THE EFFECT OF ORALLY ADMINISTERED *AEROBACTER AEROGENES* AND *ESCHERICHIA COLI* CULTURES ON CHICK GROWTH AS INFLUENCED BY PENICILLIN

By

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

The finding that certain antibiotics stimulate the growth of chicks, poults, and other rapidly growing animals when included in their diets has aroused much interest among investigators in the fields of nutrition, chemistry, and biology regarding the exact mode of action involved. The elucidation of the mechanism of antibiotic growth stimulation, aside from the nutritional implications, would indeed be a valuable contribution to the field of human and veterinary medicine.

In a relatively short period, after the announcement by Stokstad and Jukes (1950) that crystalline aureomycin was effective in promoting the growth of chicks, numerous reports became available on the efficacious use of such antibiotics as penicillin, aureomycin, terramycin, bacitracin, and streptomycin in animal feeds. The beneficial effects of these antibiotics on the growth of chicks, poults, and swine led to their wide acceptance as dietary supplements by the feed industry. The value of antibiotic feed supplements approaches the figure of $30 million per year and sales of antibiotics in the purified form for veterinary use are estimated at $5 million (Lazier, 1951).

Several antibiotics which differ chemically have been reported to serve as growth promotants. Some work has shown that injected moieties of antibiotics, including those having antibacterial activity and those which have been rendered inactive by heat or enzymatic activity, are devoid of growth promoting properties. Accordingly, it is believed that antibiotics exert their effect indirectly through alterations of the intestinal microflora of the host instead of functioning as a metabolite per se.

The main theories pertinent to the mode of action of antibiotics in

promoting growth may be listed as follows: (1) antibiotics have a static effect on certain intestinal microorganisms, the metabolic by-products of which are detrimental to the growth of the animal, (2) the drug prevents the excessive proliferation of certain intestinal microorganisms which compete with the host for essential nutrilites, (3) as a result of dietary administration of antibiotics an imbalance of intestinal bacteria is established whereby certain surviving organisms produce an as yet unidentified growth factor, (4) large amounts of known nutrients are synthesized for intestinal adsorbtion thereby beneficially affecting the host, and (5) antibiotics function to bring about physical changes within the intestinal tract to enhance the absorption of nutrients.

Early studies by Komoser et al. (1952) revealed that three intestinal microorganisms appeared to be affected when antibiotics were administered to chicks. When antibiotics, especially procaine penicillin G, were included in the diets of chicks at a level of 150 ppm, an increase in the number of Aerobacter aerogenes and Escherichia coli normally present in the ceca was noted. When no antibiotics were administered, and when chick growth was especially poor, high numbers of Lactobacillus bifidus were observed. This organism was rarely seen in birds which responded favorably to antibiotic treatment.

Consequently, this investigation was initiated in an effort to determine if these three organisms could be definitely related to the overall mechanism of antibiotic growth stimulation, or if their appearance was only coincidental.

Experiments were performed wherein various mashes were fermented with Aerobacter aerogenes and used as supplements in chick diets. The effect on chick growth of orally administered preparations containing viable, pure

cultures of A. aerogenes, E. coli, and L. bifidus was investigated. Studies were also performed to determine the effect of environmental and dietary influences on the growth response of chicks to antibiotics, viable microorganisms, and a combination of antibiotics and viable microorganisms.

REVIEW OF THE LITERATURE

The Use of Antibiotics in Poultry Nutrition

Following the discovery that vitamin B_{12} was active in replacing a substantial portion of the factor present in animal proteins which is necessary for the hatchability and growth of chickens, an intensive search was begun for a readily available source of this vitamin which would profitably lend itself to incorporation into animal feeds. The Lederle group, using a chick assay, found that the residue from mashes which had been fermented with Streptomyces aureofaciens, in the preparation of aureomycin, was an excellent source of vitamin B_{12} and, in fact, found that this substance gave better growth than did crystalline B_{12} (Stokstad et al., 1949). This added response was ascribed to a second "animal protein factor" but further investigation led to the announcement by Stokstad and Jukes (1950) that the additional factor present in the fermented product was due to the presence of residual amounts of aureomycin which was not removed by the extraction process.

The effect of various antibiotics on the growth of chicks and poults. In an early report, Briggs et al. (1944) stated that sulfasuxidine and high levels of ascorbic acid stimulated the growth of chicks when these drugs were added to purified rations which were adequate in all nutrients then known to be required. Moore et al. (1946) also found that streptomycin, as well as sulfasuxidine, could stimulate the growth of chicks. These early accounts appeared at a time when cost and availability of antibiotics rendered non-profitable their inclusion into animal feeds. However, interest in the use of antibiotics as feed supplements was renewed when Stokstad and Jukes (1950) reported that crystalline aureomycin favorably affected the

growth of chicks. Whitehill et al. (1950) added 100 ppm of penicillin to a corn-soybean type diet and found at the end of four weeks that chick growth was increased. McGinnis et al. (1950) showed that streptomycin gave supplemental growth stimulation to chicks and poults above that which was obtained with the control diet. Moore et al. (1946), and later Groschke and Evans (1950) also reported that crystalline streptomycin would stimulate the growth of chicks. In addition, the effectiveness of bacitracin in the stimulation of the growth of chicks was demonstrated by Kramke and Fritz (1951). These investigators found that this antibiotic afforded optimum growth stimulation of chicks to four weeks of age when the level included in the diet was 10 ppm.

Based on this preliminary work, experimentation with levels and other conditions which would elicit optimum results with various crystalline antibiotics was initiated.

Bird et al. (1951) found that the addition of 250 ppm of crystalline aureomycin to an all-plant protein ration stimulated the rapid growth of chicks to six weeks of age. After this time, however, the growth rate was sharply reduced by the complete withdrawal of the antibiotic. Coates et al. (1951a), using 25 ppm of crystalline aureomycin in the diet, obtained excellent results and Ingram and Edgar (1951) reported that this antibiotic gave a growth response of 20 per cent over the basal ration at four weeks. Scott et al. (1951) obtained no response to 15 ppm aureomycin when it was added to a test ration consisting of ground corn, soybean oil meal, DL methionine and minerals and known vitamins. However a definite response to the antibiotic was obtained when corn distillers' solubles, condensed fish solubles, and whey and butyl fermentation solubles were also present. These results seem to indicate that the crude supplements were supplying a factor which

was necessary for the antibiotic response. A similar relationship was found to exist between antibiotic response and the presence of crude supplements in the diet by Bird (1951), who reported that streptomycin gave an optimal response at a level of 40 ppm during the first five weeks of growth if fish meal was present in the diet but much higher levels of the antibiotic were needed on an all-vegetable protein ration. Elam et al. (1951a) reported that chickens at ten weeks of age which had been fed an all vegetable ration supplemented with 33 ppm of procaine penicillin were larger than those birds which received no antibiotic supplement.

Reynolds et al. (1951) compared levels of 2 and 5 ppm of penicillin and terramycin in chick diets and concluded that the two antibiotics were optimally effective at the lowest level used and compared identically with each other, gram for gram, in so far as growth promoting activity is concerned. Franke and Fritz (1951) fed graded levels of several antibiotics in practical starting rations to chicks and poults. Aureomycin, bacitracin, penicillin, and terramycin gave optimum stimulation at a level of 10 ppm. Penicillin at a level of 5 ppm was more effective than any of the other antibiotics. Maximum percentage gains, amounting to about 20 per cent with chicks and slightly higher with turkeys, were observed at about four weeks of age. When streptomycin was added at a level of 66 ppm to a corn-soybean oil meal type ration, Atkinson and Couch (1951) observed that the growth of poults was stimulated by about 15 per cent at eight weeks. In a series of experiments involving five replications, Matterson et al. (1952) found that male chicks fed procaine penicillin at a level of 2.2 ppm for eight weeks weighed approximately 16 per cent more than their respective controls. The response obtained with penicillin was greater than that obtained with comparable levels of aureomycin, streptomycin, terramycin, and bacitracin. The percentage gain in every instance was decreased when 2.5 per cent fish meal

was included in the diet. The data suggest that the better the quality of the ration with respect to protein and vitamins, the less the response to antibiotic.

The exact optimum level of any of the antibiotics necessary to elicit maximum growth response has not been conclusively determined. However, 2-5 ppm of penicillin and 5-10 ppm of terramycin, bacitracin, aureomycin, or streptomycin are now generally used when the presence of an antibiotic in a practical poultry ration is desirable.

Among many other investigators who have reported that antibiotics promote the growth of chicks and poults are: Branion and Hill (1951), Heuser and Norris (1951), and Waibel et al. (1952a).

Aside from influencing growth, antibiotics have also been reported to exert other effects when included in the diets of poultry. Some of the more important aspects of these features of antibiotics will be reviewed.

The effect of antibiotics on the efficiency of feed utilization of chicks and turkey poults. Some investigators have reported that antibiotics favorably influence the efficiency of feed utilization by poultry while others have reported negative results. After 11 weeks, Berg et al. (1950) found that chicks which were fed a corn-soybean oil meal type diet containing 6.8 per cent fish meal with or without .25 per cent aureomycin fermentation product required approximately 3.0 grams of feed per gram gain in weight. However, they concluded that the antibiotic supplement enhanced feed utilization since those birds which were fed this product were one-fourth pound heavier than the controls. Biely et al. (1951a) also showed that definite increases were obtained in the efficiency of feed utilization by chicks which were fed diets containing an aureomycin fermentation product. Similar results were obtained by Scott and Glista (1950) and Groschke and Evans (1950).

Bird et al. (1951) presented results which indicated that crystalline aureomycin (20 ppm) improved the feed conversion rate of groups of chicks at the end of four and six weeks which were fed protein levels of 15, 17, 19, and 21 per cent.

Negative effects of penicillin, streptomycin, and aureomycin on the feed efficiency of chicks after eight weeks were reported by Hewang (1952). No differences were obtained by Carpenter and Duckworth (1952) in feed efficiency rates between groups of birds which were fed diets with and without aureomycin for 21 weeks.

The sparing effect of antibiotics on the requirement of poultry for protein, known vitamins, and unidentified growth factors. McGinnis (1951) reported that poults which were fed diets containing 24 per cent protein and supplemented with penicillin weighed approximately the same at four weeks as those birds which were fed diets containing 28 per cent protein but no antibiotic. Jones and Combs (1951) noted a sparing effect on the dietary requirement of the chick for tryptophane but not lysine when aureomycin was added to diets which were sub-optimal in these amino acids. Scott et al. (1952) also studied the influence of antibiotic on the protein requirement of the chick. Diets were fed which contained 20, 17, and 14 per cent protein respectively with and without 15 ppm of aureomycin. In the presence of the antibiotic, gains were more rapid at the 14 and 17 per cent levels of protein than they were in the absence of the antibiotic. However, the improvement in protein efficiency was no better than the improvement in over-all feed efficiency. It was concluded, therefore, that there was no evidence of a protein-sparing action of the antibiotic. When an aureomycin feed supplement was added to chick diets which contained 15, 17, 19, and 21 per cent protein, Machlin et al. (1952) noted that at four and six weeks maximal weights were obtained at the 19 per cent protein level. On the other hand,

when no antibiotic was included in the diet, greater gains were observed in the groups of birds which were fed the 21 per cent protein diets, when they were compared with those birds which were fed lower levels of protein.

The function of antibiotics in promoting the growth of chicks is, in all probability, multiple in nature. Hence, the statement that protein is spared *per se* is not justifiable. The apparent sparing of dietary protein could result from an alteration of the intestinal microflora by the antibiotic. If this does occur, a synthesis of individual amino acids which are limiting in the low protein diets would appear likely. This theory is further substantiated by experimental evidence which suggests that antibiotics also spare the dietary requirement for vitamins.

Oleson *et al.* (1950) and Stokstad and Jukes (1951) reported that aureomycin exerts a sparing effect on the requirement for vitamin B_{12} by the chick. Poor growth was observed by Biely and March (1951b), when a semi-purified ration deficient in folic acid, riboflavin, and nicotinic acid were fed to chicks. However, the addition of aureomycin to the deficient diets produced chicks having weights similar to those birds which were fed the complete basal ration. Slinger *et al.* (1952a) observed that the progeny of dams which were fed penicillin grew more rapidly than progeny from dams which received no antibiotic. From this they suggested that penicillin may bring about increased synthesis of vitamins in the intestinal tract of the dam which are subsequently deposited in the yolk and utilized by the developing chick. Waibel *et al.* (1952b) indirectly measured the sparing effect of penicillin on the requirement for biotin and folic acid by finding increased amounts of these vitamins in the eggs of dams fed this antibiotic. Common *et al.* (1950) found an increase in riboflavin content of the blood of pullets which were fed aureomycin. Ross and Yacowitz (1952) used diets sub-optimal in

vitamin D and reported that penicillin seemed to decrease the amount of this vitamin necessary for normal bone calcification.

Additional evidence which indicates that antibiotics spare the dietary requirement for certain B vitamins was presented by Blaylock et al. (1952). They found that aureomycin and penicillin increased the growth rate of chicks which were fed diets inadequate in riboflavin, choline, and vitamin B_{12} but, the antibiotics were without effect when the diets were inadequate in pyridoxine, pantothenic acid, or niacin. When diets which were deficient in niacin were fed to chicks, Nelson and Scott (1952) concluded that neither penicillin nor aureomycin spared the niacin requirement or accentuated niacin deficiency.

Unidentified factor activity has been ascribed to the following crude supplements: fish meal (Weise et al., 1949); fish solubles (Sunde et al., 1950); dried brewers' yeast, whey, and certain liver products (Menge et al., 1949); water extracts of leafy green vegetables (Kohler and Graham, 1951); and recently, in dehydrated alfalfa (Vavich et al., 1953). Some reports have appeared which indicate that antibiotics spare the factors present in some of these supplements.

Jones and Combs (1951) reported that either penicillin or aureomycin were effective in sparing the unidentified factors supplied by fish meal, molasses fermentation solubles, and/or dried brewer's yeast. Likewise, Scott and Jensen (1952) obtained data which indicated that aureomycin spared the requirement of poults for an unidentified factor not present in fish meal to a greater extent than for the unidentified factor supplied by fish meal. Matterson and Singsen (1951) found that greater growth responses could be obtained with antibiotics when no fish meal was present in the ration. Aureomycin, terramycin, and streptomycin gave responses which were statistically equivalent to the basal which was supplemented with 2.5 per cent fish meal.

Slinger et al. (1952b) added grass juice concentrate to a corn, oat groats, fish meal type poult diet at levels of 2.5 and 5.0 per cent. Penicillin at a level of 10 ppm gave a response in terms of efficiency of feed utilization only in the case of no grass juice supplementation or when it was added at the 2.5 per cent level. They suggested that penicillin caused increased synthesis of the grass juice factor within the intestinal tract or made the factor more readily available for the poult.

The sparing effect of antibiotics for the dietary requirement of chicks for vitamins and unidentified growth factors strongly suggest that the intestinal flora of the host is involved. In both instances it would appear that either nutritionally fastidious organisms, which require these substances are eliminated, or that certain organisms responsible for the synthesis of these factors are increased in the intestine. The over-all complexity of the mode of action of antibiotics indeed warrant further research before any definite conclusions may be attained.

The Mode of Action of Antibiotics in Promoting Growth

The mechanism whereby antibiotics exert their effect on growth is still not clearly defined. Some investigators believe that antibiotics or fragments thereof exert a vitamin-like action. Others state that they eliminate toxin producing organisms from the intestinal tract. The theory has been proposed that organisms which synthesize known or unknown growth factors are increased within the intestinal tract of the animal which is being fed the antibiotic. It has also been proposed that sub-disease levels, manifested only by body weight, are eradicated either by growing the chicks in environments in which no chicks have been previously raised or by supplementing the feed with antibiotics. Some evidence has been presented to indicate that physical effects are brought about in the animal which render it more

efficient in the utilization of nutrients. A review of some of the more important papers covering the theories mentioned will be given in this section.

The effect of parenteral administration of antibiotics on growth. The hypothesis has been propounded by several investigators that the antibiotic molecule, or a fragment thereof, acts as a specific metabolite within the body and, hence, exerts a vitamin-like effect in promoting growth. However, growth is not always favorably affected when antibiotics are administered interparenterally.

Oroschko and Evans (1950), and Whitehill et al. (1950) injected aureomycin into chicks and obtained no stimulation of growth. Contrary to these reports, Dixon and Thayer (1951) found that intramuscular injections of either penicillin or aureomycin produced a growth response which was equal to that obtained when the birds were administered these antibiotics in the diet.

Further evidence which indicates that antibiotic molecules or their components might act as metabolites within the body was presented by Elam et al. (1951 b). These workers reported that the intramuscular injection of either penicillin in sesame oil, which contained aluminum monostearate, or autoclaved penicillin elicited slightly better responses in chicks than when penicillin per se was injected or administered orally. Pellets which contained 1,000 units of bacitracin were implanted subcutaneously into the neck of newly hatched chicks. However, Branion et al. (1952) found no stimulation of the growth of these birds at six weeks of age.

