Ward and Reid (1948) found that all groups excreted similar proportions of the total lipid intake regardless of the mineral supplement received.

Wells and Cooper (1958) found that both 2 and 4 percent calcium chloride levels inhibited cholesterol absorption when the diet was supplemented with lactose, while a minimum of 4 percent calcium chloride was required to produce a similar effect on a diet based on sucrose. Calcium ion in the form of 2.2 percent calcium carbonate was less inhibitory than 2 percent calcium choloride. Calcium chloride had no effect when added to fat-free diets containing bile salts.

Carroll and Richards (1958) reported that the calcium content of the diet played an important role in determining the digestiblity of fatty acids in rats. The poor digestibility of oleic, linoleic and erucic acid relative to their corresponding triglycerides was shown to be associated with increased fecal excretion of calcium and phosphorus in the lipid fraction extractable with nuetral ether.

Fedde et al. (1960 reported that apparent absorption coeffients of fats by chicks were similar for either 10 or 20 percent dietary fat levels, were high for safflower oil, corn oil and hog grease, and were low for rendered beef fat. The apparent absorbability of tallow increased

from 53 percent at one week of age to 80 percent at 12 weeks of age and was increased when 0.5 percent or more of dietary ox bile was added. The digestibility was apparently not dependent upon growth rate or feed intake and was decreased by high dietary calcium and increased by reducing the calcium intake.

An investigation conducted by Drenick (1961) using human subjects indicated that increased dietary calcium caused increased fecal fat. However, results involving other soap-forming agents such as aluminum hydroxide and magnesium sulfate were inconclusive.

Yacowitz (1962) reported a 100 percent increase in fecal fat in human subjects by raising the calcium level 2.66 grams above normal daily intake. Calcium soaps made up the major portion of the excreted fatty acids. It was also reported that serum cholesterol was reduced. This was in agreement with an investigation conducted by Iacona et al. (1960). He reported that dietary calcium was hypocholesterolemic in rabbits. In a later study, Iacona (1964) demonstrated that on a diet deficient in calcium, rabbits exhibited elevated plasma cholesterol.

Studies of Combs and Wallace (1962) with practical rations for young pigs containing 3 percent lard found no effect on the digestibility of either extract by doubling the calcium intake in one trial, while in a second trial, digestibility of ether extract was decreased from 64 to 52 percent. Average daily gain of pigs fed the lower calcium level was significantly higher than for those fed the high calcium level in the latter trial.

Yacowitz (1965) was able to demonstrate that increased calcium ingestion, in humans of both sexes, caused a significant decrease in both serum cholesterol and serum triglycerides. Both fecal dry matter and fecal lipid were increased. The exact mechanism of the hypolipemic action of calcium was undetermined.

In an attempt to elucidate the hypocholesterolemic and hypotriglyceridemic action of dietary calcium, Fleischman et al. (1966) found that blood lipids decreased with increasing dietary calcium with the major decrease occurring at the 0.2 percent calcium level. Some tissue lipids decreased and none increased. Fecal lipids were significantly increased up to the 1.2 percent dietary calcium level. Fecal fatty acids became progressively more saturated with increased calcium intake, but the preferential excretion of saturated fatty acids did not appear to be sufficient to alter significantly the blood fatty acids. Fecal bile acids significantly increased at the 1.2 percent calcium level. This data indicated that the lowering of blood cholesterol by increased dietary calcium is mediated in part by an increased excretion of bile acids.

At the conclusion of a long term study, fleischman et al. (1967) found that serum total lipids, phospholipids, cholesterol and triglycerides decreased significantly with increased calcium intake, often to levels equal to or lower than the control diet. This significance was based on a comparison to a control ration composed of 4 percent fat, 1.15 percent calcium and 0.76 percent phosphorus. Fecal lipids increased singificantly between the 0.2 percent and 1.2 percent level, primarily because of an

increase in the free fatty acids. The excretion of lipid phosphorus was unaffected by increasing dietary calcium, except at the 2 percent calcium level or by increasing dietary fat levels. Fecal bile acids rose significantly at the 0.2 percent calcium level, with no further increase due to increased dietary calcium. Excretion of 3-Beta-hydroxysterol increased as dietary calcium increased and was, at the higher calcium levels, found to equal all the exogenous cholesterol, whereas fecal lipid excretion was found to account for the weight gain differences between the various calcium levels. No significant changes were noted in liver total lipid, cholesterol, triglyceride or free fatty acid due to an increase in dietary calcium. There was a decrease at the 1.2 percent calcium level in phospholipid, no further decrease was noted at the 2 percent level. All liver lipids were significantly higher ($P < .05$) with the high fat diet than with the control regardless of calcium level. Similar results were noted with the heart lipids, except that dietary fat concentration did not affect heart lipid concentration.

Bhattacharyya et al. (1968) reported that in humans a low calcium level in a saturated fat diet caused serum cholesterol to be significantly higher than a low calcium level in a polyunsaturated diet. The addition of 2 grams of calcium daily caused a decrease in serum cholesterol and an increase in fecal fatty acid excretion. No such changes were produced when calcium was added to the polyunsaturated diet but bile excretion increased.

The beneficial effects of dietary fat upon calcium assimilation as found by Holt and Fales (1922) and the curative effect of vitamin-free

fat upon rickets as found by Zucker and Barnett (1922-23) possibly depended upon a more favorable pH of the intestinal contents, produced by the fatty food. Boyd and Lyman (1929) have shown that the utilization of calcium soaps may be high. Seventy to 80 percent of calcium oleate was absorbed. In direct contrast to these results, Cronheim and Muller (1908); Bahrdt (1910); Rothberg (1907); and Birk (1907), found that as the amount of fat in the diet increased above a moderate level, calcium soaps in the stools also increased.

Jones (1940) found that by adding lard at levels of 5 to 25 percent in the diet of chicks he could induce definite antirachitic properties which were not associated with the non-saponifiable fraction. Lard increased calcification on diets containing 1.1 or 0.38 percent calcium, but the effect was less pronounced when the diet was low in phosphorus. Oleic acid increased calcification also, but sodium oleate and calcium soaps showed no antirachitic action.

French (1942) demonstrated excellent growth in rats fed diets containing 5, 15 and 28 percent fat, but 45 percent of fat in the diet resulted in less growth. The high fat level exerted a definite depressive effect on the growth rate as well as feed consumption. The utilization of calcium was depressed moderately and consistently in the order of the increasing fat content of the diets from 5 to 28 percent, and then decreased considerably for the 45 percent fat diet. A more acid condition in the intestine resulted from the 5 percent fat diet than from diets richer in fat. The utilization of calcium paralleled the acidity of the

intestional tract, the most efficient utilization accompanying the most acid reaction. The most efficient calcium utilization was obtained from the diet containing 1 gram of fat to 0.06 gram of calcium; and the efficiency of calcium utilization decreased in the order of the increase in the ratio of dietary fat to calcium. It was suggested that there are two basic physiological relationships involving fat and calcium: (1) the acidity of the intestinal tract, and (2) the formation of readily absorbable bile-fatty acid-calcium complexes.

Calverly and Kennedy (1949) studied various sources of fat in relation to calcium utilization. These authors concluded that poorly absorbed fats, long chain fats and saturated fatty acids increased fecal calcium, probably through formation of insoluble calcium soaps. The more soluble triglycerides resulted in less calcium excretion. Excretion of calcium and phosphorus in urine was affected indirectly by the presence of fat. When fecal calcium increased, there was a decrease in urinary calcium accompaned by a marked increase in urinary phosphorus. Inclusion of 5 percent fat in a normal diet well supplied with calcium and phosphorus in an optimum ratio, did not affect bone formation in healthy growing rats.