The work with injected antibiotics has not conclusively demonstrated that these substances function as vitamins in the metabolic processes of the test animal. The strong possibility exists that even after injection,

antibiotics may still be capable of producing changes in the intestinal bacteria. Favorable evidence to support this view has been presented in at least two reports. Abraham et al. (1941) reported that injected penicillin was excreted in the intestinal tract and Larson and Carpenter (1952 a) demonstrated that aureomycin, after injection, is excreted in the feces of pigs.

A direct attempt was made by Luckey (1952) to determine if antibiotics act as metabolites. He found that the administration of sterile antibiotics in sterile diets were ineffective in stimulating the growth of germ-free chicks. On the other hand, contaminated birds responded favorably to dietary antibiotics. From these observations, it was concluded that antibiotics are more likely to function through indirect effects on the intestinal microflora.

Aside from the observations reported above, some work has been performed which implies that antibiotics eliminate toxin-producing anaerobic bacteria in the intestine of the host and thereby permit the animal to grow at a normal rate. Reports have also been presented which intimate that other bacteria are influenced and, consequently, act indirectly to promote growth.

The effect of antibiotics on intestinal bacteria. There appear to be no reports in the literature which signify that toxins are produced in the intestinal tract of chickens by Clostridium perfringens. Nevertheless, some investigators have attempted to correlate the reduction or complete elimination of these bacteria, normally present in the intestinal tract, with increased growth as a result of administering antibiotics to chickens and turkeys. Williams (1950) observed that the oral administration of penicillin or of aureomycin caused a marked depression in the number of hemolytic toxin-producing anaerobes in the intestinal tract of the chick.

Similarly, Sieburth et al. (1951) reported that penicillin and terramycin inhibited the growth of C. perfringens in the ceca of turkeys. Williams et al. (1951) administered sterile toxins of C. perfringens, (type A), C. septicum, C. histolyticum, C. tetani, C. novyi, and C. oedematoides either inter cloacally, or orally. These toxins failed to have an effect on the growth of chicks in the presence or absence of aureomycin.

Johansson et al. (1952) found that there was a decrease in the intestinal clostridia of rats which were fed aureomycin. Larson and Carpenter (1952 b) in work with pigs found that the number of fecal clostridia was reduced but that there was no correlation between this observation and the growth of the host.

Observations made by Smyser et al. (1952) failed to lend support to the enterotoxemia concept since, over a six week period, they observed only a slight depression in the numbers of C. perfringens in the feces of chicks which were fed aureomycin supplemented diets. Moreover, an increase in the number of these organisms was noted when penicillin was fed. It is difficult to conclude from any of these papers that the reduction of the numbers of clostridia is anything other than an effect rather than a cause. Aside from alterations in the numbers of these organisms, many workers have presented data to indicate that other species of bacteria and their numbers are altered materially by the feeding of antibiotics.

Much evidence has been presented which shows that intestinal bacteria can synthesize certain vitamins. Dam et al. (1937) and Greaves (1939) have shown that the synthesis of vitamin K takes place in the intestinal tract of rats. The inclusion of 1 per cent sulfasuxidine in a vitamin K free diet produced a high incidence of hypoprothrombinemia in cecectomized rats. The fact that this syndrome is absent when no sulfasuxidine is fed indicates that vitamin K is synthesized by organisms which are susceptible to the

drug. (Day et al. 1943). Miller (1945) was able to induce nutritional deficiency symptoms by feeding rats highly purified diets which contained succinylsulfathiazole at levels of 0.2 and 5.0 per cent. The symptoms were corrected by feeding biotin and folic acid. A decrease in the number of coliform organisms in the feces of the rats was observed when they were fed the drug. Less biotin, folic acid, and pantothenic acid was excreted by these rats than was excreted by those animals which were not fed the drug.

Bechdel et al. (1928) reported that cattle, and possibly all other ruminants could synthesize the vitamin B complex in the rumen. Further work on this particular problem has been presented by Fairbanks and Krider (1944 a, b).

Couch et al. (1950) found that the concentration of riboflavin, niacin, pantothenic acid, biotin, and folic acid was higher in the cecal feces of mature chickens and turkeys than it was in the intestinal content of the same birds. Perhaps there is a greater degree of bacterial synthesis of vitamins in the ceca than there is in the small intestine. Since, comparatively higher numbers of bacteria were found in the ceca by Shapiro and Sarles (1949).

The extreme susceptibility of certain intestinal bacteria to some of the antibiotics known to promote the growth of chicks would indeed lend some support to the theory that antibiotics function through an alteration of the intestinal microflora.

Peppler et al. (1950) observed a marked decrease in the number of coliform bacteria in the cecal feces of pullets which were fed a carbon-adsorbed polymyxin preparation. This inhibition persisted for several days after the antibiotic was withdrawn from the diet. Anderson et al. (1952a) studied the effect of protein levels on the cecal bacterial flora of chickens in

the presence and absence of penicillin and aureomycin. Increasing the protein level had no effect on the aerobic bacterial types but the anaerobic types were decreased. Penicillin enhanced the coliform and lactobacilli counts and reduced the enterococci. Aureomycin likewise brought about an increase in the coliform types but decreased the lactobacilli up to the third week after which time these organisms increased in number within the ceca.

An increase in the counts of A. aerogenes and E. coli was observed in the ceca of chicks fed diets which contained 150 ppm procaine penicillin G Romoser et al. 1952). These investigators also correlated the appearance of high numbers of L. bifidus in the ceca with poor growth. This organism was decreased or totally eliminated upon the addition of penicillin to the diet. Rosenberg et al. (1952) were unable to eliminate or even materially reduce the bacterial flora of the intestinal tract of chicks when they fed levels of terramycin 16 times in excess of the recommended amount. In fact, the enterococci counts were significantly greater among the groups receiving the higher levels of the antibiotic.

March and Biely (1952) studied the effect of feeding aureomycin on the bacterial content of chick feces. These investigators found that the level of aureomycin which is customarily added to chick starting rations produced a statistically significant decrease in the lactic acid bacteria. Contrary to these findings, Anderson et al. (1952b) fed to chicks diets which contained terramycin and found increases in the intestinal lactobacilli which were correlated with improved growth.

The effect of antibiotics on the intestinal microflora was also studied by Kratzer et al. (1951) who noted that the inclusion of streptomycin in the diets of chicks and poults resulted in a 5-10 fold increase in the yeast population of the intestinal contents. Further experimentation pertinent

to the effect of antibiotics on intestinal bacteria has been performed by Cook et al. (1952). These workers fed diets containing penicillin to turkey poults and found that lactobacilli counts were reduced by this treatment in the duodenum and in the small and large intestine. Penicillin feeding brought about increases in the coliform bacteria of the small and large intestines which ranged from 10-10,000 fold.

From the reports reviewed in this and other sections it may be seen that antibiotic influence on the intestinal bacteria are indeed diverse and some of the conclusions are in direct conflict. In view of the multiplicity of these reports and their wide variability, only generalizations may be made. The four groups of intestinal bacteria which seem to be implicated with antibiotic feeding are the coliforms, enterococci, clostridia, and lactobacilli.

Further evidence to strengthen the "bacterial" concept has been presented by several workers who have studied the influences of various rearing environments on the response of poultry to antibiotics.

The influence of environment on the growth response of chicks. It was shown by Coates et al. (1951 b, 1952) that chicks which were raised from one day to three weeks in quarters not previously used for rearing chicks failed to show a response to penicillin. The theory was proposed by these investigators that an "infection" was present in those birds which were housed in old quarters and, hence, responded to the antibiotic. No bacteriological data were presented to confirm this postulate. Likewise, Davis and Briggs (1951) were unable to obtain a response from antibiotics when chicks were housed in new quarters.

Bird et al. (1952) also presented evidence which supported these observations. They showed that chicks housed in old environment responded to aureomycin after one week but no response was apparent until after three

weeks in a new environment. Similar relationships, relative to the growth response of chicks to antibiotics in old and new quarters, were observed by Hill et al. (1953). In addition, these investigators showed that the total aerobic bacterial count in the ceca of the chicks in the old environment was higher than in the ceca of those birds reared in a new environment. Combs et al. (1952, unpublished data) have also obtained evidence which show that environmental influences are extremely important in determining the effect of antibiotics on the growth of chicks.

Therefore, one may conclude that all investigators are in agreement with the observations of Coates et al. (1951 b, 1952). Although these reports further substantiate the theories of the microbial effects in antibiotic growth response, more work is needed to elucidate the causative agents of the so-called "infections".

The effect of antibiotics on the absorption of nutrients. Another approach relative to the mechanism of antibiotic growth stimulation aside from the influences of antibiotics on the microbial flora has been proposed by several investigators. Gordon (1952) morphologically compared birds which were fed antibiotics and no antibiotics in germ-free and conventional environments. No special effects of antibiotics were apparent in the germ-free group. However, preliminary histological findings revealed that the intestinal wall of contaminated, conventional birds fed antibiotics was thinner than it was in those birds which received no antibiotic. This finding suggests that perhaps absorption of nutrients through the intestinal wall is more efficient when antibiotics are fed.

The work of Migicovsky et al. (1951) has shown that penicillin enhances the absorption of calcium. In addition, Pepper et al. (1951) noted that aureomycin lowered the incidence of perosis in chicks which were fed diets limiting in manganese. Ross and Yacowitz (1952) fed chicks a corn-soybean

oil meal type diet with graded levels of 4, 8, 30, and 600 I.U. of vitamin D_3 per 100 grams of diet with and without penicillin. At three weeks there was no evidence of a sparing effect on the vitamin D requirement. Penicillin did cause a significant increase in the per cent bone ash at the 8 and 30 I.U. levels of vitamin D. These results indicate that penicillin decreased the vitamin D requirement for normal bone calcification. The increased absorption of minerals may be related to the observations of Anderson et al. (1952a) who reported that antibiotics in chick diets lowered the pH of the cecal contents.

Other evidence has been presented that the rate or completeness with which nutrients are absorbed has a direct effect on the growth of the chick. Ely (1951) presented data which ascribed a growth promoting action to strong surface active agents. Since these materials alter surface tension, it is logical to assume that the rate of absorption is affected. Stern et al. (1952) were not able to obtain a stimulation of growth with surfactants, although penicillin in vitro did not lower the surface tension of feed, the antibiotic significantly lowered the surface tension of the intestinal contents. These workers concluded that the action of antibiotics which stimulates growth also lowers surface tension.

The effect of dietary supplementation or oral administration of microorganisms, feces, and fecal preparations on the growth of chicks and turkey poults. The oral administration of either prepared microorganisms or certain dietary supplements which ultimately alter the intestinal bacteria are by no means new methods of studying the relationships between intestinal microorganisms and nutrition. The work of Rodella (1905), Sittler (1908), and Bahrdt and Beifeld (1910) among many others, led these authors to the conclusion that the diet plays a very important role in the determination of the type of intestinal microorganisms which will predominate. Rettger

and Horton (1914) reported that organisms of the acidophilus and bifidus types constituted 85-90 per cent of the flora of rats which were fed a diet consisting of starch, lard, and purified protein. Hill and Rettger (1917) noted that the daily administration of 2-3 grams of lactose to human subjects resulted in an aciduric intestinal microflora within 2 or 3 days.

Since Kratzer et al. (1952) observed a 5-10 fold increase in the intestinal yeast population of chicks and poults which had been fed diets supplemented with streptomycin, they performed experiments to further determine the role, if any, which these organisms played in the growth promoting action of the antibiotic. A yeast tentatively identified as Candida tropicalis was isolated from the intestinal contents and feces of poults which had been fed a diet containing 50 ppm streptomycin. When large masses of the cultivated yeast cells were fed back to chicks and poults very slight increases, if any, were noted in some trials.

Sieburth et al. (1952) investigated the effects of fecal supplements, cultures of fecal organisms, and antibiotic treatments on the growth of turkey poults. When poults were allowed access to feces from month-old poults which were fed a basal diet, no growth depressing action was observed. Dietary supplementation with a culture of the turkey feces tended to depress growth. This depression was overcome by autoclaving the culture or adding to it 5 ppm of procaine penicillin. These results indicate that one or several microorganisms present in the turkey feces was increased in the broth culture and exerted a growth depressing effect.

It was found by Nomoser et al. (1952) that E. coli and A. aerogenes were increased in number in the ceca of chicks which had been fed procaine penicillin G at a level of 150 ppm. Higher numbers of these microorganisms were coincident with increased growth. On the other hand, high numbers of L. bifidus were coincident with poor growth. Lactose further increased the

number of these organisms in the ceca when it was fed in the diet at levels of from 10-15 per cent, and chicks growth was further depressed. When lactose was fed in combination with penicillin, these organisms were almost completely eliminated from the cecal contents and the growth of chicks on this diet excelled that of chicks which were fed penicillin alone. If E. coli and A. aerogenes are responsible in whole or in part for the growth response of chicks fed antibiotics, then it is logical to assume that the oral administration of large numbers of these organisms to the chicks might show a definite stimulation of growth. If the high lactobacilli count, on the other hand, is responsible for a depression in growth, likewise, the administration of large numbers of these organisms with subsequent growth depression would further imply that they are denying the host of essential nutrients because of their fastidious nature, and are, therefore, depressing the growth of the host.

The majority of reports in the literature at the time of inception of this problem (June, 1951) ascribed an important role to intestinal microorganisms in the over-all mode of action of antibiotics in promoting growth. Based on these reports and, in particular, preliminary findings which seemed to associate E. coli, A. aerogenes, and L. bifidus with antibiotic growth stimulation, the investigation reported in this thesis was initiated.

EXPERIMENTAL PROCEDURE

I. Methods Used in Rearing Chicks

Throughout this investigation, unless otherwise indicated, 15-20 day-old New Hampshire chicks of both sexes were used per experimental group. The chicks used in experiment 2 were the progeny of dams which were housed on raised wire floors and fed a ration which was complete in all known nutrients but contained no animal protein supplements. The designation "deficient chicks" has been assigned to this type chick because, based on other studies, they are considered to have sub-optimal "carry-over" of unidentified growth factors. The chicks used in all other experiments, however, were progeny of dams maintained on litter and fed a complete breeder ration.

Except in experiment 15, the chicks were raised in electrically heated, wire-floored battery brooders. In experiment 15, the chicks were reared at the University of Maryland poultry farm, in floor pens and fresh wood shavings were used for litter. In addition, heat was supplied by electrically heated hovers.

The day-old chicks were individually wing-banded, divided into groups of uniform weight, and placed in battery brooders. Weighings were made of individual chicks at weekly intervals. Except in experiment 15, a four week experimental period was used. In experiment 15, an eight week trial period was used.

The basal diets and the experiments in which they were used are presented in tables 1, 2, 3, and 4. Diets CS-1, CS-2, and CS-3 are chick starter rations which are composed of corn, soybean oil meal, and the necessary vitamins and minerals which are required by the chick for normal growth. These rations are considered to supply sub-optimal amounts of two

Table 1

Composition of basal diets employed in experiments with chicks (experiments 1-10, 15-18)

Ingredients	Corn-Soybean Oil Meal Type CS-1* %	CS-2** %	CS-3** %
Ground yellow corn	60.85	59.70	62.85
Soybean oil meal, solvent, 44% protein	34.00	33.00	26.00
Fish meal, menhaden			5.00
Dehydrated alfalfa meal		2.50	2.50
Limestone	1.00	1.00	1.00
Bone meal	3.25	3.00	1.85
Sodium chloride, iodized	0.30	0.30	0.30
Cod liver oil (2250 IU Vit. A, 300 ICU Vit. D/g)	0.25	0.20	0.20
Manganese sulfate	0.025	0.025	0.025
DL-methionine	0.05	0.05	0.05
Nitrofurazone, 11.2% mix		0.05	0.05
"D" activated animal sterol (1500 ICU Vit. D/g)		0.025	0.025
Vitamins	**milligrams per pound**		
Riboflavin	1.60	2.00	2.00
Niacin	8.00	10.00	10.00
Calcium pantothenate	2.00	2.50	2.50
Choline chloride, 25% mix		0.10	0.10
Choline chloride, C.P.	200.00		
2-methyl-1, 4 naphthoquinone	0.20		
Vitamin B_{12}, crystalline	0.01		
Vitamin B_{12}, feed supplement, (5 mg/lb)		0.05	0.05

*Diet CS-1 was used in experiments 1-10, and 18.

**Diets CS-2 and CS-3 were used in experiments 15-17.

Table 2

Composition of basal diets employed in experiments with chicks (experiments 11-14)

Oat Groat-Fish Meal Type (diet OG-F)	
Ingredients	%
Oat groats	83.00
Fish meal, menhaden	15.00
Calcium carbonate	1.25
Sodium chloride, iodized	0.30
Dry Vit. A & D supplement (4000 IU Vit. A, 750 ICU Vit. D/g)	00.20
Manganese sulfate	0.02
Copper sulfate	0.001
DL-methionine	0.08
Glycine	0.30
Vitamins	**milligrams per pound**
Riboflavin	1.60
Niacin	16.00
Calcium pantothenate	5.00
Choline chloride, C.P.	200.00
2-methyl-1, 4-naphthaquinone	0.20
Alphatocopherol acetate	3.00
Pyridoxine HCl	1.60
Biotin	0.025
Folacin	0.20
p-aminobenzoic acid	10.00
Vitamin B_{12}, crystalline	0.002

Table 3

Composition of basal diet employed in study with poults (experiment 19)

Corn-Soybean Oil Meal Type	
Ingredients	TS-1 %
Ground yellow corn	38.65
Soybean oil meal, solvent, 44% protein	53.00
Di-calcium phosphate	2.50
Limestone	2.75
Sodium chloride, iodized	0.50
Manganese sulfate	0.05
Ferric sulfate	0.025
Copper sulfate	0.001
Cobalt sulfate	0.0001
Dry Vit. A & D supplement (4000 IU Vit. A, 750 ICU Vit. D/g)	0.30
DL-methionine	0.10
Corn oil	2.00
Vitamins	**milligrams per pound**
Riboflavin	2.00
Niacin	20.00
Calcium pantothenate	4.00
Choline chloride, C.P.	450.00
2-methyl-1, 4-naphthaquinone	1.00
Alphatocopherol acetate	3.00
Pyridoxine HCl	1.50
Biotin	0.05
Folacin	0.50
Vitamin B_{12}, crystalline	0.015

Table 4

Composition of purified basal diet employed in experiment with chicks (experiment 20)

Purified Type Diet (diet PD-1)	
Ingredients	%
Cerelose	61.82
Casein (crude)	22.00
Gelatin	8.00
Corn oil	2.00
Dry Vit. A & D supplement (4000 IU Vit. A, 750 ICU Vit. D/g)	0.20
DL-methionine	0.30
Mineral misture	5.37*
Vitamins	**milligrams per 100 grams**
Riboflavin	1.00
Niacin	5.00
Calcium pantothenate	2.00
Choline chloride, C.P.	200.00
2-methyl-1, 4-naphthaquinone	0.50
Alphatocopherol acetate	0.50
Pyridoxine HCl	0.60
Biotin	0.02
Folacin	0.30
p-aminobenzoic acid	0.20
Thiamin	0.50
Inositol	100.00
Vitamin B_{12}, crystalline	0.002

*The composition of the mineral mixture expressed as the percentage of each ingredient in the total diet when added at this level supplied:

KI	0.004	$Ca_3(PO_4)_2$	1.30
$ZnCl_2$	0.002	K_2HPO_4	0.90
H_3BO_3	0.0009	Na_2HPO_4	0.73
$CoSO_4.7H_2O$	0.0001	$MgSO_4.7H_2O$	0.25
NaCl	0.50	$Fe(C_6H_5O_7).6H_2O$	0.14
$CaCO_3$	1.50	$MnSO_4.4H_2O$	0.041
$CuSO_4$ (anhydrous)	0.0013		

different growth factors based upon previous work in our laboratory, Arscott and Combs, 1950). Diet OG-F was designed to supply ample quantities of the unidentified factor present in fish meal. However, soybean oil meal, which has been reported to contain unidentified growth factor activity (Hill, 1948; Hill and Briggs, 1950), was omitted. Diet TS-1 is a corn-soybean oil meal type turkey ration. Diet PD-1 is a purified chick ration which contains all of the known vitamins, minerals, and amino acids required by the chick for normal growth.

In experiments 21 and 22, diets R-138 and R-134 were used respectively. Although the percentage of wheat gluten and sodium proteinate which are supplied in these diets contain an adequate amount of lysine by analysis, it has been shown that only a portion of it is available for absorption (see Arscott, Thesis, University of Maryland, 1953). Consequently, when the data obtained in experiments 21 and 22 are evaluated, the possibility of a lysine deficiency in the chicks can not be overlooked.

All diets were mixed by weighing the ingredients separately and then combining them in a conventional type feed mixer. The vitamins were combined and mixed with a small amount of water. This solution was then evenly distributed throughout approximately 15 pounds of feed by means of a Hobart mixer, and then combined with the total feed. For each group of 20 chicks, 30 pounds of practical type diets and 22 pounds of the purified type diets were sufficient to feed ad libitum for the usual four week duration of the experiment. The diets were always prepared fresh for each experiment, and, after the supplements were added, each diet which was being employed in a particular experiment was stored at 10°C in a tinned lard can and covered with a tight-fitting lid. Feed and water were allowed to be consumed ad libitum in every experiment.

In experiment 1, the chicks were removed from the hatching trays and placed in unused chick boxes. Surfaces of the batteries with which the chicks came in contact were washed with Compound Germicidal Rinse RH 988 (Fink Roselieve Co., Inc., New York, N.Y.). These precautions were taken in order to decrease the possibility of contaminating the birds with micro-organisms other than those which were used in the experiment.

II. Methods Used in the Preparation of Samples

The cultures of A. aerogenes and E. coli which were used in this investigation were isolated from the cecal feces of a four week old chick which had been fed diet CS-1 supplemented with 10 per cent lactose and 150 ppm of procaine penicillin G.

Two strains of L. bifidus were used. One strain was isolated from the cecal feces of a three week old turkey which had been fed a corn-soybean oil meal type ration supplemented with 10 ppm of procaine penicillin G (Veltre, 1953). The other strain of L. bifidus was isolated from the cecal feces of a four week old chick which had been fed diet CS-1 containing a 10 per cent lactose supplement (McCarthy, 1953). The respective symbols assigned to these strains are Vt 3 and C 31 (Veltre, 1953).

In experiment 1, chicks were orally inoculated with A. aerogenes. The suspension of the organism was prepared by inoculating two-1 liter Erlenmeyer flasks, each of which contained 100 ml of trypticase soy agar, with 1 ml of a trypticase soy broth suspension of the organism. The inoculated agar surfaces were incubated for 24 hours at 37° C. At the end of this time, the surface growth was removed with sterile physiological saline. The suspension was then centrifuged at 2,500 r.p.m. for 30 minutes. The centrifugate was resuspended in 200 ml of a 5 per cent lactose solution (Seitz filtered) which contained 50 units of procaine penicillin G per ml. Each chick in the experimental groups received orally 1 ml of this preparation. The suspension contained 160 x 10^8 viable organisms per ml, as revealed by a plate count using trypticase soy agar.

Several fermentation products were prepared with A. aerogenes. These products were tested in experiments 2 through 5 and 21 to determine their effect on chick growth in the presence and in the absence of penicillin. The method which was used in the preparation of these samples and the number of viable organisms per gram of the finished product are presented in table 5.

Table 5

Methods used in preparing Aerobacter aerogenes fermentation supplements (experiments 2 through 5)

Sample Number	Used in experiment number	Method of Preparation	Viable bacteria/gm. final product
1.	2	16 liters of a 5 per cent chick growing mash saccharified with malt and fermented for 3 days anaerobically at 35°C. Coarse solids screened off and air-dried; supernatant vacuum-dried to a syrup and spray dried. Dried coarse solids (300 gms) mixed with spray-dried supernatant (50 gms).	65×10^6
2.	2	300 ml skimmilk - $CaCO_3$ culture of A. aerogenes dried on 200 gms sterile ground corn.	40×10^6
3.	3, 4	1000 gms distillers' solubles, 200 gms cerelose, 17 liters distilled water inoculated with 1 liter of a 48 hr culture of A. aerogenes grown in the same medium. Incubated for 40 hrs at 95°F. Pasteurized for 1 hr at 150-160°F. Dried by gradual addition of 1 pound of corn meal in an air drier at a temperature of 45°C over a period of 30 hours.	None
4.	3, 4	Mash and fermentation conditions identical to above. Mash dried on 1 lb steamed peat moss under same conditions as above. No pasteurization.	60×10^6
5.	5	2250 ml 2 per cent skimmilk, 1 per cent $CaCO_3$ culture of A. aerogenes dried on 1500 gms corn meal.	60×10^6

In experiment 8, two Koser's citrate broth cultures (see appendix) of A. aerogenes were prepared in an attempt to determine the effect of these organisms, per se, on the growth of chicks with and without penicillin in their diets. For the preparation of one sample, two 6-gallon Pyrex bottles, each of which contained 10 liters of sterile citrate medium, were inoculated with 2 per cent of a 24 hour culture of the organism which also had been grown in citrate medium. After aeration at 37° C for 48 hours, the organisms were separated from the broth by passing it through the Sharples centrifuge. The bowl was driven by an air pressure of approximately 40 p.s.i. which caused it to revolve at 38,000 r.p.m. The affluent was limited to a flow of 5 liters per hour. The effluent material was found to be rather cloudy, which indicated that complete removal of all of the cells was not accomplished. The creamy, white yield, which amounted to a total of 3 grams, was suspended in sterile 10 per cent Difco skimmilk and lyophilized. The method used for lyophilization of the cultures is described later in this section.

For the preparation of the other sample used in this experiment, Koser's citrate medium which contained 100 units per ml of procaine penicillin G, Merck (approximately 100 gamma) was fermented with the organism. During the fermentation of this substrate, large quantities of foam were apparent on the surface of the medium. After fermentation was completed, the broth seemed to be more viscous than that broth which was fermented without the antibiotic. The rate of affluent flow into the Sharples centrifuge, when penicillin was included in the medium, had to be limited to 2.5 liters per hour in order to obtain an effluent broth of the same turbidity as that which was obtained when citrate broth containing no antibiotic was centrifuged. The cell concentrate was yellow in appearance and the yield, which amounted to 4 grams, was suspended in 40 ml of sterile, 10 per cent Difco skimmilk prior to lyophilization.

The apparatus used for the aeration and incubation of the substrates described on the preceding page is presented on plate 1. A water aspirator was connected to the terminal end of the system which pulled air into the culture bottles at the rate of approximately 25 liters per hour. Prior to entry of the air into the culture bottles, it was sterilized and dried by first drawing it through sterile, non-absorbent white cotton, then through concentrated H_2SO_4, and, finally, through sterile cotton.

The technique of lyophilization or "freeze-drying" was employed for the preparation of bacterial cells which were used in experiments 8 through 20. This process has been described by Flosdorf and Mudd (1935, 1938) and utilizes the principle of sublimation. The material to be dried is frozen and subjected to high vacuum which transforms the ice to a vapor, without melting. The vapor is subsequently trapped in a dry ice condenser.

An apparatus which was designed to accommodate relatively large volumes of culture is shown on plates 2 and 3. When this technique was employed, it was possible to obtain large masses of viable cells. The resultant powder was easily passed through a fine wire screen and combined into the feed.

The standardized method employed in the preparation of all viable coliform cells is outlined below:

1. Place 45 ml Eugon agar fortified with 0.5 per cent agar in Kolle flask, plug and sterilize at 15 lbs steam pressure for 20 minutes.

2. Lay flasks flat, with the indented side of the flask lip proximal to the desk top, and allow agar to harden.

3. Flood the agar surface of the flask with 2 ml of a 24 hour trypticase soy broth (B.B.L.) culture of the appropriate organism.

4. Incubate at 37°C for 24 hours.

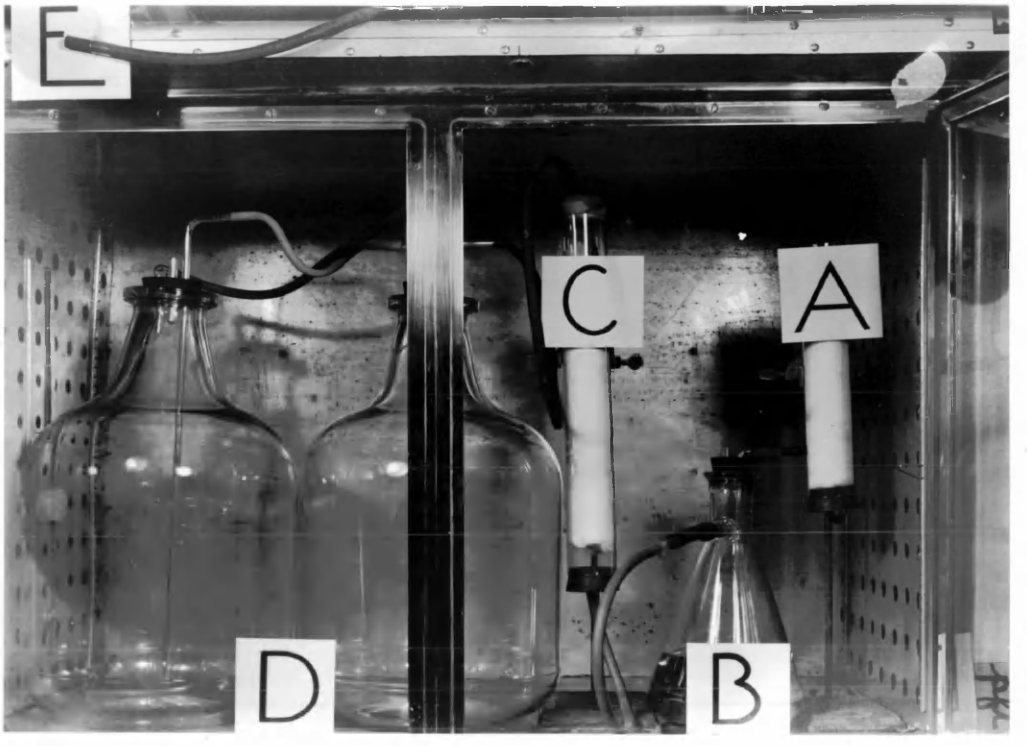


Plate 1. Apparatus used for aeration and incubation of Aerobacter aerogenes in Koser's citrate broth. A and C, cotton filters; A, concentrated H_2SO_4; F, fermentation flasks; E, to water aspirator.

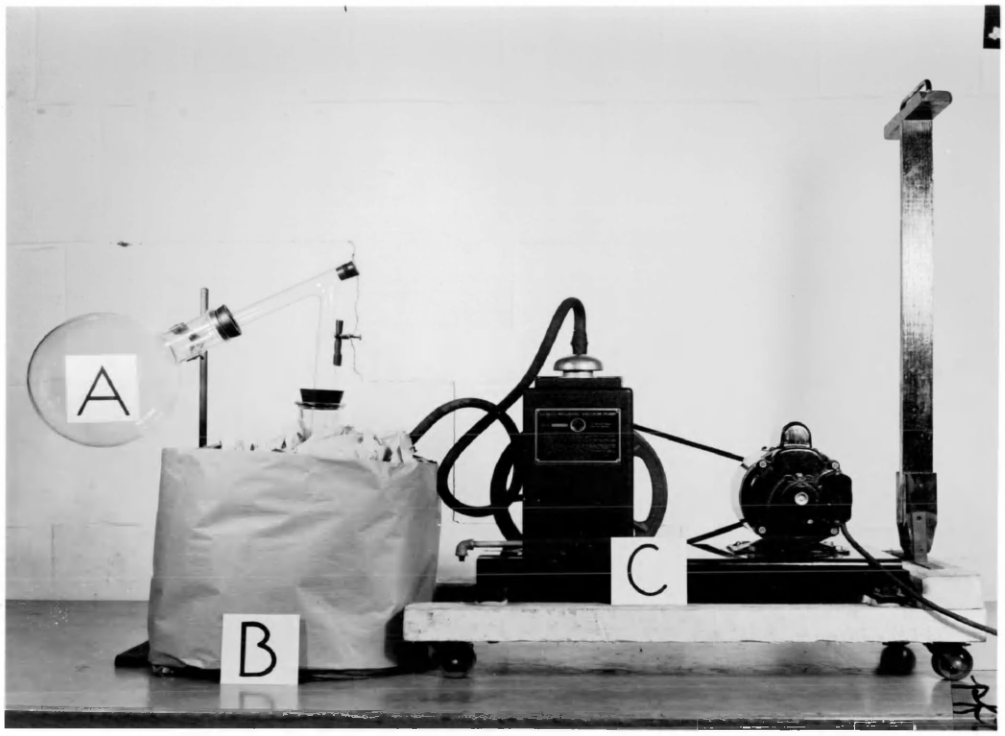


Plate 2. Apparatus used for lyophilization of cultures. A, container for shell-frozen culture; B, insulated, dry-ice condenser; C, vacuum pump.