Newman et al. (1967) reported that added tallow in swine rations had no consistent effect on calcium digestibility or dietary phosphorus. Endogenous fecal calcium loss was not affected by the level of dietary tallow. Increased dietary calcium resulted in a reduction of the digestion coefficient of ether extract.

The melting point of fat has long been considered one of the factors affecting the digestibility of fats. Cheng et al. (1949) found an inverse relationship between melting point and digestibility of triglycerides with rats. Deuel (194 in a study of 34 vegetable fats melting below 50 degrees centrigrade, found that only two had coefficients of digestibility below 95. It was also found that in the case of animal fats, 18 different varieties melting under 50 degrees centigrade were found to be digestible to the extent of 93 percent or more in man. This work is supported by the results of Langworthy and Holmes, (1915); Holmes, (1919); and Deuel and Holmes, (1922). However, Lyman (1917) suggested that the melting point is not the only factor in determining digestibility but that the fatty acid makeup of the ester is also an important consideration. Cheng et al. (1949) demonstrated that calcium and magnesium did not significantly influence the digestibility of low melting fat, although they decreased markedly that a higher melting triglycerides and hydrogenated fats. These results were supported by data collected by Crockett and Deuel (1947).

Investigations conducted by Werner and Lutwak (1963) demonstrated that primary lipid absorption is influenced both by the nature of dietary lipid and by dietary calcium concentration. The absorption increases as the melting point decreases, and is greater when triglycerides rather than free fatty acids of the same chain length are fed.

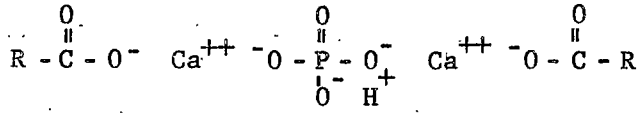
The incorporation of calcium-45 and phosphorus-32 in broiler femurs was studied by Bruggemann et al. (1961) . Their data indicated that

incorporation of calcium and phosphorus diminished in groups fed high calcium and 8 percent tallow. It was suggested that the metabolism of calcium and phosphorus on high calcium rations was not only dependent on the formation and excretion of calcium soaps but that there was a more specific interrelationship between fat and these minerals. The higher calcium diet decreased calcium-45 content in the bone by 20 percent. Tallow increased the calcium-45 content by 18 percent on the low calcium diet and decreased it by 50 percent on the high-calcium diet.

Swell et al. (1956_a) demonstrated the presence of a calcium-oleat-phosphate complex. In the case of dietary corn oil and olive oil, the phosphorus-containing lipid accounted for approximately 10 percent of the total lipid excretion. Oleic acid greatly increased the lipid-phosphorus excretion and there was a corresponding drop in the absorption of oleic acid. The study indicated that unsaturated fatty acids were more effective than saturated fatty acids in the formation of the calcium-fatty acid-phosphate complex. The data were in direct contrast to the belief that unsaturation causes better absorption. An oleic acid diet impaired calcium and phosphorus absorption. It was determined that 50 percent of the calcium and 50 percent of the phosphorus were lost in the feces as part of the calcium-fatty acid-phosphate complex.

Swell et al. (1956_b) found that rats fed a 25 percent oleic acid diet excreted large amounts of a phosphorus-containing lipid in their feces. The lipid resembled phospholipid in that it contained phosphorus and could be precipitated from chloroform solutions by acetone.

Characterization of the lipid revealed that it was a salt with the following composition: 2 atoms of calcium++, 1 mole of phosphate-, and 2 moles of oleic acid. The following is the proposed structure of the lipid:



Phosphorus-32 studies indicated that the lipid was formed in the gut from dietary phosphorus, calcium and oleic acid. The incorporation of phosphorus-32 into the lipid was about 15 times greater when the isotope was given in the diet than when given intraperitoneally. Thus, there would appear to be no turnover of phosphorus in the lipid and its formation appeared to be strictly exogenous in nature. The formation of this lipid greatly impaired the absorption of oleic acid.

Kim and Ivy (1952) and Pihl (1955) reported that rats fed an oleic acid diet excreted large amounts of phospholipid in their feces. Kim and Ivy (1952) also pointed out that palmitic acid and corn oil were found to have a mild stimulatory action on phospholipid excretion.

EXPERIMENTAL

Twenty-four 8-week-old crossbred Hampshire-Poland China pigs weighing approximately 20.4 kilograms were allotted equally to 6 pens on the basis of weight, litter and sex. Diets were formulated to contain calcium levels of 0.4, 0.8 and 1.2 percent. Two sources of calcium, ground limestone and defluorinated rock phosphate were used. The calcium to phosphorus ratio was maintained at 1:1, and all diets were fed ad libitum. The pigs were fed a 14 percent protein grower diet during the initial 30 days of the trial, by which time the pigs had reached an average weight of 45.4 kilograms. During the remainder of the investigation, the pigs were fed a 10 percent protein finisher diet. Diets and proximate analysis are described in Tables I and II, respectively. Pigs were weighed at 3-week intervals for the duration of the trial and total feed consumption by pen was recorded.

The pigs were sacrificed as they reached a weight of approximately 91 kilograms. Prior to sacrifice, blood samples were taken by vena cava puncture. Serum was obtained by centrifugation and frozen for later lipid and mineral analysis.

Intestinal acidity was determined after sacrifice to determine the approximate acidity of the intestinal contents existing during the experimental period. After sacrifice, the entire digestive tract was removed intact. The duodenum was removed and its contents expelled into a beaker for pH reading. The contents were thoroughly mixed before inserting the electrodes and determining the pH. This value was then checked by repeating the procedure. The middle 90 percent of the jejunum was removed next

TABLE I. COMPOSITION OF SWINE RATIONS.

Ration No.	39[1]	40	41	42	43	44	45[2]	46	47	48	49	50
Ingredients, %												
Barley	87.4	83.8	80.0	87.4	85.4	83.1	96.2	92.7	89.1	96.4	94.3	91.8
Soybean meal, 44%	10.0	11.0	12.0	10.0	10.5	11.0	1.0	1.8	2.6	1.0	1.5	2.0
Monosodium phosphate	---	1.6	3.3	---	---	0.4	0.2	1.9	3.6	---	---	0.6
Defluorinated rock phosphate	---	---	---	---	2.2	3.8	---	---	---	0.2	2.5	3.9
Limestone	0.9	1.9	3.0	0.9	0.2	---	0.9	1.9	3.0	0.7	---	---
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Trace Minerals[3]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin Premix[4]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Antibiotic[5]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Calcium, %[6]	0.41	0.79	0.21	0.41	0.80	1.21	0.41	0.79	1.21	0.39	0.80	1.21

1/ Rations 39-44, grower rations
2/ Rations 45-50, finisher rations
3/ Trace minerals furnished the following per kg. of complete diet: Mn., 100 mg.; Fe, 100 mg.; Cu., 10 mg.; Iodine, 3 mg.; Co., 1 mg. and Zn., 100 mg.
4/ Vitamin premix furnished the following per kg. of diet: vitamin A, 2500 I.U./kg. vitamin D, 1300 I.U./kg. vitamin E, .5 I.U.; vitamin K, 13 mg. vitamin B_{12}, 11 mg. Riboflavin, 3 mg., Niacin, 15 mg., Pantothenic Acid, 1.9 mg., Choline, 6 mg.
5/ Bacitracin, 25 gm./lb.
6/ Calculated

TABLE II. PROXIMATE ANALYSIS AND CALCIUM PHOSPHORUS CONTENT OF RATIONS

Ration No.	39[1]	40	41	42	43	44	45[2]	46	47	48	49	50
Item												
Protein, %	14.10	14.10	14.20	14.40	13.90	14.30	10.80	11.30	11.30	10.40	11.00	10.80
Ether Extract,%	2.27	2.21	2.26	2.40	1.89	1.93	2.38	2.28	2.41	2.34	2.31	2.14
Fiber, %	4.80	3.60	4.40	4.20	4.10	4.20	4.75	4.65	4.78	5.64	4.50	4.18
Nitrogen Free Extract, %	74.41	73.77	71.00	74.73	73.77	72.02	79.95	77.44	75.39	78.36	77.91	78.60
Ash, %	4.42	6.32	8.14	4.27	6.34	7.55	3.12	4.33	6.12	3.26	4.28	4.28
Calcium, %	0.48	0.90	1.16	0.48	0.91	1.22	0.48	0.90	1.16	0.48	0.91	1.22
Phosphorus, %	0.41	0.92	1.29	0.40	0.90	1.16	0.41	0.92	1.29	0.40	0.90	1.16

1/ Rations 39-44, grower rations
2/ Rations 45-50, finisher rations

and the contents treated similarly. The third determination was made on a similar portion of the ilieum. The acidity of the large intestine and stomach was determined last.

As the pigs were sacrificed, fecal samples were taken from the colon of each animal.

Because of the large size of the livers, it was impossible to process the entire organ; therefore, only a portion was used. The caudate lobe was removed from each liver, was ground and well mixed. A sample was taken for homogenization in a Vertis blender. The heart was treated in the same fashion as the liver except that the entire heart was processed. The fecal matter was prepared by grinding the undried material in a mortar and pestle.

The liver, heart, serum and feces were analyzed for total lipid, cholesterol and lipid phosphorus content using modified procedures of Williams (1962), Abdell et al. (1952) and Pons et al. (1953), respectively. The serum calcium concentration was determined using a procedure from Oser (1965). These procedures are described in detail in the appendix.

Data were analyzed by analysis of variance according to Snedecor (1956).

RESULTS

Weight gains and feed data are outlined in Table III. An average daily gain of 0.79 kilograms by the barrows was significantly ($P < .01$) greater than that of the gilts regardless of source or level of dietary calcium. An average total weight gain of approximately 74.0 kilograms was essentially the same for all treatments. Feed utilization indicated an improved feed efficiency with increased dietary calcium levels, although the differences only approached significance ($P > .05$).

Results of the serum calcium analysis are shown in Table IV. Treatment had no individual effect on the serum calcium concentration; however, a significant ($P < .05$) interaction between calcium source and level did develop.

The results of the intestinal pH trial revealed no difference in the pH of the various intestinal areas due to different dietary calcium levels or sources. The data are shown in Table V.

Lipid excretion and deposition data are shown in Table VI. Neither the dietary calcium level nor calcium source had any consistent effect on the amount of lipid excreted in the feces. Although the difference in total lipid content of both the liver and heart between gilts and barrows was not great, it approached the ($P > .05$) level of significance. (Liver: gilts = 108.74, barrows = 120.29 milligrams per gram of dry matter. Heart: gilts = 98.93, barrows = 109.14 milligrams per gram of dry matter.) These results are shown in Table VII. The calcium level-sex interaction for the total lipid content of the liver also approached significance ($P > .05$). The serum lipid concentration exhibited no effect due to treatment.

TABLE III. EFFECT OF SOURCE AND LEVEL OF CALCIUM ON BODY WEIGHT GAIN AND FEED EFFICIENCY.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level,%	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	12
Av. Initial weight, kg.	20.4	20.4	20.9	61.8	20.4	20.9	21.3	62.7	20.4	20.4	20.9
Av. Final weight, kg.	94.0	95.4	94.0	283.3	94.9	95.4	95.4	285.6	94.4	95.4	94.4
Av. total gain, kg.	73.5	74.9	73.1	226.6	74.5	74.9	74.5	223.8	74.0	74.9	73.5
Days fed	98	98	102	298	101	99	98	298	100	98	100
Av. daily gain, kg.	0.75	0.77	0.71	2.24	0.71	0.75	0.76	2.23	0.74	0.76	0.74
Feed consumed, kg.	293.3	314.2	286.9	894.4	340.0	296.5	294.2	930.7	316.9	305.5	290.6
Feed/kg. gain, kg.	1.81	1.91	1.78	5.50	2.07	1.80	1.79	5.66	1.94	1.86	1.78

TABLE IV. EFFECT OF SOURCE AND LEVEL OF CALCIUM ON SERUM CALCIUM LEVEL.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level, %	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	8
Serum Calcium mg./100 ml.	10.51	11.79	9.56	31.86	12.22	10.37	10.61	33.20	11.36	11.08	10.09

TABLE V. EFFECT OF VARYING LEVELS OF DIETARY CALCIUM ON THE pH OF THE GASTROINTESTINAL TRACT.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level, %	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	8
Stomach pH	2.83	3.49	2.70	9.02	2.35	2.99	2.95	8.29	2.59	3.24	2.83
Duodenum pH	5.80	6.06	6.10	17.96	6.03	6.24	5.89	18.16	5.92	6.15	6.00
Jejunum pH	5.84	6.25	6.31	18.40	6.14	6.18	6.30	18.62	5.99	6.22	6.31
Illeum pH	6.10	6.35	6.49	18.94	6.29	6.20	6.54	19.03	6.20	6.28	6.52
Large intestine pH	5.58	5.58	5.55	16.71	5.55	5.53	5.74	16.82	5.57	5.56	5.64

TABLE VI. EFFECT OF GRADED LEVELS OF DIETARY CALCIUM ON TISSUE, FECAL AND SERUM LIPIDS.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	8
Fecal											
Total lipid[1/]	70.43	91.41	90.75	252.59	70.67	93.65	86.25	250.57	73.59	92.53	88.50
Cholesterol[2/]	51.65	43.94	48.04	143.63	57.78	45.63	44.53	147.94	54.72	44.78	46.28
Lipid phosphorus[2/]	2.06	2.08	1.77	5.91	1.44	1.92	1.72	5.08	1.75	2.00	1.75
Serum											
Total lipid[3/]	434.94	552.84	477.81	1465.59	447.92	486.60	515.20	1449.72	441.43	519.72	496.51
Cholesterol[3/]	101.67	110.12	71.37	283.16	93.12	88.26	82.90	264.28	97.39	99.19	77.14
Lipid phosphorus[2/]	11.24	8.78	9.74	29.76	9.60	9.98	10.72	30.30	10.42	9.38	10.23
Liver											
Total lipid[1/]	118.14	120.07	106.98	345.19	119.58	111.25	111.03	341.86	118.86	115.66	109.02
Cholesterol[2/]	59.70	61.77	55.56	177.03	62.78	69.70	55.17	187.65	61.24	65.73	55.36
Lipid phosphorus[2/]	3.49	3.49	4.45	11.43	4.13	3.52	3.69	11.34	3.81	3.51	4.07
Heart											
Total lipid[1/]	108.91	111.27	98.71	318.89	98.58	109.90	96.85	305.33	103.75	110.58	97.78
Cholosterol[2/]	41.52	36.63	45.09	123.24	54.17	43.75	47.43	145.35	47.85	40.19	46.26
Lipid phosphorus[2/]	2.77	2.67	3.13	8.57	3.78	2.96	4.19	10.93	3.28	2.82	3.66