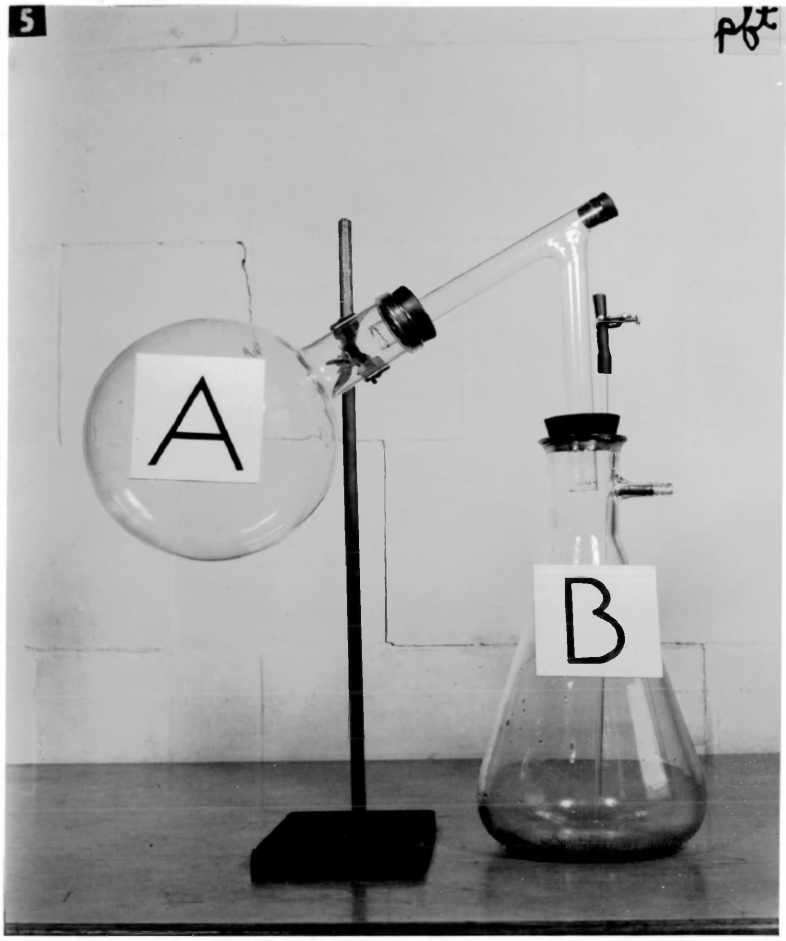


Plate 5. Detail of dry-ice condenser. A, container for frozen culture; B, flask with 6 mm glass tube inserted in side arm to pull vapor to bottom.

5. Harvest the growth from the flask by first placing 12 ml of sterile 10 per cent Difco skimmilk in the flask and then passing it over the surface with a bent, sterile glass rod.

6. Cool the pooled rinsings from all of the flasks in a beaker which has been submerged in an ice bath.

7. Place the skimmilk suspension of the organisms in a round bottom flask which is free from scratches and flaws. The ratio of culture to flask volume should be 1:10.

8. Shell-freeze the culture to the side of the flask by tilting it and revolving it in a dry ice-methanol bath at a temperature of -60 to -70°C. A dull appearance of the milk, when observed through the open mouth of the flask, is a good indication that the contents are thoroughly frozen. The flask may be connected to the chuck of an electrically driven motor and revolved in the bath. The centrifugal force will cause an even distribution of the culture on the sides of the flask; but, the hand turning is just as efficient.

9. Place the flask and shell-frozen contents on a dry-ice condenser (see plate 3) and with a high vacuum pump bring the internal pressure to 60-70 microns.

10. After about 15 minutes a frost will form on the outside surface of the flask. The complete disappearance of this frost and an equilibration between the exterior of the flask and room temperature indicates that the run has been completed.

On the average, the percentage of organisms which survived lyophilization was usually 80 for A. aerogenes, 60-70 for E. coli, and 40-60 for L. bifidus.

In experiment 14, one 225 liter tank batch each of A. aerogenes and E. coli was prepared for testing. The organisms which were removed were not resuspended in skimmilk. No viable organisms were detectable on Eugon agar when these samples were plated at a dilution of 10^2.

The same amounts of culture were prepared for testing in experiments 15 through 17. However, after the organisms were removed from the broth, they were resuspended in sterile, 10 per cent Difco skimmilk prior to lyophilization. The numbers of viable E. coli and A. aerogenes in each gram of sample were 400×10^6 and 86×10^8 respectively.

In experiment 16, the two strains of L. bifidus, which have been previously described, were lyophilized and incorporated into the basal diet as supplements. The samples were prepared by inoculating 1 per cent of each organism into two 10-liter batches of sterile modified Hassenin's medium (see appendix) contained in each of two 12-liter Pyrex, round-bottom flasks. In place of the conventional cotton plug, the mouths of the flasks were sealed with sterile, tight-fitting rubber stoppers to prevent the entry of O_2 into the medium. Incubation was allowed to proceed at 37°C for four days. At the end of this time, the culture was centrifuged in 1 liter quantities in a Servall angle centrifuge at 2,000 r.p.m. Each liter of culture yielded approximately 1 gram of cell material and the resultant 10 grams of wet cells from the complete broth were resuspended in 100 ml of sterile Difco skimmilk and subjected to the process outlined above. The lyophilized Vt 3 contained 60 x 10^6 organisms per gram; the plate count performed on C 31 showed that 50 x 10^6 viable cells were present in each gram of the dried powder.

The two strains of L. bifidus were also prepared for oral inoculation in this experiment by growing them in modified Hassenin's medium. An inoculum of 1.5 per cent of each culture was used to seed 100 ml of medium. This medium was sterilized in Boston round bottles (200 ml capacity) for 10 minutes at 121°C. The bottles were covered with screw-caps fitted with rubber gaskets. After three days incubation the cultures were centrifuged in the Servall angle centrifuge for 20 minutes at about 2,500 r.p.m. and the centrifugate from each 150 ml of broth culture was resuspended in 80 ml of physiological saline.

During the first week of the experiment, each chick receiving either of the oral preparations, was administered 1 ml of the organisms which were recovered from 100 ml of medium and resuspended in 80 ml of physiological saline. The volume of cells was doubled the second week and during

the third and fourth weeks 2 ml of the resuspended cells from 200 ml of medium were used.

When it was necessary to orally inoculate chicks with viable organisms (experiments 1 and 18), a 1 ml graduated medicine dropper with a long tip was used. The culture was deposited directly into the crop by inserting the tip of the dropper far down into the gullet and depressing the rubber bulb with a rapid motion.

In experiment 22, A. aerogenes was used to ferment a medium which was designed to supply ample quantities of the unidentified growth factor reported to be in certain liver preparations (see appendix). The medium was inoculated with 1 per cent of a 24 hour culture of the organism which had been propagated in the same medium. The seeded medium was incubated and aerated as previously described. At the end of 48 hours the culture was condensed by vacuum distillation to a volume of 800 ml and a plate count on Bugon agar (Baltimore Biological Laboratory) revealed that 250×10^7 viable A. aerogenes cells were present in each ml of the culture. The material was then pasteurized at 100°C for 60 minutes to kill the cells, and condensed further to a final volume of 500 ml. The designation BCF-1 was assigned to this sample and will be used when further reference to it is made.

During the course of these investigations, certain bacteriological tests were performed. These tests are discussed in the following section.

III. Materials and Methods Used in Bacteriological Investigations

In some of the experiments performed in this investigation it was desirable to analyze bacteriologically the cecal content of birds which were fed certain experimental diets. The bacteriological methods which were used in the performance of this test have been previously described by Romoser et al. (1952).

When various substrates which contained procaine penicillin G were fermented with either A. aerogenes or E. coli, the broth, after fermentation, was assayed for antibiotic activity using the method of Schmidt and Moyer (1944). Prior to assay, the broth cultures were passed through cintered glass filters (ultra fine) to remove the viable bacterial cells.

Preliminary experiments with chicks seemed to indicate that L. bifidus was competing with the chick for a factor which was being synthesized by E. coli and/or A. aerogenes in the intestinal tract. Consequently an in vitro test was designed to ascertain the validity of this supposition. The method used for the performance of this test is outlined below:

1. Place 4 drops of a 24 hour culture of L. bifidus strain being tested in tube containing 15 ml of sterile, melted #5 medium. Rotate to assure thorough distribution of the cells throughout the medium.

2. Pour seeded agar into sterile Petri dish and allow to harden.

3. With inoculating needle place 10 cm streak of E. coli on plate and bisect this streak with a similar inoculum of A. aerogenes. Incubate plate at 37°C and observe periodically for zone of growth below the streaks.

This method is, at best, only presumptive. However, Shorb and Veltre (1953, unpublished data) have developed a tube assay which demonstrates the need of a growth factor for L. bifidus. The potency of various preparations of E. coli cells have been tested using this assay. Presented in table 6 are the methods which were used in the preparation of the cellular material for assay.

Table 6

Methods used in preparing Escherichia coli cells for microbiological assay using Lactobacillus bifidus as the test organism

Sample number	Inoculum*	Medium in Kolle Flasks	Treatment** # (Total cells 3 flasks)
1.	E. coli grown on plain Eugon agar slant	Eugon agar	Growth removed with distilled H_2O
2.	Same	Same	Growth removed with 70 per cent ethyl alcohol. Alcohol evaporated and residue readjusted to orig vol.
3.	Same	Same	Growth removed with distilled H_2O, pH adjusted to 4.0 and suspension autoclaved at 121°C for 15 minutes
4.	E. coli grown on Eugon agar slant which contained 10 units of penicillin G per ml	Eugon agar 10± units pen/ml	Same as sample 1
5.	Same	Same	Same as sample 2
6.	Same	Same	Same as sample 3

*1.5 ml of a 24 hour trypticase soy broth culture used to inoculate each flask.

**10 ml of solution used to rinse each flask.

#Before the samples were assayed they were shaken thoroughly at 15 minute intervals over a period of an hour to assure thorough mixing.

Several crude supplements which contain unidentified growth factor activity for the chick also stimulate the growth of L. bifidus when the assay of Shorb is employed. However, evidence for a definite correlation between organism activity and chick activity for any particular sample is lacking at the present time.

Procedures other than those which have been given previously were used in several experiments. Since they apply to only one experiment, the procedure is given with the results.

EXPERIMENTAL RESULTS

I. The Effect of Dietary Composition on the Persistence of *Aerobacter aerogenes* in the Ceca of Orally Inoculated Chicks

At the outset of this investigation it was advantageous to determine if the oral inoculation of a heavy suspension of viable cells of *A. aerogenes* would implant the organism in the ceca of the chick. The effect of penicillin, lactose, and a combination of these materials on the number of organisms in the ceca after oral inoculation was also determined. At the beginning of the experiment, 10 chicks in each experimental group were inoculated with 1 ml of the suspension of *A. aerogenes* which has been previously described. In addition, a series of uninoculated chicks were raised on the same experimental diets. At the end of 24, 48, 96, and 144 hours, plate counts were performed on the cecal contents of 2 inoculated and 2 uninoculated chicks from each group.

The results of experiment 1 are presented in table 7. *A. aerogenes* was detected in the cecal contents of all orally inoculated chicks which were examined at the end of 24 and 48 hours. A comparison of the number of organisms in the ceca of the birds which were fed each of the experimental diets, however, revealed that the organisms were more numerous in the ceca of those chicks which were fed either the lactose or lactose plus the antibiotic. At the end of 96 hours, there was a marked decrease in the number of organisms in the ceca of the chicks from experimental groups 1 and 2. On the other hand, plate counts on the cecal contents of those chicks receiving the diet which contained the combination of penicillin and lactose revealed no appreciable decrease in the number of organisms. At the end of 144 hours, there were no detectable colonies of

Table 7

Bacterial colony counts of cecal contents of chicks orally inoculated with *Aerobacter aerogenes** (experiment 1)

Time in hours	Group number	Supplement	Koser' Citrate Medium** Inoc. Chicks	Uninoc. Chicks
24	1	None	180	<1
	2	15% lactose	840	<1
	3	As 2 +150 ppm pen	1230	<1
48	1	None	250	<1
	2	15% lactose	500	<1
	3	As 2 + 150 ppm pen	500	<1
96	1	None	20	<1
	2	15% lactose	110	<1
	3	As 2 + 150 ppm pen	550	<1
144	1	None	< 1	<1
	2	15% lactose	< 1	<1
	3	As 2 + 150 ppm pen	160	<1

*Counts are expressed as the number of organisms per gram of wet cecal feces x 10^8.

**2% agar added to Koser's Citrate broth. It is assumed that all of these organisms are *A. aerogenes*, since all colonies had the same morphological characteristics.

A. aerogenes in the ceca of either the birds which were fed the control diet or the diet which contained lactose. In addition, the number of cecal A. aerogenes in the chicks from experimental group 3 was approximately one-third as high at this time than it was at the 96 hour period.

Under the conditions of this experiment, it is indicated that after oral inoculation the organism survives during its passage to the ceca. It will persist at that location for at least six days if lactose and penicillin are included in the diet. If only lactose is present in the diet, the organism seems to disappear after approximately four days.

II. The Effect of *Aerobacter aerogenes* Fermentation Products on the Growth of Chicks

Five experiments were performed which involved the use of various substrates which had been fermented with *A. aerogenes*. These samples were prepared as described in table 5, page 30. The samples were used to supplement chick diets to determine if the organism could, under various conditions of fermentation, synthesize a factor which would stimulate the rapid growth of chicks. If this were possible, it would, in part, explain the role of *A. aerogenes* in the ceca as a result of the administration of penicillin to chicks.

The results of three experiments which are summarized in figure 1 indicate that, in general, increased growth was observed when samples 1, 2, and 5 were used to supplement the chick diets. In experiment 2, greater gains were observed when the air-dried *A. aerogenes*-skimmilk preparation was employed as compared with those gains observed in experiments 5 and 21 when a similar product was used. The fact that "deficient" chicks were used in experiment 2 may account for the apparent difference in the responses.

In all three experiments, the gains which were observed with the fermentation product and the antibiotic together were greater than the gains which were observed when penicillin was used alone. This phenomenon was later observed in most other experiments in which the antibiotic was included in the diet with viable organisms or their fermentation products.

In the two remaining experiments of this series, a distillers' solubles-cerelose substrate was used in the preparation of the samples. The method of preparation is also described in table 5, page 30. Part of the sample was pasteurized and dried on sterile corn meal (sample 3) and the other part was not pasteurized and dried on steamed peat moss (sample 4).

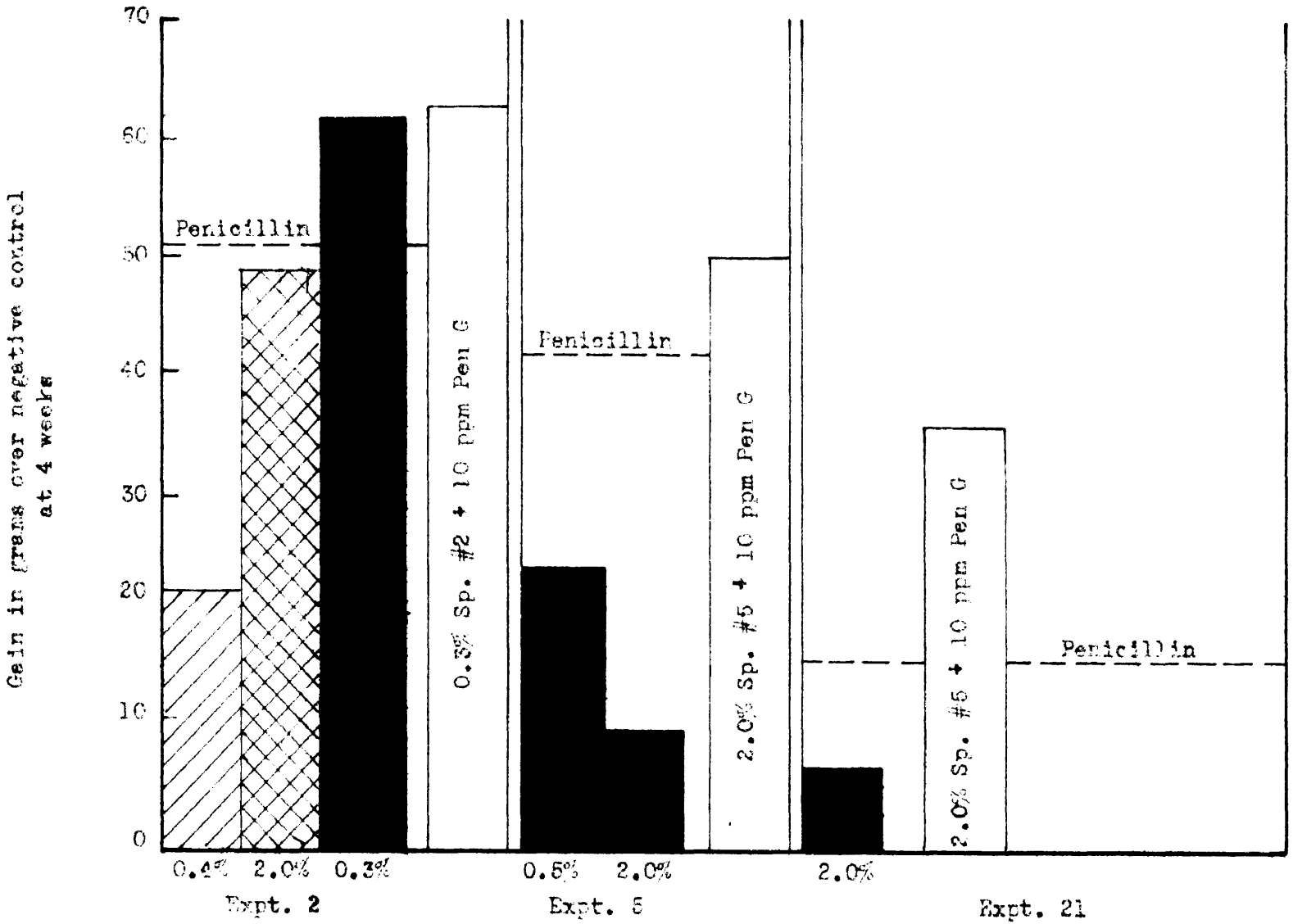


Fig. 1 Effect of Aerobacter aerogenes fermentation products on growth of chicks to 4 weeks (experiments 2, 5, and 21). Cross-hatched bar, sample 1; double cross-hatched bar, sample 2; solid bar, sample 5.