1/ Expressed as mg./gm. dry matter
2/ Expressed as mg./gm. lipid
3/ Expressed as mg./100 ml. serum

TABLE VII. EFFECT OF SEX ON CALCIUM-LIPID RELATIONSHIPS.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level, %	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	8
Fecal											
Total lipid[1]											
Gilt	69.10	102.95	78.67	250.72	82.94	108.57	93.14	284.65	76.02	105.76	85.91
Barrow	71.76	79.86	102.86	254.48	58.40	78.72	79.34	216.46	65.08	79.29	91.10
Cholesterol[2]											
Gilt	50.37	47.37	41.77	139.51	54.67	49.44	38.44	142.55	52.52	48.41	40.11
Barrow	52.94	40.52	54.30	147.76	60.90	41.81	50.62	153.33	56.92	41.17	52.46
Lipid phosphorus[2]											
Gilt	1.39	1.77	1.43	4.59	1.64	2.51	1.44	5.59	1.52	2.14	1.43
Barrow	2.75	2.40	2.11	7.26	1.25	1.34	2.00	4.59	2.00	1.87	2.05
Serum											
Total lipid[3]											
Gilt	386.0	556.75	471.1	1413.9	456.7	451.32	577.9	1485.1	421.3	504.0	524.1
Barrow	483.9	548.9	484.5	1517.3	439.2	521.9	453.3	1414.4	461.5	535.4	468.9
Cholesterol[3]											
Gilt	97.66	108.23	67.29	273.18	83.73	82.55	84.06	250.34	90.17	95.39	75.68
Barrow	105.66	112.02	75.45	293.13	102.51	93.97	81.75	278.23	104.09	103.00	78.60
Lipid phosphorus[2]											
Gilt	14.61	8.23	8.44	23.28	7.67	9.59	13.49	30.75	11.14	8.91	10.97
Barrow	7.88	9.34	11.04	28.26	11.53	10.37	7.95	29.85	9.71	9.86	9.50

TABLE VII. (CONTINUED)

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level, %	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	8
Liver											
Total lipid[1]											
Gilt	113.05	101.09	110.62	324.76	119.97	102.44	109.77	332.18	116.51	101.77	110.20
Barrow	123.22	139.05	103.35	365.62	119.20	124.58	112.33	356.11	121.21	131.82	107.84
Cholesterol[2]											
Gilt	66.39	71.19	59.13	196.71	60.84	77.35	53.17	191.36	63.62	74.27	56.15
Barrow	53.02	52.35	51.98	157.35	64.73	62.06	57.17	183.96	58.88	57.21	54.58
Lipid phosphorus[2]											
Gilt	3.59	4.08	3.94	11.52	2.69	3.36	3.86	9.91	3.14	3.72	3.90
Barrow	3.29	2.90	4.96	11.15	5.57	3.78	3.58	12.93	4.43	3.34	4.27
Heart											
Total lipid[1]											
Gilt	95.92	100.03	96.74	292.69	105.86	114.15	80.89	300.90	100.89	107.09	88.82
Barrow	121.90	122.51	100.69	345.10	91.33	105.64	112.82	309.79	106.62	114.08	106.76
Cholesterol[2]											
Gilt	52.59	36.36	50.06	139.01	60.48	34.04	58.74	153.26	56.54	35.20	54.40
Barrow	30.46	36.91	40.12	107.49	47.87	53.46	36.12	137.45	39.17	45.19	38.12
Lipid phosphorus[2]											
Gilt	2.89	2.50	2.93	8.32	3.57	2.78	5.61	11.96	3.23	2.64	4.27
Barrow	2.66	2.85	3.33	8.84	3.99	3.14	2.77	9.90	3.33	3.00	3.05

1/ Expressed as mg. of lipid per gram of dry matter.
2/ Expressed as mg. of cholesterol per gram of lipid.
3/ Expressed as mg. of phosphorus per gram of lipid.

The high calcium level caused a significant ($P < .01$) decrease in serum cholesterol content. The second calcium source, defluorinated rock phosphate, when compared to limestone (calcium carbonate), caused a significant ($P < .05$) reduction in the serum cholesterol level. It was established that gilts had a significantly ($P < .05$) lower serum cholesterol level than did the barrows. The data resulting from the sex comparison are shown in Table VII. A significant interaction ($P < .01$) occurred involving the level and source of dietary calcium and their combined effect on serum cholesterol. A comparison of the two calcium sources and their effect on heart cholesterol levels showed that, contrary to its magnified effect on serum-cholesterol levels, the limestone calcium source now caused a drop in the cholesterol level ($P < .05$). It was revealed that gilts had a significantly higher ($P < .05$) cholesterol concentration in heart tissue than did the barrows; this is in direct contrast to serum cholesterol results, where the gilts had the lower level ($P < .05$). The dietary calcium level and the sex of the animal combined to induce an effect on the cholesterol content of the heart tissue ($P < .05$). Treatment failed to influence liver or fecal cholesterol levels.

DISCUSSION

Calcium and Phosphorus Requirements

On the basis of growth rate and physical appearance, there appeared to be no evidence of a calcium deficiency of the lower level of intake (0.4%). The intake of calcium and phosphorus was in a 1:1 ration which appeared to be adequate for skeletal maintenance and growth at all levels. These observations are in accord with the earlier work of Bethke et al. (1932), Theiler et al. (1937), and Chapman et al. (1962). Although there was no significance shown in average daily gain of pigs due to level of calcium, the average daily gain of those fed 0.8 percent calcium was slightly higher than those fed the 0.4 and 1.2 percent calcium levels. This is supported by earlier data (Aubel et al. 1936, Miller et al. 1960, Rutledge et al. 1961 and Chapman et al. 1962).

Overall feed efficiency was not grossly affected by calcium content of the diet. This conclusion is supported by Rutledge et al. (1961) and Newman et al. (1967). Combs et al. (1962) reported similar results involving dietary phosphorus.

The data suggested an increase in feed efficiency with increment increases in dietary calcium levels. Average data on feed efficiency resulted in an apparent trend for increased efficiency with increased increments of calcium; however, this is not consistent within calcium source. These results contrast with data reported by Chapman et al. (1962). However, Chapman's results came from a study utilizing only a 0.2 percent dietary phosphorus level. This could confound the data as an increase in dietary calcium levels would cause a servere corresponding increase in the

calcium:phosphorus ratio.

Haldt et al. (1939) observed that amounts of dietary calcium and phosphorus in excess of 1.2 percent, interfered to a slight extent with the utilization of other nutrients. This is contrary to the data presented, but it must be pointed out that Haldt found these results only with excessive dietary calcium and phosphorus levels. It has been pointed out by Davis (1959) that an excessive calcium intake will induce an adverse effect on the utilization of magnesium, iron, iodine, manganese, zinc and copper, which in turn may cause poor growth and efficiency. These researchers indicate that moderate calcium levels (0.5 - 0.8 percent) do not cause adverse effects on nutrient digestibilities; however, when excessive amounts (over 1.2 percent) of calcium are introduced, there is, indeed, detrimental effects.

The serum calcium data from the present investigation demonstrated no individual effect due to treatment. These results are in agreement with Dunlop (1935), Aubel et al. (1936), Theiler et al. (1937) and Rutledge et al. (1961). However, an interaction developed between the level and source of dietary calcium ($P < .05$). This interaction resulted from opposite flucuations in serum calcium concentration at the 0.8 percent level. The limestone source caused an increase while the defluorinated rock phosphate induced a decrease in the serum calcium level. Further investigation is necessary to elucidate a possible mechanism for this interaction.

Calcium-Phosphorus Source Effect

The average daily gain data collected in this study did not demonstrate

any significant differences in the efficacy of the two calcium sources. These results are substantiated by Thomas et al. (1933), McCampbell and Aubel (1934), Colovos et al. (1958), Combs and Wallace, (1962) and Van Zante et al. (1967).

Calcium-Fat Relationship

Calcium and Fecal Total Lipid

Evidence has been reported as far back as 1917 (Givens, 1917) suggesting that high dietary calcium levels cause a decrease in the lipid absorption which was demonstrated by increased fecal lipid levels. This theory is supported by the later work of Telfer (1921, 1930), Bassett et al. (1939), Carroll and Richards (1958), Fedde et al. (1960). Drenick (1961) and Bhattacharyya et al. (1968). However, occasionally data from the literature does not conform entirely to this idea. Holt et al. (1919_a, 1919_b) for example, concluded from a study using infants that a high calcium intake did not necessarily cause a large loss of fat in the feces. Ward and Reid (1948) found no effect on fecal lipid levels due to increased calcium ingestion in cattle. Wells and Cooper (1958) found that calcium chloride inhibited cholesterol absorption but had no consistent effect on fecal total lipids. Combs and Wallace (1962) using pigs, demonstrated no difference in the digestion coefficient for ether extract regardless of dietary calcium level.

Data collected from the present investigation are in agreement with the later group, which showed that increased calcium ingestion caused no effect on fecal lipid levels. It should be noted that the diets fed in

this study contained no exogenous fat other than that common to barley. The soybean meal included as a source of protein was solvent extracted and contained less than one percent lipid by analysis. The diets contained approximately 2.5 percent lipid.

Research supporting this data was conducted by Cheng et al. (1949). They found that with rats there was an inverse relationship between melting point and digestibility of triglycerides. Calcium did not influence the digestibility of low melting point fats, although it markedly reduced the digestibility of higher melting triglycerides. Most natural fats and oils melting below 50° centigrade have long been recognized as being almost completely digestible. Deuel (1948), in a study of 34 vegetable fats and oils melting below 50° centigrade, found that only two had coefficients of digestibility below 95 percent. These results are supported by Langworthy and Holmes (1915), Holmes (1919) and Deuel and Holmes (1922). This leads to the assumption that unless the diet contains a source of fat other than lipids common to all grain diets, little effect on fat digestibility can be expected as a result of dietary calcium levels. An investigation of the methods used by the researchers advocating an inhibitory effect upon fat digestibility by dietary calcium reveals that in every case there was a high melting fat added to the diets.

Calcium and Serum Total Lipids:

Fleischman et al. (1966, 1967) found that increased dietary calcium caused a corresponding decrease in serum total lipids. The results of the present investigation support these findings only in part. Data

revealed that increased calcium ingestion caused a significant drop in serum cholesterol but not in total lipid. Boyd (1935) substantiated these results with human subjects on somewhat reduced fat intakes. He found also that increased dietary calcium levels had no lasting effect on serum total lipid levels. A comparison of the melting points of the dietary fats used in the present study and that of Fleischman's may help to explain the differences in findings. Fleischman et al. (1966, 1967) obtained his results using diets supplemented with high melting point fats; consequently, they were poorly digestible. The majority of the fats present in these experimental diets were of the low melting variety, characteristic of grains, and readily digestible regardless of calcium level. Since calcium has a negligible effect on low melting point fats, it would be logical to expect little or no effect on the serum total lipid concentration due to increased dietary calcium levels. However, cholesterol seems to be inhibited by calcium; therefore, the cholesterol level could be significantly changed by increased dietary calcium without inducing a corresponding change in serum total lipids.

Calcium and Tissue Total Lipids:

Data indicated no effect on liver and heart total lipid levels by increasing increments of dietary calcium. This is in agreement with results reported by Fleischman et al. (1966, 1967).

Calcium and Serum Cholesterol:

Increased calcium ingestion caused a significant decrease in serum cholesterol ($P < .01$). This lowering of serum cholesterol by calcium is

interesting from two points of view. First, Wells and Cooper (1958) reported that calcium inhibited cholesterol absorption in rats in the presence of dietary fat. They also pointed out that calcium chloride had a more pronounced inhibitory effect on cholesterol absorption than did calcium carbonate. Calcium was also reported to be hypocholesterolemic in rabbits (Iacona et al., 1960), humans (Yacowitz, 1962, 1965; Bhattacharyya et al.,1968), and rats (Fleischman et al., 1966, 1967). The hypocholesterolemic activity of calcium may be due in part, to a reduced absorption of exogenous cholesterol. The second reason for interest in the hypolipemic action of calcium is repeated reports of a negative relation between mortality from cardiovascular disease and the hardness of drinking water (Schroeder, 1960_a, 1960_b; Morris et al., 1961). As calcium is the major cation associated with water hardness, it is not unexpected that a similar negative correlation would be found between calcium-ion concentration and cholesterol.

Calcium and Fecal Cholesterol:

Treatment had no effect on the level of cholesterol excreted in the feces. Fleischman et al. (1967) reported an increase in fecal cholesterol corresponding to an increase in dietary calcium. One possible explanation for this lies in the types of cholesterol analysis employed. Fleischman et al. (1967) employed an anthrone procedure to determine 3-Beta-hydroxysterols. Anthrone reagent reacts with any number of structures. In this case it was the ring hydroxyl group, characteristic of cholesterol. The fallacy with this procedure is that many sterols other than choles-

erol, such as coprostanol and sitosterol, plus such things as glycolipids will also react with anthrone. Therefore, it is possible that the significance resulting from Fleischman's investigation may have been due to material somewhat foreign to cholesterol. The procedure used in the present investigation was the Leiberman-Burchard reaction which does not differentiate between cholesterol, sitosterol or coprostanol. As sitosterol is a major sterol for plants, the barley-base diets probably contributed sitosterol to the feces. Also coprostanol has been shown to occur as the chief fecal sterol component of practically all animals; therefore, it, too, might be present in large quantities in the feces. These two sterols, sitosterol and coprostanol, could have been present in such quantities as to have masked the actual cholesterol flucuations resulting from increased dietary calcium levels, thus accounting for these nonsignificant results. Also Fleischman employed diets containing high melting point lipids, in contrast to the diets used in this study.

Calcium and Tissue Cholesterol:

The limestone calcium source when compared to defluorinated rock phosphate appeared to cause a significant ($P \leq .05$) decrease in heart cholesterol content. The limestone calcium source supplied the calcium in the form of calcium carbonate compared to tricalcium phosphate from the defluorinated rock phosphate. The difference in anion form may explain the difference in effect. However, results such as these have never been reported before with any type of tissue and it is felt that more data are necessary to verify this point.