These experiments were designed to ascertain if a substrate of this nature would support the *in vitro* synthesis of a growth factor by *A. aerogenes*, and if the elimination of viable organisms from the fermented product would be reflected in its effect on the growth of chicks.

The results of experiment 3 are presented in table 8. No gains were observed when either product was included in the basal diet. As a matter of fact, the chicks grew less rapidly when either product was employed. The addition of penicillin to the diet which contained 2.0 per cent of the unpasteurized sample caused a significant response over and above that which was obtained with penicillin.

These samples were again tested in experiment 4. The results, which are presented in table 9, show that the apparent depressing action of these samples was again exerted. In group 4, the depression of growth which was observed when 2.0 per cent of the unpasteurized sample was employed was found to be statistically significant at the 5 per cent level when the data were analyzed by the T test. In this experiment, when penicillin was added to either the pasteurized or the unpasteurized products, growth was no better than that obtained with penicillin alone.

Plate counts were performed on the cecal contents of representative chicks from three experimental groups used in experiment 3 and from all groups which were used in experiment 4. The results of these examinations are presented in table 10. In experiment 3, the *A. aerogenes* counts were only slightly higher in the ceca of the birds which were fed the unpasteurized fermentation product with added antibiotic than they were in the birds from the control group. However, in experiment 4, 270×10^7 *A. aerogenes* colonies were found in the cecal contents of those birds which were fed 2.0 per cent pasteurized fermentation product and penicillin, whereas the plate count revealed that there were no *A. aerogenes* present

Table 8

The effect of pasteurized and unpasteurized Aerobacter aerogenes fermentation products on the growth of chicks to 4 weeks (experiment 3)

Group number	Supplement	Avg. wt., 4 weeks in grams			Avg. gain in grams over neg. control
1.	None	303	± 7.62	(19)	-----
2.	0.4% ferm prod (past.)	286	± 9.95	(19)	- 17
3.	2.0% ferm prod (past.)	296	±10.62	(18)	- 7
4.	0.4% ferm prod (unpast.)	305	± 9.35	(18)	2
5.	2.0% ferm prod (unpast.)	294	±11.38	(18)	- 9
6.	10 ppm penicillin	318	± 8.58	(20)	15
7.	As 5 + 6	355**	± 9.31	(17)	52

( ) represent number of surviving chicks.

** the difference between the average gain of this group and that of group 1 is statistically significant at the 5% level.

Table 9

The effect of pasteurized and unpasteurized Aerobacter aerogenes fermentation products on the growth of chicks to 4 weeks (experiment 4)

Group number	Supplement	Avg. wt., 4 weeks in grams			Avg. gain in grams over neg. control
1.	None	316	± 9.99	(17)	-----
2.	0.4% ferm prod (past.)	315	±10.93	(18)	- 1
3.	2.0% ferm prod (past.)	300	±10.63	(18)	- 15
4.	2.0% ferm prod (unpast.)	285**	±10.15	(17)	- 31
5.	10 ppm penicillin	371	±12.70	(18)	55
6.	As 3 + 5	362	±11.76	(18)	46
7.	As 4 + 5	333	±17.76	(15)	17

( ) represent the number of surviving chicks.

** the difference between the average weight of this group and that of the negative control group is statistically significant at the 5% level.

Table 10

The effect of pasteurized and unpasteurized Aerobacter aerogenes fermentation products on the predominating bacteria in the ceca of 4 week old chicks* (experiments 3 and 4)

Group number	Supplement	Media used for counts	Spore-formers	A. aerogenes	E. coli
		(Experiment 3)			
1.	None	Eugon agar	< 1	200	200
		Eugon agar + pen**	< 1	150	200
5.	2% ferm prod (unpast.)	Eugon agar	500	100	10
		Eugon agar + pen	<1	100	10
7.	As 5 + 10 ppm pen	Eugon agar	100	300	<1
		Eugon agar + pen	<1	200	<1
		(Experiment 4)			
1.	None	Eugon agar	<1	<1	200
		Eugon agar + pen	<1	<1	300
2.	0.4% ferm prod (past.)	Eugon agar	150	<1	150
		Eugon agar + pen	<1	<1	200
3.	2.0% ferm prod (past.)	Eugon agar	100	<1	350
		Eugon agar + pen	<1	<1	200
4.	2.0% ferm prod (unpast.)	Eugon agar	1000	<1	300
		Eugon agar + pen	10	<1	250

Table 10 (continued)

The effect of pasteurized and unpasteurized Aerobacter aerogenes fermentation products on the predominating bacteria in the ceca of 4 week old chicks* (experiments 3 and 4)

Group number	Supplement	Media used for counts	Spore-formers	A. aerogenes	E. coli
		(Experiment 4)			
5.	10 ppm penicillin	Eugon agar	< 1	10	700
		Eugon agar + pen**	< 1	10	1000
6.	As 3 + 5	Eugon agar	< 1	250	500
		Eugon agar + pen	< 1	300	650
7.	As 4 + 5	Eugon agar	800	< 1	200
		Eugon agar + pen	< 1	10	200

*Counts expressed as the number of organisms per gram of wet cecal feces x 10^6.

**Plates contained 6 units of penicillin per ml of medium to obtain a measure of the penicillin resistant microorganisms.

in the same dilution of cecal feces of birds from the control. Since the fermentation product contained no viable organisms after it was prepared, this higher count of A. aerogenes can not be ascribed directly to feeding of the product, although it may have indirectly influenced the number of these organisms.

In experiments 3 and 4, a spore-forming, broad, heavily stained gram-positive rod was found in the cecal contents of those birds which were fed the unpasteurized fermentation product. Colonies of this organism when examined on Eugon agar had morphological characteristics which were similar to A. aerogenes. Closer observation of these colonies revealed that they possessed a dull, rough surface and a flat edge, whereas, typical colonies of A. aerogenes appear on Eugon agar with a smooth, glistening surface. Since this organism appeared only in these experiments, it was suspected that the samples may have been contaminated. Consequently, one gram of the pasteurized and unpasteurized samples were plated on Eugon agar and incubated at 37°C for 48 hours. When the plates were examined, it was found that both samples were contaminated with an organism which had the same gross and microscopic morphological characteristics as those which appeared in the ceca of the birds which were fed diets supplemented with these samples. The number of organisms in each sample was approximately 60 x 10^6 per gram. It is apparent from table10 that the inclusion of penicillin in the diet with either supplement had little effect on the proliferation of the contaminating organism in the ceca. However, when 5 units of penicillin were included in each ml of the plating medium, a decided inhibition of the organism was found to occur.

The over-all results which were obtained from this series of experiments indicate that slight increases in weight of the chicks were coincident with the administration of the fermented chick mash (sample 1),

(samples 2 and 5) and the air-dried skimmilk-$CaCO_3$ preparation of A. aerogenes. The dietary administration of a distillers' solubles-cerelose fermentation product, which appeared to be contaminated with a spore-forming aerobic rod, had no effect on chick growth in either the pasteurized or unpasteurized state. The inclusion of penicillin with either sample number 1, 2, or 5 in experiments 2, 5, and 21 appeared to enhance the stimulatory effect of these preparations (see figure 1, page 46).

In view of the wide variability which was encountered in these experiments, the large scale preparations of fermented mashes with A. aerogenes was abandoned. In the remaining experiments, which were designed to study the effect of viable organisms on chick growth, lyophilized preparations of the organism were employed. Lyophilized preparations of pure cultures of the organism which was being studied not only reduced the possibility of contamination but also increased the number of viable organisms per pound of feed by about 10 times. This increase in viable organisms was observed when only 0.022 per cent of the lyophilized preparation was used, compared with a level of 2.0 per cent of some of the fermented mashes. Whereas the number of viable organisms present in a gram of fermented mash was usually about 10×10^6, one gram of lyophilized culture of A. aerogenes usually contained, on the average, approximately 180×10^8 viable cells per gram. In addition, the preparation of organism supplements by the standardized procedure permitted the elimination of substances such as distillers' fermentation solubles which, in itself, is reported to contain unidentified factor activity.

III. The Effect of Lyophilized Cultures of *Aerobacter aerogenes* and *Escherichia coli* on the Growth of Chicks

At the outset of this phase of the investigation, the selection of a suitable medium for the large scale production of masses of cells and their resistance to lyophilization presented a problem. However, a series of preliminary trials ultimately led to the development of the process of lyophilisation which has been previously described.

The first experiment in this series, experiment 8, was designed to exploit the possibility of large scale preparation of the organism in deep-culture, by aerating it with sterile air. Koser's citrate broth, with and without penicillin, was aerated and incubated according to the method which has been described on page 32 and plate 1, page 33. Koser's citrate broth was used since it was desirable to ascertain if the organism, when grown in a synthetic medium, would be capable of promoting growth. Penicillin was included in one batch of broth to determine if the organism, in the process of fermentation, could convert the antibiotic by molecular rearrangement or destruction, into an entity which would cause a rapid growth of the chicks.

After fermentation of the Koser's citrate broth which contained 100 units (0.1 mg) per ml, viable bacterial cells were removed by passing the broth through a sintered glass filter and assayed for antibiotic activity according to the method of Schmidt and Moyer (1944). However, in place of the conventional stainless steel cylinders, filter paper pads (5 mm in diameter) were employed. The standards were prepared by soaking the pads in solutions of penicillin made up in Koser's citrate broth which contained 0.5, 1.0, and 10 units of penicillin per ml. After the prepared plates were incubated for 18 hours, the pad representing the 10 unit concentration produced a zone of inhibition of 48 mm. No

inhibition was observed surrounding the pad which had been soaked in the filtered, fermented broth.

In order to test the potency of the inactivated penicillin, 2,000 ml of the fermented broth which contained the penicillin were dialyzed in running tap water for 3 days. The material was then lyophilized and the dry powder, which was obtained weighed 7 grams. Assuming that none of the original constituents of the penicillin molecule were lost either during fermentation or dialysis, the 7.0 grams of lyophilized dialysate, when added to the necessary 30 pounds of feed, supplied the equivalent of 14 ppm of antibiotic by weight.

The cell-free filtrate from 2,000 ml of the fermented broth which contained no antibiotic was treated in the same manner and was used as a dietary supplement.

From the results of this experiment, which are presented in table 10a, it may be seen that the supplementation of chick diets with 0.022 per cent of either preparation of A. aerogenes had no effect on chick growth. Even in the presence of added penicillin, no gains were observed. Likewise, both non-dialyzable fractions exerted no appreciable effect. In three additional experiments, similar preparations of fermented Koser's citrate broth with and without penicillin, had no effect on chick growth.

Beginning with experiment 9, E. coli, the other organism which seemed to be influenced when antibiotics were administered to chicks, was also studied. Moreover, beginning with this experiment, a standardized procedure for preparing the organism supplements was adopted. They were grown on Kolle flasks and lyophilized according to the procedure which has been described on pages 32 and 36, and plates 2 and 3, pages 34 and 35. In every experiment, the dried powder was used to supplement each experimental diet with a level of 0.022 per cent.

Table 10a

The effect of lyophilized Aerobacter aerogenes, grown in Koser's citrate broth on the growth of chicks to 4 weeks (experiment 8)

Group number	Supplement	Average weight in grams, 4 wks		Gain in grams over negative control
1.	None	345	(18)	-----
2.	0.022% A. aerogenes (no penicillin in broth)	329	(18)	- 16
3.	0.022% A. aerogenes (100 U penicillin/ml of broth)	339	(16)	- 6
4.	10 ppm penicillin	370	(18)	25
5.	As 2 + 4	361	(18)	16
6.	As 3 + 4	364	(18)	9
7.	0.044% dialyzed cell-free filtrate (equivalent to 14 ppm penicillin)	331	(17)	- 14
8.	0.044% dialyzed cell-free filtrate (no penicillin in broth)	364	(15)	19

( ) represent number of surviving chicks.

The results of experiments 9 through 13 and 20 are summarized in figure 2. With the exception of experiments 12 and 13, both A. aerogenes and E. coli seemed to exert a slight effect on growth. The average gains which were observed when either organism was used seemed to be about the same. When penicillin was included in the diet with either organism in experiment 9, and with E. coli in experiment 20, the growth responses which were obtained were significantly higher than the responses which were obtained with penicillin alone. Although the data in the other experiments were not treated statistically, it may be seen in figure 2 that, in general, the addition of penicillin to most diets which were supplemented with viable organisms resulted in greater gains than were observed in those groups of birds which were fed penicillin alone. However, the exceptions to this generalization were: E. coli with penicillin in experiment 10, and A. aerogenes with penicillin in experiments 12 and 13.

Since it is obvious from figure 2 that there were no appreciable differences in the activity of either E. coli or A. aerogenes in promoting growth, the data were summarized and part of it was evaluated statistically by the analysis of variance. These data are presented in table 11. In the columns designated "organisms", the values represented may have been obtained from groups of birds which were fed either A. aerogenes or E. coli. Although slight gains, if any were observed when no antibiotic was fed, these cultures did improve growth in every case when 10 ppm of procaine penicillin G were also fed with the organism supplement. The average growth increase obtained with the addition of 10 ppm of penicillin alone to rations CS-1 and OG-F was 30 and 54 grams, respectively. Further addition of viable organisms in the presence of the antibiotic resulted in average growth increases of 54 and 77 grams over that obtained with basal rations CS-1 and OG-F. Consequently, the growth promoting effect of the antibiotic

Part. V

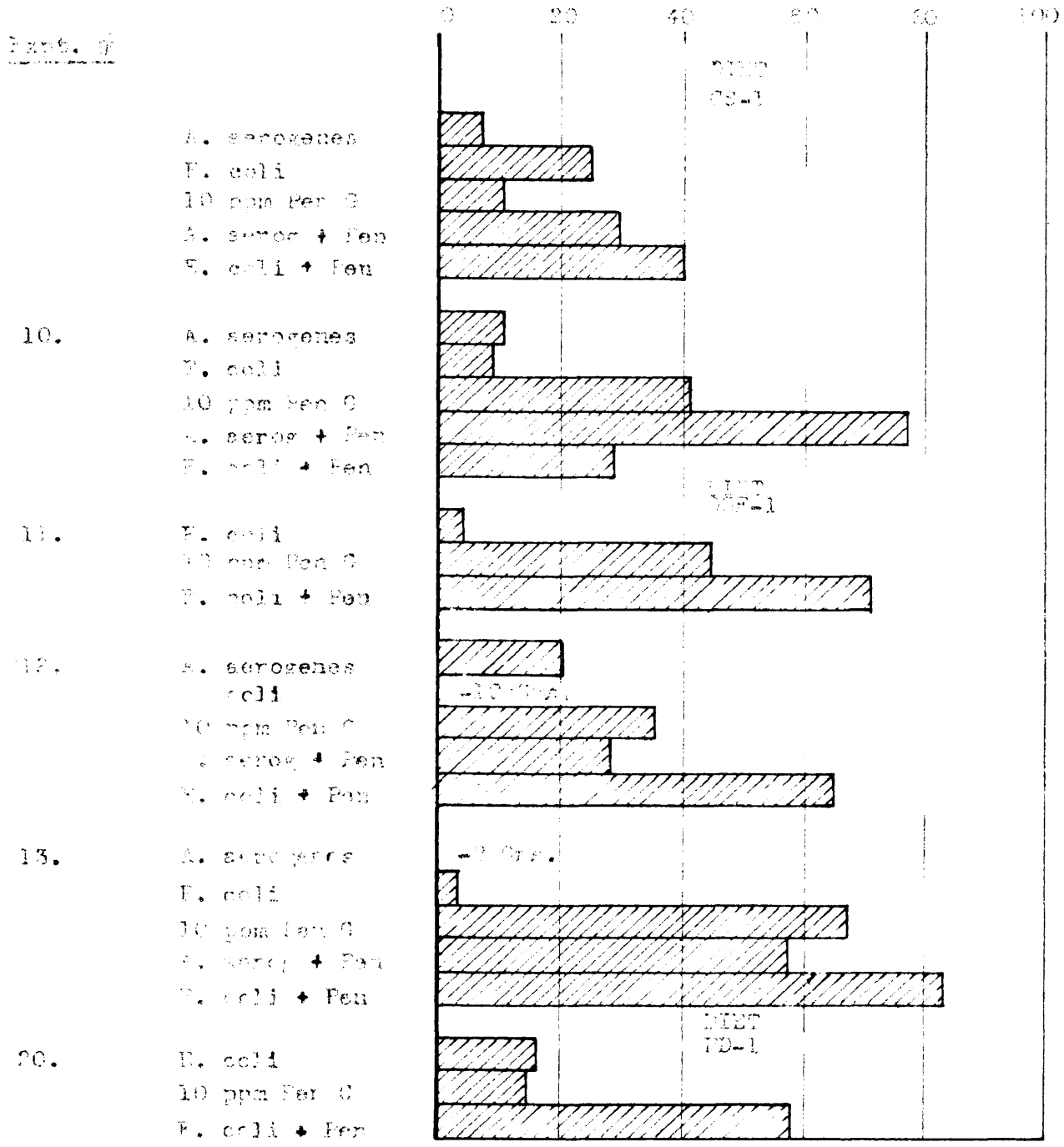


Fig. 2 Effect of viable penicillin resistant microorganisms, and penicillin on chick growth at 4 weeks.