Treatment had no effect on liver cholesterol concentrations.

Calcium-Fecal Lipid Phosphorus:

Treatment caused no effect on the excretion of lipid phosphorus. Fleischman et al. (1967) reported a corresponding nonsignificant effect on fecal lipid phosphorus due to increased dietary calcium. However, in an earlier report, Fleischman et al. (1966) did find a significant increase in fecal lipid phosphorus concentrations due to increased dietary calcium levels.

Calcium-Tissue Lipid Phosphorus:

Data indicated that tissue lipid phosphorus levels were unaffected by increased dietary calcium levels. In a 1966 investigation, Fleischman et al. found these same results in relation to increasing dietary calcium level. However, in a later experiment, Fleischman et al. (1967) found a significant decrease in liver lipid phosphorus levels due to increased dietary calcium ingestion. This decrease was noted only at the 1.2 percent calcium level not at higher or lower levels. These same results were noted with heart lipid phosphorus concentrations.

Calcium-Serum Lipid Phosphorus:

Treatment apparently had no noticeable effect on serum lipid phosphorus concentrations. This is in contrast with results found by Fleischman et al. (1966, 1967) who found that increased dietary calcium caused a decrease in serum lipid phosphorus levels. Yacowitz et al. (1965) found that he was unable to induce a reduction in serum lipid phosphorus levels because of increased dietary calcium ingestion.

Sex Effect

This investigation indicated that gilts had a lower serum cholesterol level than did barrows. Although it only approached significance, the total lipid content of the liver and heart also supported this finding. The cholesterol concentration of the heart, however, indicated a lower concentration for the barrows. The sex hormones of each respective animal may be involved in causing this difference. Okey et al. (1934) found that females may have a higher liver total lipid level but lower cholesterol content when compared to males. This same result was found for phospholipids. Although the present investigation did not yield significant results in the liver and heart lipid phosphorus concentration, it did appear that the barrows had higher lipid phosphorus concentration than the gilts. This sex effect is supported by Deuel et al. (1934) and Barnes et al. (1941). It should be emphasized that the present study was conducted using females and castrated males rather than males.

Interactions

Cholesterol

Data from the calcium-serum cholesterol relationship resulted in a two-way interaction between calcium level and calcium source. This significant ($P < .05$) interaction was the result of a corresponding drop in cholesterol level at the 1.2 percent dietary calcium level for both sources. The difference originated from the magnitude of the decrease. The limestone source induced a drop in cholesterol level almost twice that of the defluorinated rock phosphate calcium source. This variation may be the

result of the different form in which the calcium is supplied by each source: limestone, calcium carbonate and defluorinated rock phosphate, tricalcium phosphate.

The cholesterol data revealed a second interaction involving the effect of dietary calcium level and sex on the heart cholesterol concentration. This significant ($P \leq .05$) interaction was the result of opposite flucuations in cholesterol levels at the 0.8 percent dietary calcium level. At the 0.8 percent dietary calcium level, the heart cholesterol level decreased for gilts and increased for barrows. The cholesterol level remained unaffected by either the lower or higher dietary calcium levels.

Lipid Phosphorus

There was a significant ($P < .05$) three-way interaction found in analyzing the effect of calcium level, calcium source and sex on serum lipid phosphorus. As there were no significant effects due to main treatments (calcium level, calcium source and sex) on this parameter, it may be assumed that the interaction was an artifact and resulted from random variation.

SUMMARY

One trial involving 24 pigs was conducted to study the effects of varying levels of dietary calcium from two conventional scources, limestone and defluorinated rock phosphate, upon lipid excretion and deposition in swine. Comparisons were made on the effect of dietary calcium levels, sources and animal sex.

Feedlot performance was essentially the same for all treatments. The barrows exhibited the traditional increased average daily gain over the gilts ($P < .01$). There was a trend toward increased feed efficiency with increased increments of dietary calcium. Intestional pH remained unaffected by calcium level, calcium source and sex. The 0.8 percent dietary calcium level and the two calcium sources combined to cause an opposite flucuation in the serum calcium concentration ($P < .05$).

There was no significant effect on the total lipid concentration of the liver, heart, feces and serum due to treatment.

Serum cholesterol content was significantly ($P < .01$) decreased by the 1.2 percent level of dietary calcium. The defluorinated rock phosphate calcium source caused a lower ($P < .05$) serum cholesterol level than did the limestone calcium cource. Gilts exhibited a lower serum cholesterol level than did the barrows ($P < .05$). The high dietary calcium level from the limestone source caused a larger drop ($P < .05$) in serum cholesterol level than did the corresponding dietary calcium level from the defluorinated rock phosphate calcium source. A significant ($P < .05$) drop in heart cholesterol levels was noted as a result of the limestone dietary calcium source.

Gilts had a higher cholesterol concentration in the heart tissue ($P < .05$) and a lower serum cholesterol level ($P < .05$) than did the barrows. The 0.8 percent dietary calcium combined with the sex of the animal to induce an opposite flucuation in the heart cholesterol level ($P < .05$). Treatment failed to effect liver or fecal cholesterol levels.

The serum lipid phosphorus level was subject to effect by the combined interaction of calcium source, calcium level and sex. The lipid phosphorus levels of the liver, heart and feces were uninfluenced by treatment.

APPENDIX A

APPENDIX TABLE I. ANALYSIS OF VARIANCE OF THE EFFECT OF VARYING LEVELS OF DIETARY CALCIUM ON LIPID EXCRETION AND DEPOSITION; SERUM CALCIUM LEVEL, INTESNTINAL pH AND AVERAGE DAILY GAINS IN SWINE.

Source of Variation	Degree of Freedon	Mean Squares					
		Average Daily Gain	Blood Calcium	Total Lipid			
				Fecal	Serum	Liver	Heart
Total	23						
Level (A)	2	.0081	3.5426	1,095.3843	12,936.0300	201.8146	328.0514
Source (B)	1	.0001	1.1396	2.8085	167.9070	7.2161	122.3113
Sex (C)	1	.0726	.5490	691.9782	178.3260	799.9531	626.4860
A X B	2	.0142	5.3840	24.1468	5,870.0320	92.6245	50.4874
A X C	2	.0277	.5965	501.3174	5,559.5620	670.7649	90.2905
B X C	1	.0060	4.5851	862.9198	5,048.3170	25.7501	315.6635
Z C B X C	2	.0088	1.3163	125.7273	7,035.9160	73.2393	688.1019
Error	12	.0106	1.1467	458.7694	8,436.0001	215.9773	205.1748

APPENDIX TABLE I. (CONTINUED)

Source of Variation	Degree of Freedom	Mean Squares					
		Average Daily Gain	Blood Calcium	Cholesterol			
				Fecal	Serum	Liver	Heart
Total	23						
Level (A)	2	.0081	3.5426	229.3719	1,200.0271	216.4609	130.5783
Source (B)	1	.0001	1.1396	12.3554	237.3846	75.2250	325.6804
Sex (C)	1	.0726	.5490	60.2934	381.9228	364.3383	373.5915
A X B	2	.0142	5.3840	46.4844	565.2406	34.9332	53.2419
A X C	2	.0277	.5965	194.2912	55.0196	133.9238	479.7781
B X C	1	.0060	4.5851	1.0752	10.4538	170.2936	41.1601
A X B X C	2	.0088	1.3163	3.0013	65.6769	277.6274	131.3949
Error	12	.0106	1.1467	182.7733	58.9669	129.5742	79.8482

APPENDIX TABLE I. (CONTINUED)

Source of Variation	Degree of Freedom	Mean Squares					
		Average Daily Gain	Blood Calcium	Lipid Phosphorus			
				Fecal	Serum	Liver	Heart
Total	23						
Level (A)	2	.0081	3.5426	.6601	2.4597	.6422	1.4313
Source (B)	1	.0001	1.1396	.0630	.1873	.0046	3.6817
Sex (C)	1	.0726	.5490	2.6070	2.5611	1.0542	.3953
A X B	2	.0142	5.3840	.2616	4.9866	.9784	.3740
A X C	2	.0277	.5965	1.7787	3.8207	1.5781	.5568
B X C	1	.0060	4.5851	.3385	.7561	1.7442	1.1008
A C B X C	2	.0088	1.3163	.7041	44.1832	2.5214	3.0537
Error	12	.0106	1.1467	2.0293	7.2885	1.3661	1.2769

APPENDIX TABLE I. (CONTINUED)

Source of Variation	Degree of Freedom	Mean Squares: Gastronintestinal Tract Intestinal pH				
		Stomach	Duodenum	Jejunum	Illeum	Large Intestine
Total	23					
Level (A)	2	.8652	.1166	.2147	.2195	.0207
Source (B)	1	.3505	.0235	.0301	.0051	.0084
Sex (C)	1	3.7605	.2710	.1001	.2301	.1001
A X B	2	.3631	.1264	.0808	.0576	.0341
A X C	2	.7781	.0678	.0313	.0719	.0802
B X C	1	.9599	.0084	.1752	.0551	.0010
A C B X C	2	.3236	.0167	.0582	.0456	.0322
Error	12	.9594	.1276	.0782	.0747	.0574

APPENDIX TABLE II. EFFECT OF SOURCE AND LEVEL OF CALCIUM ON BODY WEIGHT GAIN AND FEED EFFICIENCY.

Calcium source	Limestone			Source Total	Defluorinated Rock Phosphate			Source Total	Average Both Sources		
Calcium level, %	0.4	0.8	1.2		0.4	0.8	1.2		0.4	0.8	1.2
No. animals	4	4	4	12	4	4	4	12	8	8	12
Av. Initial weight, lbs.	45	45	46	136	45	46	47	138	45	45	46
Av. Final weight, lbs.	207	210	207	624	209	210	210	629	208	210	208
Av. Total gain, lbs.	162	165	161	488	164	165	164	493	163	165	162
Days fed	98	98	102	298	101	99	98	298	100	98	100
Av. daily gain, lbs.	1.66	1.69	1.58	4.93	1.58	1.66	1.67	4.91	1.62	1.68	1.63
Feed consumed, lbs.	646	692	632	1970	749	653	648	2050	698	673	640
Feed/lb. gain	3.99	4.20	3.92	12.11	4.56	3.97	3.96	12.46	4.28	4.09	3.94

APPENDIX B

ANALYTICAL PROCEDURES

Total Lipid Determination: (Williams, 1962)

Reagents:

1. Chloroform, reagent grade, redistilled.
2. Methanol, reagent grade, redistilled
3. Chloroform-methanol mixed in ratio of 2:1.
4. Glacial Acetic Acid, reagent grade.

Procedure:

The solvent mixture and the homogenized undried sample, containing up to 3 grams of dry material, were mixed together in a glass-stoppered flask to give a final liquid volume of 45 milliliters. An estimate of the water content of the sample was made first and the solvent mixture prepared to give a ratio of chloroform methanol: water: acetic acid of 1.0:0.35:0.90, with the water already present in the samples being considered. (The water:chloroform methanol ratio may vary from 0.3 to 0.4 with maximal extraction still obtained.) This mixture was then shaken vigorously for 5 minutes and left standing for an additional 25 minutes. With fecal, liver, and heart material, the mixture was next filtered to remove all particulate matter. This was not necessary for serum, however. After filtering, duplicate 20 milliliter aliquots were transferred to graduate 50 milliliter glass-stoppered centrifuge tubes. Chloroform-methanol was then added to give a final volume of 35 milliliters. After mixing by inversion several times, the tubes were centrifuged at 1500 rpm's for 15 minutes. Twenty-five milliliters of the supernatants were transferred to 50 milliliter graduated glass-stoppered centrifuge tubes. Distilled water was then added to make a final volume of 50 milliliters. The tubes were stoppered, shaken vigorously for 10 seconds,

and centrifuged for 10 minutes at 1600 rpm's. The water layer, on top, was withdrawn with suction and discarded (with care so as not to remove any of the interface solids that may have formed). The washing was performed two more times using 14 milliliters of water each time. The washed extracts were then brought to 45 milliliters with chloroform-methanol, and the contents of the tubes were mixed by inversion. The extracts were then filtered through a medium porosity ground glass funnel. (It is important that filter paper not be used for the filtration as it was found that up to 30 percent of the phospholipids could be lost by absorption in Whatman Number 1 filter paper, Williams, 1962). The filter was washed 3 times with 5 milliliter aliquots of chloroform-methanol and the extract was made to 50 milliliters. A 5 milliliter aliquot was placed in a preweighed vial and taken to complete dryness in an oven and weighed again.

Calculations:

A= Amount of lipid component per gram of wet sample.

B= Amount of lipid component in aliquot of final extract taken for analysis as determined by weight.

C= Volume of final extract taken for analysis (milliliter).

D= Weight of sample taken for homogenization (grams).

$$A = \frac{35}{25} \times \frac{45}{20} \times B \times \frac{50}{(C)\ (D)}$$

Cholesterol Determination: (Abdell et al., 1952)

Reagents:

1. Absolute alcohol, redistilled.
2. Petroleum ether, redistilled.
3. Acetic acid, reagent grade.
4. Sulfuric acid, reagent grade.
5. Acetic anhydride, reagent grade, free from hydrochloric acid.
6. Potassium hydroxide, reagent grade, 33 percent. (10 grams of reagent grade potassium hydroxide dissolved in 20 ml. of water.)
7. Alcoholic potassium hydroxide solution, prepared just before using. (6 ml. of 33% potassium hydroxide to 94 ml. of absolute alcohol.)
8. Standard cholesterol solution. (1 mg./1 ml.)
9. Modified Liebermann-Burchard reagent. (20 volumes of acetic anhydride chilled to a temperature lower than 10 degrees centigrade in a glass-stoppered container, 1 volume of concentrated sulfuric acid was added, the mixture well shaken and kept cold for 9 minutes; 10 volumes of glacial acetic acid were added and the mixture was warmed to room temperature.) The reagent had to be used within one hour after preparation.
10. Blank: Liebermann-Burchard, reagent.

Procedure:

A 0.5 milliliter aliquot of the lipid extract was measured into a 50 milliliter glass stoppered centrifuge tube, and 5 milliliters of alcoholic potassium hydroxide were added to each tube. The tubes were stoppered, well shaken, and incubated in a water bath at 37-40 degrees centigrade for 55 minutes. After cooling the tubes to room temperature, a 10 milliliter volume of petroleum ether was added and mixed well with the contents of each tube. Five milliliters of water were then added and the tubes were shaken vigorously for 1 minute. If an emulsion did not break, the tubes were centrifuged at 1,000 rpm's for 5 minutes, at which time two clear layers should have formed. A 4 milliliter aliquot

of the petroleum ether layer was transferred to a dry 40 milliliter erlenmeyer flask. (The analysis could be interrupted at this point, and the flasks stoppered and kept until it was convenient to complete the procedure.) The petroleum ether was evaporated by placing the flasks in a pan of water at room temperature and gradually heating to 80 degrees centigrade. A stream of air was blown over the flasks while they were drying. After the water had reached 80 degreees centigrade, the samples were removed and placed in a drying oven at 100 degrees centigrade for 10 minutes to insure complete dryness. After cooling to room temperature, the flasks were stoppered with clean, dry corks.