Table 11

Summary of experiments showing the effects of viable penicillin-resistant microorganisms on chick growth to 4 weeks with and without antibiotic (experiments 3 and5, 7-13)

Expt. no.	Without antibiotic				10 ppm penicillin			
	No added organisms		Organisms added		No added organisms		Organisms added	
				Diet CS-1				
5.	219	(15)	282	(14)	270	(17)	285	(15)
6.	312	(20)	328	(16)	356	(19)	361	(16)
7.	309	(27)	320	(14)*	349	(15)	334	(13)
			321	(14)**			384	(14)
			351	(16)***			353	(15)
8.	304	(18)	284	(19)	318	(20)	355	(17)
9.	310	(18)	319	(20)	353	(19)	356	(20)
10.	345	(18)	329	(18)	350	(18)	361	(14)
Average	299		316		329		353	
				Diet CS-F				
11.	261	(17)	267	(17)	307	(18)	333	(17)
12.	269	(18)	258	(19)	307	(19)	339	(18)
13.	282	(15)	309	(13)	359	(15)	374	(13)
Average	270		278		324		347	

( )represent the number of surviving chicks.

*A lyophilized culture of A. aerogenes obtained from Kelle flask surface was added to the diet.

**A lyophilized skimmilk culture of A. aerogenes which contained 100 γ per ml of penicillin prior to fermentation added to diet (0.022%).

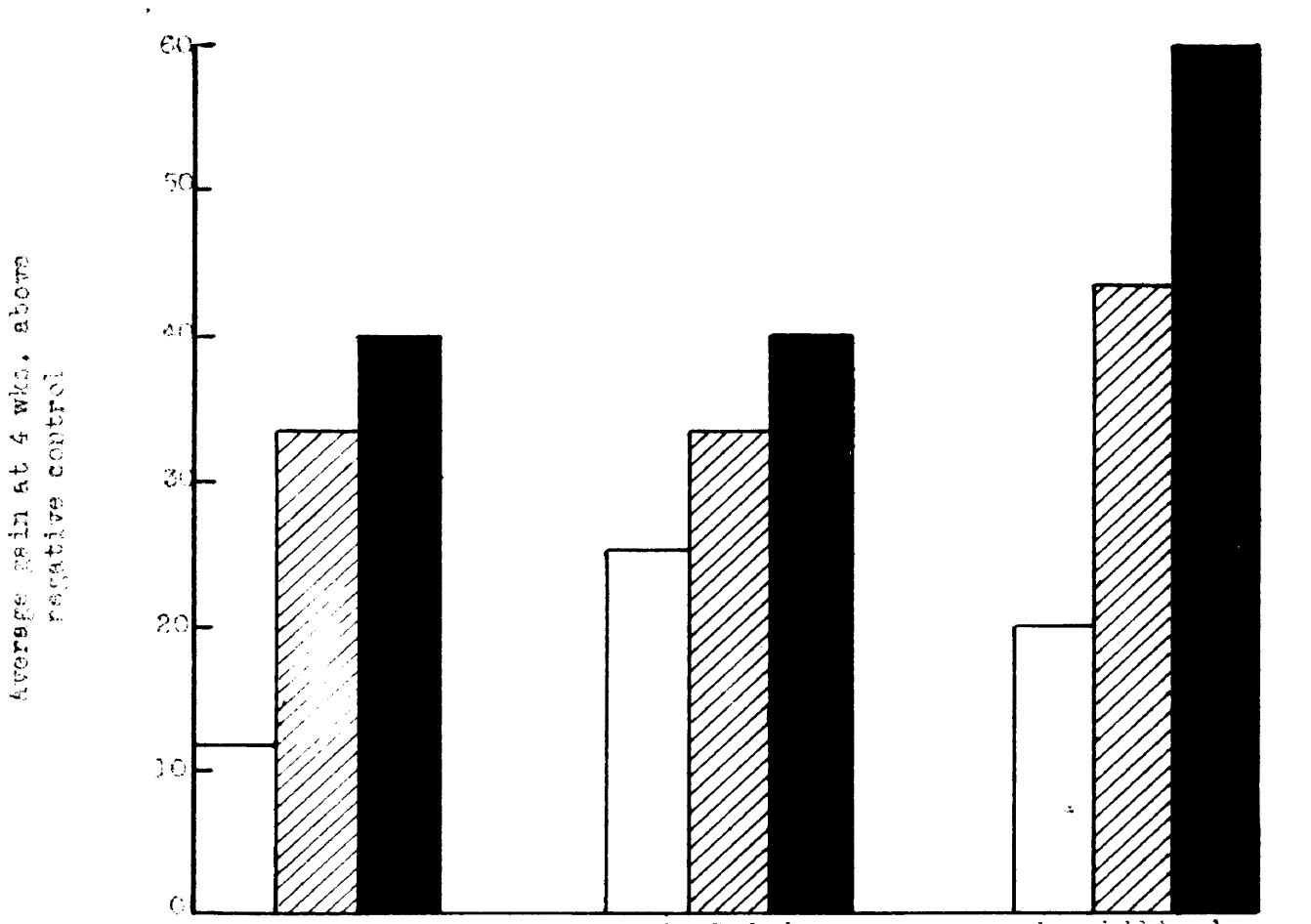
***Same as above without antibiotic prior to fermentation.

was increased an average of 80 and 84 per cent when viable cultures of A. aerogenes and/or E. coli were added to basal rations CS-1 and OG-F, respectively.

The average weight of the chicks which were fed diet CS-1 in six experiments was 29 grams higher than the average weight of those birds which were fed diet OG-F in three experiments. Likewise, the average weight of the chicks fed diet CS-1 with added organisms was 40 grams higher than the average weight of those birds which were fed the organism in diet OG-F. However, these apparent differences in weight were not observed when penicillin or penicillin and organism supplement were fed together in either diet.

An analysis of variance which was performed on the data from all six experiments in which diet CS-1 was used, revealed that the response from the addition of viable penicillin-resistant microorganisms to the diets which contained 10 ppm of penicillin was statistically significant to the 1 per cent level.

At the end of this series of experiments, the 4 week gains of birds which were fed the lyophilized organism, penicillin, or organism and penicillin together, were averaged and compared with the average gains of those birds fed viable organisms prepared in a different manner. From the results which are presented in figure 3, it may be seen that the number of viable organisms supplied by either the air-dried fermented mash or the skimmilk culture of the organism was only 30 and 36 x 10^7 per pound of feed, when added to the diet at a level of 2.0 per cent. The number of viable organisms per pound of feed when they were grown on Kolle flask surfaces and resuspended in skimmilk and added at a level of 0.022 per cent, on the other hand, was 200 x 10^7. It is interesting to note that the average gains which were observed with all three supplements, regardless of the



Replicates	4	3	5
Viable Org. lb. Feed at Start x 10^7	35	30	200

Fig. 3 Effect of various A. aerogenes preparations on the growth of chicks to 4 weeks. (white bar, no antibiotic; cross-hatched bar, 10 ppm procaine penicillin G; solid bar, supplement plus 10 ppm procaine penicillin G).

number of viable organisms, was about the same. However, the addition of the antibiotic to the diet which contained the highest number of viable organisms per pound of feed seemed to have a greater effect on the growth rate than when it was added to diets which contained the lower number of viable organisms.

IV. The Effect of Non-Viable Preparations of *Escherichia coli* and *Aerobacter aerogenes* on the growth of Chicks

Experiment 14 was performed to test the effect of lyophilized cells of *E. coli* and *A. aerogenes* which were non-viable, on the growth of chicks in the presence and in the absence of penicillin.

The method of preparation of the supplements has been described on page 36.

Each supplement was added to the diet at levels of 0.066 and 0.122 per cent both in the presence and in the absence of 10 gms of procaine penicillin G.

The results of experiment 14 are presented in table 12. It is apparent from the data that when either of these preparations was included in the diet at either level, no real gains were observed. Furthermore, addition of the antibiotic to the diets which contained these preparations, at either level, was no more effective than penicillin alone.

A supplemental experiment, 14-a, was performed to further test the effect of non-viable preparations of *E. coli* and *A. aerogenes* on the growth of chicks. In this experiment, each organism was supplied in the feed at the same concentration used for viable preparations, (i.e. the total growth from 3 Kelle flasks per 50 pounds of feed). However, the growth from each flask was removed with 12 ml of 70 per cent ethyl alcohol in place of the usual skimmilk. The alcohol suspensions of each organism were held for 24 hours. At the end of this time, no viable cells were detected by plate counts performed on a 10^2 dilution of both suspensions.

The alcohol suspensions were placed in the feed and mixed in a large feed mixer. This process tended to remove the alcohol from the feed by evaporation since, after mixing, no odor of alcohol was detected in the feed.

The results of experiment 14-a are presented in table 13. Fair gains

Table 12

The effect of non-viable preparations of Escherichia coli and Aerobacter aerogenes bacterial cells on the growth of chicks to 4 weeks (experiment 14)

Group number	Supplement	Average weight in grams, 4 weeks		Gain in grams over negative control
1.	None	310	(20)	-----
2.	0.066% E. coli preparation	312	(20)	2
3.	0.122% E. coli preparation	323	(20)	13
4.	0.066% A. aerogenes preparation	307	(19)	- 3
5.	0.122% A. aerogenes preparation	313	(19)	3
6.	10 ppm penicillin	323	(20)	13
7.	As 2 + 6	332	(19)	22
8.	As 3 + 6	304	(20)	- 6
9.	As 4 + 6	329	(19)	19
10.	As 5 + 6	322	(18)	12

( ) represent number of surviving chicks.

Table 13

The effect of alcohol treated cells of Escherichia coli and Aerobacter aerogenes on the growth of chicks to 4 weeks (experiment 14-a)

Group number	Supplement	Average weight in grams, 4 weeks		Gain in grams over negative control
1.	None	268	(18)	-----
2.	0.022% E. coli (lyophilized)	258	(19)	-10
3.	Equivalent cell mass (alcohol)	306	(18)	38
4.	0.022% A. aerogenes (lyophilized)	290	(18)	22
5.	Equivalent cell mass (alcohol)	293	(18)	25
6.	10 ppm penicillin	307	(19)	39
7.	As 2 + 6	335	(18)	67
8.	As 3 + 6	311	(19)	43
9.	As 4 + 6	294	(19)	26
10.	As 5 + 6	366	(19)	98

( ) represent the number of surviving chicks.

were observed when alcohol treated E. coli or viable and non-viable preparations of A. aerogenes were fed. In this experiment, the addition of lyophilized, viable E. coli had no effect on growth. The addition of penicillin to the lyophilized preparation of E. coli caused the chicks to grow better than they did when the antibiotic was excluded from the diet. In addition, the antibiotic exerted an even more marked effect on growth rate when it was fed with the alcohol treated A. aerogenes preparation. These results seem to suggest that possibly the alcohol treatment was liberating a factor from A. aerogenes which exerted its effect in the presence of the antibiotic.

V. The Influence of *Escherichia coli* and Penicillin on the Growth of Chicks Fed Diets Deficient in Certain B Vitamins

Experiment 20 was performed to determine if lyophilized *E. coli*, penicillin, or a combination of the organism and antibiotic would influence the growth of chicks which were fed purified diets from which certain B vitamins were omitted.

The sparing effect of the supplements listed above on the dietary requirement of the chick for thiamin, biotin, calcium pantothenate, and riboflavin was studied. Moreover, a control series was used which was fed the complete basal diet (PD-1), supplemented with either the organism, the antibiotic, or the two together. In each series, an unsupplemented control was also included. Skimmilk powder at a level of 0.022 per cent was added to all diets which received no organism preparation.

The results of experiment 20 are presented in table 14. The addition of organism and penicillin to the complete basal diet caused a significant stimulation of growth. When thiamin was omitted from the diet, all of the chicks from each of the four experimental groups were dead by the tenth day. However, at 1 week of age, there was no apparent difference in the average weights between any of the supplemented groups of the thiamin series.

When no biotin was added to the diet, the chicks grew better if the organism was added to the diet. However, the T test showed that the gain fell short of being significant at the 5 per cent level.

The organism significantly depressed growth when it was added to the diet low in calcium pantothenate. Penicillin, on the other hand, elicited a response of 28 grams. When the organism was added with penicillin, growth was again depressed significantly when the response was compared to that obtained with the antibiotic alone.

Table 14

The effect of lyophilized Escherichia coli, penicillin, or the organism and antibiotic together on chick growth to 4 weeks in the absence of thiamin, biotin, calcium pantothenate, or riboflavin (experiment 20)

Group number	Vitamin omitted from basal diet	Average weight 4 weeks, in grams			Average gain in in grams over negative control
1.	None	313	±17.08	(13)	-----
2.	None + E. coli	332	±17.90	(14)	19
3.	None + 10 ppm penicillin	331	± 9.78	(14)	18
4.	None as 2 + 3	370[a]	±19.52	(14)	57
5.-8.	Thiamin	(all chicks dead at 10 days)			
9.	Biotin	185	±12.82	(9)	-----
10.	Biotin + E. coli	209	±12.70	(7)	24
11.	Biotin + 10 ppm penicillin	206	±21.04	(4)	21
12.	Biotin as 10 + 11	198	±17.58	(7)	13
13.	Calcium pantothenate	135[b]	± 8.48	(10)	-----
14.	Calcium + E. coli	105	± 6.64	(12)	- 30
15.	Calcium + 10 ppm penicillin	163[c]	± 7.79	(11)	28
16.	Calcium as 14 + 15	140	± 7.38	(9)	5
17.	Riboflavin	105	± 5.16	(15)	-----
18.	Riboflavin + E. coli	118	± 5.01	(15)	13
19.	Riboflavin + 10 ppm pen.	127[d]	± 6.15	(15)	22
20.	Riboflavin as 18 + 19	182[e]	±12.41	(8)	77

( ) represent the number of surviving chicks.
[a] significantly better than 1 at 5% level.
[b] significantly better than 14 at 5% level.
[c] significantly better than 13 and 16 at 5% level.
[d] significantly better than 17 at 5% level.
[e] significantly better than 17, and 19 at 5% level.

Significance based on T test.

When the organism preparation was added to the diet to which no riboflavin was added, growth was again slightly improved, although not significantly so. In addition, a significant response was obtained with penicillin. The addition of the organism and antibiotic to the riboflavin-deficient diet caused a response which was greater than that obtained with either the organism or the antibiotic when they were added separately to the diet.

Livability seemed to be poorest in those chicks which were fed the basal diet without added thiamin; next in line were those chicks fed the ration without biotin. Livability was somewhat better in those chicks which were fed the diet without calcium pantothenate, and mortality in the series fed the basal diet without riboflavin was observed only in that group which was fed the organism with added antibiotic. During the course of this experiment, only the chicks which were fed the thiamin deficient ration manifested the characteristic syndrome which appears in cases of deficiency of this vitamin. A logical explanation for this can, in all probability, be based on the fact that the deficiencies, when they were present, were so severe that the chicks died before the characteristic symptoms could be observed.

VI. The Influence of Environment on the Growth of Chicks Fed Viable, Penicillin-Resistant Microorganisms, With and Without Added Antibiotic

This series of experiments (15 through 17) was performed to determine if different environmental conditions would influence the growth rate of chicks which were fed viable, penicillin-resistant microorganisms, penicillin, and the two supplements together. Eight experimental groups of chicks were reared in each of three environments. One series consisted of four experimental groups which were fed basal diet CS-2, which contains no fish meal. Sub-group 1 of this series was fed the basal diet with no supplement; 10 ppm penicillin, 0.088 per cent lyophilized A. aerogenes with 0.055 per cent E. coli, and the two organisms plus the antibiotic were added to sub-groups 2, 3, and 4, respectively. A series of four parallel experimental groups was also included in these experiments, but diet CS-3 was used. This diet contains 5 per cent fish meal, which has been reported to contain unidentified factor activity for the chick. This substance was included in the diet in an effort to determine if it would influence the growth rate of the birds which were fed the viable organisms.

Day-old Barred Plymouth Rock chicks of both sexes were used in the three experiments of this series. In experiment 15, (environment A) 70 chicks were used per experimental group. The method of rearing these birds has been described on page 22. The walls of the floor pens were cleaned only with cold water, and, during the experimental term, the birds had access to their droppings. In experiment 16 (environment B) 20 chicks were used per experimental group. The chicks were reared at the Poultry Nutrition Laboratory in battery brooders which were equipped with raised wire floors.