The flasks containing the dry residues from the samples and the standards were arranged so that a standard came at the beginning, one in the middle, and one at the end of the run. A clean, dry flask was placed at the beginning to receive the blank. Six milliliters of the Liebermann-Burchard reagent were added to the flasks at one minute intervals. Once the Liebermann-Burchard reagent had been added to a tube, care was taken to wash down the entire inner surface of the flask with the reagent. The flasks were tightly stoppered and placed in a dark drawer for color development. Contact between the flasks and light was kept at a minimum. The optical density of each flask was read against a blank in a Beckman DB-G spectrophotometer at 620 millimicrons, 30 minutes after the reagent was added.

Calculations:

1. Volume of sample x Concentration of Standard = A

2. 4/10 x A = B

3. $\frac{B}{\text{Optical Density of Standard}} = K$

4. K x Optical Density of Unknown x $\frac{100}{B}$ = mg/100 milliliters

Lipid Phosphorus Determination: (Pons et al., 1953)

Reagents:

1. Molybdate reagent: Dissolve 50 grams of ammonium molybdate in 400 milliliters of 10 normal sulfuric acid and dilute to 1 liter with distilled water. Store in a paraffin-lined bottle.

2. Sulfuric Acid, approximately 1 N : Dilute 280 milliliters of concentrated sulfuric acid to one liter to get 10 N, then take one part of this to nine of water to get 1 N.

3. Stannous Chloride, stock solution: Dissolve 10 grams of stannous chloride dihydrate in 25 milliliters of concentrated hydrochloric acid. Stored in a glass-stoppered brown bottle.

4. Stannous Chloride, dilute solution: 1 milliliter of stock solution plus 200 milliliter of 1 N sulfuric acid, prepare just prior to use.

5. Isobutyl alcohol, reagent grade.

6. Ethyl alcohol, 95 percent which can be used to make 70 percent by diluting 73.5 milliliters of 95 percent to 100 milliliters with distilled water.

7. Magnesium nitrate, saturated solution: Magnesium nitrate hexahydrate in 95 percent alcohol.

8. Standard Phosphate solution: Recrystallize A.C.S. grade monobasic potassium phosphate (KH_2PO_4) three times from water, dry at 110° C., and store in a desiccator over concentrated sulfuric acid. Dissolve 4.3929 grams of dry salt in 300 milliliters of water

and 200 milliliter of 1 N sulfuric acid contained in a 1 liter volumetric flask, add several drops of 0.1 normal potassium permanganate, and dilute to volume with distilled water. This stock solution, 1.0 milligram phosphorus per milliliter, is stable.

Procedure:

The material was first extracted using the before-mentioned total lipid extraction procedure. A 10 milliliter volume of the lipid extract was taken to dryness in a rotary evaporator and then redissolved in a volume of 95 percent ethyl alcohol. Following the addition of the 95 percent ethyl alcohol, the solution was heated to insure complete solvation of all phospholipids. A second and third washing was completed using 95 and 70 percent ethyl alcohol, respectively, heating each time. After each washing, the alcohol was poured into an ashing crucible. The crucibles were placed on a hot plate and subjected to a flow of air to enhance evaporation of the alcohol. Once the volume of alcohol reached a volume of 1 to 3 milliliters, 3 milliliters of magnesium nitrate were added to each crucible. The crucibles were allowed to continue heating until the samples were dry, at which time the heat was increased to effect a partial ashing of the sample. The crucibles were then placed in a muffle furnace and heated at 600 degrees centigrade for 4 to 5 hours or until a white ash was obtained. The crucibles were allowed to cool to room temperature. The ash was dissolved in 5 milliliters of 10 sulfuric acid. It was necessary to heat the acid and ash together in order to get all the ash in solution. The acid solution was then transferred quantitatively to a 100 milliliter volumetric flask using distilled water to effect the transfer.

The solution was then brought to volume using distilled water.

A 5 milliliter aliquot of the extract was quantitatively measured into a 60 milliliter separatory funnel. Five milliliters of the molybdate reagent were added to the extract and the solution was diluted to 20 milliliters with distilled water. Ten milliliters of isobutyl alcohol were added and the contents were shaken for two minutes. The lower, aqueous, layer was discarded and the extract was washed by shaking for one-half minute with 10 milliliters of 1 Normal sulfuric acid; again the water layer was discarded. The solution was then shaken for one minute with 15 milliliters of dilute stannous chloride, the water being discarded. The blue isobutyl alcohol layer was quantitatively transferred to a 50 milliliter volumetric flask using 95 percent ethyl alcohol. The optical density was determined by comparing the blue solution against a blank containing all reagents. A Beckman DB-G spectrophotometer set at 730 millimicrons was used at any time from 40 minutes to 19 hours after the color had developed, to determine the optical density.

Calculations:

1. $$\frac{1}{\text{Volume of unknown phosphorus solution}} \times \frac{\text{Volume of the phosphorus solution taken for analysis}}{1} = A$$

2. $$\frac{\text{Concentration of Standard}}{\text{Optical Density of Standard}} = \frac{\text{Concentration of Unknown}}{\text{Optical Density of Unknown}} = B$$

3. B x A = Milligrams of phosphorus per gram of material.

Serum Calcium Determinations: (Oser 1965)

Reagents:

1. Standard calcium solution (2.0000 milligrams calcium

per milliliter)
2. Saturated ammonium oxalate
3. Ammonium hydroxide (2 N) saturated with calcium oxalate (wash solution)
4. 10 percent sulfuric acid (v/v)
5. Potassium permanganate concentrated (6.9009 grams per liter = 2 N)
6. Potassium permanganate dilute solution (1 part of concentrate to 98 of distilled H_2O)
7. Acetic acid diluted 1-4 with H_2O
8. Ammonium hydroxide diluted 1 to 4 with H_2O
9. Methyl red indicator
10. 2 N hydrochloric acid.

Procedure:

A 4 milliliter aliquot of serum was diluted with an equal volume of distilled water. Three milliliters of saturated ammonium oxalate was added to the sample and allowed to stand for a minimum of four hours, preferrably overnight. The solution was then centrifuged at 2000 rpm's for 20 minutes. The supernate was decanted and the remaining precipitate was washed 2 times using the wash solution: Five milliliters of 10 percent sulfuric acid was added to dissolve the precipitate. The samples were then heated to 90° centigrade and titrated with the dilute potassium permanganate solution. The standard was prepared in the range of .4 milligrams per milliliter and treated exactly like the serum.

Calculations:

1. $\frac{\text{Concentration of Standard}}{\text{ml } KMnO_4 \text{ for Standard}} = \frac{\text{Concentration of Unknown}}{\text{ml } KMnO_4 \text{ for Unknown}} = \text{mg Ca/ml.}$

2. $\frac{\text{mg Ca/ml}}{\text{ml of serum}} \times 100 = \text{mg \%}$

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