The battery room was used only for 4 out of every 6 weeks, with a

2 week period for cleaning. No floor drains are in this room, hence, thorough cleaning presents somewhat of a problem. The birds which were used in experiment 17 (environment C) were reared in a new battery brooder in a temperature controlled room. Chicks had been raised in this room only once, one year prior to this experiment. For this reason, environment C was more ideal from the standpoint of contaminating microorganisms than either environments A or B.

In addition to the eight experimental groups which were used in three environments, 4 additional groups of 70 birds each were included in experiment 15. The diets were supplemented in the same manner as described, but 15 mg per pound of orotic acid were added to the basal diet (CS-3).

The results of the experiments which were conducted under the three environmental conditions described above, are presented in table 15. With the exception of group 1, the chicks which were fed diet CS-2 and reared in environment C grew more rapidly than those birds which were raised in either of the other two environments. Moreover, the birds grew at a more rapid rate in every experimental group reared in environment A than those birds in environment B. These data seem to suggest that, in general, environment B had the most pronounced effect on the growth rate of the birds. The chicks in this series, regardless of the treatment, grew less rapidly than those birds in either of the other two environments. The chicks reared at the Poultry Farm (environment A) showed no increased average gain when the organism was added to diet CS-1; whereas, when this supplement was added to the same diet in environments B and C, gains of 25 and 26 grams were observed, respectively. The greatest gain in the presence of added antibiotic, to diet CS-1, was observed in those chicks in environment C. This is of interest since reports in the literature indicate that

Table 15

The influence of environment on the growth of chicks to 4 weeks fed viable penicillin-resistant micro-organisms, and Penicillin (experiments 15, 16, and 17)

Group number	Supplement	Average 4 week weight (gms) ENVIRONMENT A			B			C			Avg. of treatments (all environments)
					Diet CS-2						
1.	None	287	±5.13	(69)	261	±11.39	(17)	275	±11.58	(17)	274
2.	0.088% A. aerogenes + 0.055% E. coli	291	±5.39	(67)	286	± 8.57	(20)	301	± 6.82	(19)	293
3.	10 ppm penicillin	290	±5.14	(69)	276	± 7.14	(18)	307	± 9.16	(19)	291
4.	As 2 + 3	290	±5.79	(70)	287	±13.16	(18)	298	±10.85	(20)	292
Average of environments (all treatments)		290			278			295			
					Diet CS-3						
1.	All groups supplemented as 1,2,3, and 4 above	314	±4.51	(70)	298	±10.94	(19)	302	±11.31	(18)	305
2.	0.088% A. aerogenes +0.055% E. coli	309	±6.32	(69)	310	± 8.72	(16)	285	± 9.80	(18)	301
3.	10 ppm penicillin	308	±5.04	(70)	333	±19.12	(18)	324	± 9.36	(18)	321
4.	As 2 + 3	311	±5.86	(70)	274	±15.28	(19)	318	± 9.63	(17)	301
Average of environments (all treatments)		311			304			307			

( ) represent the number of surviving chicks.

in "new" environments, no growth response is observed when antibiotics are fed. When both organism and antibiotic supplement were added together to diet CS-2, the average gains which were observed, regardless of environment, were no better than those gains observed in the groups of birds which were fed the same diet supplemented with either the organisms or antibiotic alone.

If all treatments and all environments are compared (table 15), it will be noted that, with one exception, the chicks which were fed diet CS-3 grew more rapidly than those birds fed diet CS-2. The group which failed to show any increased gain when the diet containing fish meal was fed, was group 4 in environment B.

When diet CS-3 was fed, the chicks which were reared in environments A and C again grew more rapidly than those chicks reared in environment B. Also, when this diet was used in the presence of the antibiotic, the chicks in environment A once again failed to show any increased gain over the negative control.

These data suggest that rearing the chicks on litter may have negated the antibiotic effect, since, increased gains were noted when penicillin was added to either diet when the birds were reared in batteries. The addition of organisms to the diet which contained both penicillin and fish meal failed to increase the rate of growth of the chicks reared in either environments A, B, or C. As a matter of fact, the addition of organisms to the diet which contained the antibiotic seemed to cause a slight decrease in the growth rate of the chicks raised in environments B and C. Likewise, no increased gains were noted when only the organism was added to the diets of chicks, in the presence of fish meal, in environments A and C, and only a slight increased gain was observed when the organism was added to the diet of the chicks reared in environment B.

If the growth response of chicks to dietary microorganisms in the presence of penicillin is due to the synthesis of a growth factor in the intestinal tract, the data obtained in this study suggest that it might possibly be related to the factor present in fish meal. This idea is supported by the observation that no additional gains were observed when the organisms were added to the diets which contained fish meal either in the presence or in the absence of the antibiotic.

In order to test the significance of certain treatments, an analysis of variance was performed on the data obtained in this series of experiments. The results of this analysis which are presented in table 16, show that the environmental conditions exerted a significant effect on the growth of the chicks. More specifically, these data indicate that the environmental conditions under which the chicks were reared in either floor pens with fresh wood shavings for litter, or in the new battery brooder in a room in which chicks were raised only once previously, seemed to be more favorable for the rapid growth of the birds than those existing environmental conditions in the Poultry Nutrition Laboratory. This is not surprising, since the chicks were reared in an environment which was, in all probability, the more highly contaminated of the three.

The analysis of variance also revealed that the environmental conditions exerted a significant effect on the response of the chicks when penicillin was added to their diets. Considering all treatments and both diets, the addition of penicillin to the diets of birds reared in environment A had no effect on the growth rate; however, the antibiotic elicited responses of 50 and 44 grams in environments B and C, respectively.

Experiments 16 and 17 were terminated at the end of 4 weeks, but experiment 15 was continued to 8 weeks to determine if any of the treatments would have an effect on the growth rate of birds raised on litter for a longer experimental period.

Table 16

Results of analysis of variance considering treatment and environmental effects on the growth of chicks to 4 weeks (experiments 15, 16, and 17)

Source of variation	Degrees of freedom	Sum of squares	Mean square
	Effect of Treatment		
Fish meal	1	2508.2	2508.2
Penicillin	1	350.4	350.4
Viable organisms	1	0.1	0.1
Fish meal x penicillin	1	3.3	3.3
Fish meal x organisms	1	703.6	703.6
Penicillin x organisms	1	381.1	381.1
Fish meal x penicillin x organisms	1	6.9	6.9
Environment	2	657.0	328.5[a]
	Effect of Environment x Treatment		
Environment x fish meal	2	174.2	87.1
Environment x penicillin	2	734.6	367.3[a]
Environment x organism	2	13.4	6.7
Remainder	8	1953.7	244.2[b]
Individual chicks (error)	818	1640499.0	83.47*

[a]significant at the 5% level.

[b]highly significant at the 1% level.

*MEAN SQUARE CORRECTED FOR UNEQUAL NUMBERS BY USING HARMONIC MEAN.

The average 8 week weights of all of the various groups of this phase of experiment 15 are presented in table 17. Except in group 2, fed diet CS-3, where a gain of 21 grams was noted, the organisms failed to increase the growth rate when they were added to any of the diets either in the presence or in the absence of penicillin. The group of chicks which were fed orotic acid in diet CS-3 with and without penicillin, weighed respectively, 63 and 82 grams more than those birds which were fed comparable diets with added organisms. From this, it appears that dietary microorganisms may exert a detrimental influence on chick growth in the presence of orotic acid.

Increased gains of 39, 33, and 44 grams were noted when penicillin was added to diets CS-2, CS-3, and CS-3 with orotic acid, respectively. Also, when the unsupplemented basal groups are compared, it will be seen that those chicks which were fed the diet supplemented with only fish meal weighed 53 grams more than those birds fed the diet without fish meal. In addition, when fish meal and orotic acid were present in the basal diet, the chicks weighed 77 grams more than those birds fed the diet without either of these supplements. These data tend to suggest that the rate of gain is influenced by the presence of fish meal when the chicks are raised on litter, and the addition of orotic acid further magnifies the rate of gain in the presence of fish meal.

Table 17

The effect of viable organisms, penicillin, and orotic acid on the growth of chicks to 8 weeks (experiment 16)

Group number	Supplement	Average gain at 8 weeks					
		Diet CS-2		Diet CS-3		Diet CS-3 15 mg/lb orotic acid	
1.	None	823	(68)	876	(70)	900	(64)
2.	0.038% lyophilized *A. aerog.* + 0.055% lyophilized *E. coli*	802	(64)	897	(67)	837	(66)
3.	10 ppm procaine penicillin	862	(67)	909	(70)	944	(68)
4.	As 2 + 3	853	(70)	867	(70)	862	(67)
Average of all treatments		843		884		886	

( ) represent the number of surviving chicks.

VII. The Effect of Lactobacillus bifidus on the Growth of Chicks and Turkey Poults

Two strains of L. bifidus were administered to chicks in experiment 18. Both strains, Vt 3 and C 31, which have been described on page 29, were administered to certain experimental groups of chicks in the lyophilized state, in the feed. In addition, the chicks in other experimental groups were orally inoculated with either strain of the organism on alternate days. The methods used for the preparation of each strain and the routine which was employed in administering the organisms to the chicks of the orally inoculated series have been described on page 37.

The lyophilized preparations were employed at a level of 0.022 per cent and, this amount of dried powder supplied approximately 60×10^5 viable organisms per pound of feed.

In this experiment, both strains of the organism were tested with the basal diet CS-1 in the presence and in the absence of condensed fish solubles. This substance has been found to stimulate the growth of L. bifidus, in vitro. Consequently, it was included in the basal diet with the expectation that this treatment would cause the proliferation of the organism in the intestinal tract of the chick and, hence, further magnify any effect high numbers of this organism might have on growth. Since, in earlier studies, the presence of lactose in the diet caused an increase in the number of these organisms which are normally present in the ceca, this carbohydrate was included in the study with lyophilized preparations of each strain of the organism.

During the course of this experiment, a control group of birds was included in both series which involved the basal diet with and without fish solubles. Physiological saline was administered orally to the birds in these control groups in place of the suspensions of viable organisms.

The results of this study, which are presented in table 18, indicate

Table 18

The effect of viable preparations of Lactobacillus bifidus on the growth of chicks to 4 weeks (experiment 18)

Group number	Supplement	Average weight 4 weeks in grams			Average gain in gms. over neg. control
		No fish solubles			
1.	None (saline)	327[a]	±10.32	(17)	-----
2.	Vt 3 (inoculated)	316	±14.00	(20)	- 11
3.	C 31 (inoculated	302	± 8.32	(20)	- 25
4.	10 ppm penicillin (saline)	345	±10.73	(20)	18
5.	As 2 + 4	340	±22.80	(16)	13
6.	As 3 + 4	346	± 8.80	(19)	19
7.	None	305	± 8.31	(20)	-----
8.	Vt 3 (lyophilized)	311	±11.14	(20)	6
9.	C 31 (lyophilized)	323	±11.74	(20)	18
		4% fish solubles			
10.	None (saline)	339	± 7.96	(20)	-----
11.	Vt 3 (inoculated)	319	± 8.40	(19)	- 20
12.	C 31 (inoculated)	337	±10.68	(19)	- 2
13.	10 ppm penicillin (saline)	349	± 7.32	(20)	10
14.	As 11 + 13	336	± 9.68	(18)	3
15.	As 12 + 13	349	± 9.16	(19)	10
		10% lactose			
16.	None	316	± 9.20	(16)	-----
17.	Vt 3 (lyophilized)	292	±11.40	(17)	- 24
18.	C 31 (lyophilized)	312	±11.27	(19)	- 4

( ) represent the number of surviving chicks.

[a] close to significance at 5% level when compared with 3. but significant at the 5% level when compared with 17.

Significance based on T test.

that those chicks which were inoculated orally with either organism seemed to grow less rapidly than their controls, when fish solubles was either added to or omitted from the basal diet. The apparent depression which was observed in group 3 approached the 5 per cent level of significance. The addition of penicillin to the diets with and without fish solubles apparently negated the effect of the organism. When the lyophilized organisms were employed, no difference in the growth rate was noted (compare groups 8 and 9). However, when the lyophilized preparations were added to the diet in the presence of 10 per cent lactose, a decrease in the growth rate was noted. When compared with group 1, the decrease in the growth rate of the chicks which were fed lyophilized Vt 3, in the presence of 10 per cent lactose, was significant. The depression in growth in this instance was 35 grams. On the other hand, when the average weight of the birds fed Vt 3 and lactose were compared with the basal diet with added lactose (group 16), the apparent decrease in the rate of growth was not significant.

The effect on chick growth of either organism was not further magnified by the presence of fish solubles in the diet. However, if groups 1 and 10 are compared, it will be noted that a slight increase in the growth rate occurred when fish solubles was present in the diet of the chicks which received only saline.

The number of L. bifidus which were present in the ceca of representative birds from certain experimental groups was estimated. Appropriate dilutions of the cecal content of these birds were plated on Rogon agar and incubated anaerobically in Brewer jars. After 4 days incubation at 37°C, the small pin-point colonies, which appeared on the plates, were counted. Since both organisms form this type colony on plates of Rogon agar which are incubated anaerobically, it was felt that this was a fair measure of the actual number of organisms.

The results of the bacteriological examination of the ceca of the birds which were selected from several of the experimental groups are presented in table 19. The addition of organisms to the diets in the lyophilized state, or orally inoculating them into the chicks, seemed to be reflected by the number of organisms which were detected in the cecal contents. Furthermore, the addition of 10 per cent lactose seemed to exert the most noticeable effect especially when lyophilized C 31 was added to the diet. The addition of 5 per cent fish solubles to the diet seemed to be ineffective in increasing the number of organisms in the ceca when either organism was inoculated orally into the chicks.

When penicillin was included in the diet of the birds which were orally inoculated with organism strain Vt 3, in place of the expected decrease in the number of L. bifidus, there was an increase in these organisms (compare groups 2 and 5).

Although, in general, the number of organisms in the ceca were increased when they were administered either in the feed or by medicine dropper, it is difficult from the data to state that any correlation existed, in this experiment, between the number of organisms which were actually present in the ceca and the growth rate of the birds.

In experiment 19, an attempt was made to influence the growth of turkey poults by the addition to diet TS-1 of lactose and fish solubles either alone or in combination. In this study, 11 day-old Broad Breasted Bronze poults were used per experimental group.

The results of experiment 19 are presented in table 20. The addition of 10 per cent lactose to the diet resulted in an increase in the rate of gain. The response which was observed with the antibiotic and also the combination of the antibiotic and lactose were both significant at the 5 per cent level. Furthermore, the addition of 4 per cent condensed fish

Table 19

The effect of administration of Lactobacillus bifidus on the number of these organisms present in the ceca of 4 week old chicks (experiment 18)*

Group number	Supplement	Pin-point colonies Eugon agar	(anaerobic)
	No fish solubles		
1.	None	50	
2.	Vt 3 (oral)	90	(-11)
3.	C 31 (oral)	770	(-25)
5.	As 2 + 10 ppm penicillin	620	(18)
	5% fish solubles		
10.	None	360	
11.	Vt 3 (oral)	50	(-20)
14.	Vt 3 (oral) ± 10 ppm penicillin	120	(- 2)
	10% lactose		
16.	None	350	
17.	Vt 3 (lyophilized)	940	(-24)
18.	C 31 (lyophilized)	4260(estimated)	(- 4)

*counts expressed as the number of organisms per gram of cecal feces on wet basis. x 10^7

( ) difference in average gain between group and negative control.

Table 20

The effect of lactose and penicillin on the growth of turkey poults to 4 weeks (experiment 19)

Group number	Supplement	Average weight 4 weeks in grams			Average gain in gms. over neg. control
		No fish solubles			
1.	None	492	±27.14	(10)	-----
2.	10% lactose	546	±29.14	(11)	54
3.	10 ppm penicillin	617[a]	±22.89	(10)	125
4.	As 2 + 3	664[b]	±27.60	(11)	172
		4% fish solubles			
5.	None	587[c]	±18.78	(11)	-----
6.	10% lactose	547	±20.76	(8)	- 40
7.	10 ppm penicillin	618	±11.11	(9)	30
8.	As 6 + 7	629	±37.99	(10)	42

( ) represent the number of surviving chicks.

[a] significantly better than 1 at 5% level.

[b] significantly better than 1 at 5% level.

[c] significantly better than 1 at 5% level.

Significance based on T test.

solubles to the basal diet resulted in a significant increase in the growth rate (compare groups 1 and 5). When lactose was added to the diet in the presence of fish solubles, the growth rate seemed to be retarded. Coincident with this observation was the appearance of high numbers of L. bifidus in the ceca of the poults. In both instances where the antibiotic and the carbohydrate were added to the diet together, the average weight of the poults was higher than that of each of the respective control groups (compare group 1 and 4; 5 and 8). Bacteriological analysis of the ceca revealed that the antibiotic was not effective in eliminating L. bifidus from the ceca.

VIII. The Effect of *Aerobacter aerogenes* and *Escherichia coli* on the Growth of *Lactobacillus bifidus*

Since, in earlier studies, the appearance of high numbers of *L. bifidus* in the ceca seemed to be correlated with poor chick growth, interest in the nutritional requirements of this organism was aroused. The organism could not be cultivated in several of the complex media which were employed by Romoser (1951). Recent work by ~~Shorb~~ YELTRE *et al.* (1953) ~~unpublished data~~) has shown that the organism requires an unidentified factor for growth. This factor is present in fish solubles. It is of interest that this same substance contains an unidentified factor necessary for the rapid growth of chicks.

On the basis of this work, the possibility exists that in the presence of penicillin, *E. coli* and/or *A. aerogenes* synthesize a factor in the intestinal tract of the chicks which promoted their growth. In the absence of the antibiotic, on the other hand, *L. bifidus* may utilize this factor at the expense of the host and hence, depress its growth.

With this theory in mind, two exploratory trials were performed to determine if either *E. coli* or *A. aerogenes* will produce a factor which stimulates the growth of *L. bifidus*.

The presumptive test, which has been described on page 39, was used to test the effectiveness of each organism, in the viable state, on the growth of *L. bifidus*. A dense zone of growth of *L. bifidus* was produced directly below the streaked organisms in the number 5 medium (see appendix). This test, while only presumptive, indicated that the organisms, in some way, exerted an effect on the growth of *L. bifidus*.

With the development of the tube assay, in which *L. bifidus* is utilized as the test organism, one very recent test has been performed using cells of *E. coli* which were prepared as described in table 6, page 40.

Each sample was neutralized and added to duplicate assay tubes at levels of 0.1, 0.5, 1.0, and 2.0 ml. At the end of 48 hours incubation, the contents of each tube was titrated with 0.1 N NaOH. While there was no difference in the activity of any of the samples, progressively increased activity was observed with the 0.1, 0.5, and 1.0 ml levels. Maximum stimulation of the organism seemed to be elicited by the 1.0 ml level of the samples.

Since the organism was grown on a medium which contains phytone, and this substance contains the "L. bifidus factor", further work is being performed to determine the activity of E. coli and A. aerogenes treated in a similar manner but grown on a synthetic medium.

DISCUSSION

The results which have been obtained in this investigation indicate that Escherichia coli and Aerobacter aerogenes beneficially influence the growth rate of chicks when added to diets which contain 10 ppm of procaine penicillin G. In the absence of the antibiotic only slight increases in growth rate, if any, were observed.

While this investigation was in progress, three accounts appeared in the literature which are essentially in agreement with the observations which are reported here. Anderson et al. (1952 b, and 1953 a) administered broth cultures of E. coli to chicks and observed a slight increase in the growth rate which was further significantly increased when penicillin was also present in the diet. Addition of the cell-free filtrate per se had no effect on the growth of chicks in the absence of penicillin. However, when the antibiotic was included in the diet with the cell-free filtrate, growth was significantly better than when the uninoculated sterile broth was fed in the presence of the antibiotic.

When non-viable preparations of either E. coli or A. aerogenes were added to the diets of chicks no effect on their growth rate was observed. Similarly, Anderson et al. (1953 b) added killed cells of E. coli to poult diets in the presence and in the absence of penicillin. These investigators reported that this treatment was ineffective. This would indicate that viable organisms must be supplied in order to obtain a growth response. This observation is further supported by the fact that diets which contained a high number of viable coliform cells per pound were more effective in promoting the growth rate of chicks than those diets which contained fewer organisms. Wide variability was encountered when bacteriological

examinations were performed on the ceca of birds which were fed diets containing viable organisms. Hence, this difference in activity between diets which contained high numbers of viable organisms and those which contained fewer organisms can not be explained only on the basis of implantation of the organism in the intestinal tract.

The effectiveness of dietary microorganisms seemed to be influenced by the environment in which birds were reared. When the chicks had access to their droppings, no increased gain resulted from the addition of viable penicillin-resistant organisms. In fact, the chicks which were raised on litter without dietary organisms grew as well as those birds which were fed the organisms, but raised in batteries. This may have been a result of the ingestion of feces by the birds raised on litter, since Johannson et al. (1948) found high numbers of E. coli in the feces of chicks.

The growth rate of chicks was also definitely influenced by the environmental conditions in which they were reared. A more rapid rate of growth was observed in an environment which was used only once previously for rearing chicks when compared with the rate of growth of those birds which were reared on litter or in a battery in a room which was used for 4 weeks out of every 6 for growing chicks. This is surprising since reports by Coates et al. (1951 b, 1952) and Bird et al. (1952), indicate that chicks which are reared in a "new" environment fail to show a response to added antibiotics.

The fact that the chicks grew better in an environment which was less likely to be contaminated with undesirable microorganisms stresses the importance of sanitation in rearing chicks.

It was observed that the birds which were raised in all environments and fed the viable penicillin-resistant microorganisms grew as well as the birds which received penicillin. In addition, no increase in growth rate

occurred when penicillin and the organisms were added together. It is possible that, in this study, there was no competition between the type of organisms which were added and the type which were present in the environment as a normal contaminant.

In three experiments the addition of viable penicillin-resistant microorganisms to a diet which contained fish meal, in addition to corn and soybean oil meal, had no effect on growth. However, the addition of organisms to a diet which was formulated to contain sub-optimal quantities of the unidentified factor present in soybean oil meal (Hill, 1948; Hill and Briggs, 1950) but which contained ample quantities of the unidentified factor reported to be present in fish meal (Weise et al. 1949) slightly improved the growth rate of the chicks. Greater gains were obtained when penicillin was also included in this diet with viable organisms. Improved growth was also observed when a corn-soybean oil meal ration, without fish meal, was used.

If A. aerogenes and/or E. coli supplied a factor which caused a growth response in the chicks, these observations imply that this factor was involved in an interrelationship between the factors reported to be present in fish meal and soybean oil meal. The presence of both substances in the diet seemed to spare the factor which was supplied by the microorganisms. On the other hand, when either substance was absent, the growth rate was favorably influenced by the presence of viable penicillin-resistant microorganisms in the diet.

Some evidence was obtained in this investigation which indicates that E. coli, when added to a purified diet can spare the dietary requirement of the chick for certain vitamins of the B complex. The addition of viable organisms or penicillin to a diet which contained no added biotin increased the growth rate of the chicks. This observation is in agreement with

Waibel *et al.* (1952) who found an increase in the biotin content of eggs as a result of feeding penicillin. However, when both the antibiotic and organism were added in combination, growth was no better than that observed when either supplement was used alone. This suggests that increased synthesis of biotin by the organism did not occur in the presence of the antibiotic.

No evidence was obtained to indicate that the organism could synthesize pantothenic acid. In fact, a depression of the rate of growth was noted when organisms were added to a pantothenic acid deficient diet. This depression approached significance. The antibiotic, when used alone in the pantothenic acid deficient diet, caused an increase in the growth rate. It is interesting that the addition of organisms to the diet which contained the antibiotic caused a depression in the growth rate when the average gain of this group was compared with the average gain of that group which received only the antibiotic. It is possible that, in the presence of penicillin, pantothenic acid may have been synthesized by an organism other than *E. coli*. The addition of *E. coli* to diets which contain sub-optimal quantities of pantothenic acid, under the conditions of this experiment, seems to be detrimental to the growth of the animal. This would indeed be a factor worthy of consideration if the inclusion of viable organisms in poultry feeds is eventually proven to be of practical importance.

Blaylock *et al.* (1952) found that the dietary requirement for riboflavin by the chick was spared by penicillin. Their observation was again confirmed during this investigation. *E. coli*, when added to the diet alone, caused a slight increase in the growth rate. However, a significant response was noted when the antibiotic and organism were included together, in the diet which was deficient in riboflavin. Actually, the response was greater than that which was observed when both supplements were added to the basal

ration which was complete in all vitamins known to be required by the chick.

The indication that E. coli spares the dietary requirement of the chick for certain B vitamins lends some support to the postulate that antibiotics promote growth by increasing the availability of essential nutrients. The evidence obtained in this study indicates that at least two essential vitamins, biotin and riboflavin, may be involved. Since antibiotics have been shown to reduce the number of fastidious organisms in the intestinal tract of the chick by Anderson et al. (1952 b), March and Biely (1952), and others, it is possible that the competition between intestinal organisms and the host for certain metabolites is abolished by the addition of antibiotics to the diet of the host. The addition of both organism and antibiotic supplements together to a practical type diet, which contains adequate amounts of the B vitamins known to be required for the chick, causes an increase in the growth rate. Hence, the complete mechanism whereby antibiotics and dietary microorganisms exert an effect on growth, can not be explained only on the basis of the findings as discussed above.

When A. aerogenes was grown in Koser's citrate broth which contained 100 units of penicillin per ml, the antibiotic did not inhibit the growth of the test organism, Micrococcus pyogenes var. aureus (strain 209 P), after fermentation. No increased growth was observed when a portion of the broth, which supplied the equivalent of 14 ppm of penicillin, was added to the diet. Hence, this indicates that the organism, under the conditions of this experiment, did not convert the penicillin molecule into an active entity. Also, these data indicate that in order to be effective, the antibiotic must be in an active form. This then, would lend support to the observations of Luckey (1952) who found that antibiotics were ineffective

in promoting the growth of germ-free chicks, and concluded that they function through a change in the intestinal microflora.

When A. aerogenes was grown in Koser's citrate broth, no evidence was obtained to support the view that the organism could synthesize a growth factor for the chick in vitro in a synthetic medium. The filtrate from the broth cultures had no effect on chick growth. Likewise, the cells which were grown in this medium failed to exert any effect on chick growth. Yet, in most instances, when the organism was grown on the surface of Bugon agar, the growth rate of chicks was favorably influenced. Since Bugon agar is a complex medium, it is possible that certain precursers of a factor are supplied by this medium and enzymatic processes within the cell make possible the synthesis of the factor. When cells of either organism were rendered inactive by treating them with 70 per cent ethyl alcohol, no increase in the growth rate was noted. However, when penicillin was added to the diets which contained the cells of A. aerogenes which had been treated with ethyl alcohol, growth was considerably better than that observed with the chicks which were fed diets with the two supplements added separately. This is of interest since it indicates the possibility that a factor within the cell may be liberated by ethyl alcohol.

In contrast to the favorable effects which were exerted on the growth of chicks by the dietary administration of viable penicillin-resistant microorganisms, L. bifidus appeared to exert a detrimental influence. A decrease in the growth rate of the chicks coincided with the oral inoculation of this organism.

The strain of the organism which was isolated from the cecal feces of a chick (C 31) when it was inoculated into the chicks, caused a decrease in the growth rate which approached significance. Similarly, the chicks which received the strain of the organism which was isolated from a poult (Vt 3)

also showed a decrease in the rate of gain but it was not as marked as that produced by strain C 31. When penicillin was included in the diet of the birds which were orally inoculated with either organism, the growth rate was similar to that which was observed when penicillin was fed to a control group of uninoculated birds. However, no reduction in the number of L. bifidus was found in the ceca of the orally inoculated birds fed penicillin. A decrease in the number of organisms was expected, since the organism was found to be highly susceptible to penicillin in vivo by Romoser et al. (1952). Penicillin-resistant forms of the organism were detected in some instances, however. The presence of high numbers of L. bifidus in the ceca of orally inoculated chicks which were fed the antibiotic was due, in all probability, to the presence of resistant strains of the organism. The frequency with which the organisms were administered and the high number of viable cells per dose would certainly promote the development of resistant strains.

Lyophilized preparations of either strain Vt 3 or C 31 exerted no effect on the growth rate. The birds which were fed diets supplemented with these materials grew as rapidly as the control group. However, a significant depression of the growth rate took place when one strain (Vt 3) was administered to the chicks in a diet which contained 10 per cent lactose. Also, the count of L. bifidus was slightly higher in the ceca of representative chicks from this group than in those chicks which were fed the diet containing lactose but no added organisms.

An early report which mentioned the nutritionally fastidious nature of the avian strain of L. bifidus (Romoser, 1951), has since been confirmed by Veltre et al. (1953). On the basis of these reports, a microbiological assay has been developed by Shorb and Veltre (1953, unpublished data) which uses the avian strain of L. bifidus as the test organism. Various

preparations, which have been shown to contain unidentified factor activity for the chick, especially condensed fish solubles, have also been shown to stimulate the growth of the test organism. At the present time, no correlation exists between the chick assay or the microbiological assay when certain fractionation procedures are performed on the crude supplement. In exploratory trials, viable cells of E. coli and A. aerogenes have both stimulated the growth of L. bifidus in vitro. In addition, cells of E. coli which were treated with 70 per cent alcohol or autoclaved at pH 4.0 stimulated the growth, in vitro, of L. bifidus.

These very recent trials suggest that the coliform organisms contain an intracellular growth factor for L. bifidus. If this factor, at a later date, is found to be responsible also for the stimulation of chick growth when these organisms are added to the diet, a satisfactory explanation of the role of E. coli, A. aerogenes, and L. bifidus in the over-all mechanism of the growth stimulation of poultry by antibiotics will be available.

SUMMARY

The addition of lyophilized preparations of Escherichia coli and Aerobacter aerogenes to chick diets which contained 10 ppm procaine penicillin G caused a significant increase in the growth rate of the birds to 4 weeks of age. The addition of the organisms to diets which contained no added antibiotic was of little consequence.

A chick starting mash was fermented with A. aerogenes, and the dried preparation which contained viable organisms of A. aerogenes, was also found to exert a beneficial effect on the growth of chicks in the presence of the antibiotic. This material, when it was added to the diet, supplied about 30×10^7 viable organisms per pound of feed when used at a level of 2 per cent. When several replications of trials in which fermented products and lyophilized preparations were compared, the latter preparation was found to be more effective than the former in promoting the rate of growth, if penicillin was also included in the diet with the viable organisms.

When A. aerogenes was grown in deep, aerated culture in a synthetic medium, neither the cells nor the cell-free filtrate had an effect on the growth of the birds.

Growing A. aerogenes in Koser's citrate broth, which contained 100 units of procaine penicillin G prior to fermentation, destroyed the antimicrobial activity of the antibiotic. When an equivalent of broth, calculated to supply 14 ppm of penicillin, was added to the diet, there was no growth stimulation which is usually observed when active penicillin is used.

No definite correlation could be made between the number of organisms which were fed in the diet and the number of organisms present in the ceca

of the chicks at 4 weeks of age.

Both organisms, when they were added together to a diet which contained fish meal and soybean oil meal, caused no change in the growth rate either in the presence or in the absence of penicillin. However, significant responses were obtained when the organisms and the antibiotic were included together in diets which contained only one of the crude supplements.

The administration of viable organisms to chicks which had access to their droppings was ineffective in increasing the rate of gain of the birds. However, when birds which were grown in an environment which was used only once, 12 months previously for housing chicks, and in a new battery brooder, the administration of the penicillin-resistant microorganisms caused an increase in the growth rate.

The dietary administration of E. coli to chicks fed a purified diet which contained no added pantothenic acid significantly depressed growth in the presence of penicillin. Also, the organisms alone, caused a decrease in the rate of growth when they were added to the diet low in pantothenic acid. On the other hand, the organism stimulated the growth rate of chicks which were fed a diet to which no biotin was added. This stimulation approached significance at the 5 per cent level. Similarly, the addition of the lyophilized preparation to a diet which contained no added riboflavin increased the rate of gain. The usual increased growth rate, over that obtained with the organism alone, was again observed when penicillin was added to this diet.

The introduction of two strains of L. bifidus directly into the crop of chicks decreased the growth rate of the birds. The strain of the organism which was isolated from a turkey (Vt 3) depressed the growth rate slightly, whereas, the strain of chick origin (C 31) caused a retardation

of growth which approached significance.

When lyophilized preparations of the organisms were employed, a retardation of growth was observed only when lactose was present in the diet at a level of 10 per cent.

The growth of L. bifidus was stimulated by viable cultures of both A. aerogenes and E. coli in vitro. Also, cells of E. coli when treated with alcohol, or autoclaved at pH 4.0, exerted a stimulatory effect on the growth of L. bifidus when the tube assay of Shorb and Veltre (1953, unpublished data) was used.

Although lyophilized preparations of non-viable cells of either E. coli or A. aerogenes had no effect on chick growth, both organisms when treated with 70 per cent ethyl alcohol increased the growth rate of chicks. This phenomenon was more marked in those chicks which were fed a diet containing cells of A. aerogenes, previously treated with alcohol and 10 ppm of procaine penicillin G, than it was in the birds which were fed the E. coli preparation with the antibiotic.

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APPENDIX

Composition of Special Bacteriological Media Employed

A. Koser's citrate broth

	gms.
Sodium ammonium phosphate	0.15
Monopotassium phosphate	0.10
Magnesium sulphate (anhy.)	0.02
Sodium citrate	0.30
Distilled H_2O	100 ml

B. Number 5 medium (McCarthy, 1952)

Trypticase	1.50
Phytone	0.50
Sodium chloride	0.40
Sodium citrate	0.10
Sodium sulfite	0.02
Cysteine	0.02
Lactose	0.50
Agar agar	0.10
Distilled H_2O	100 ml

C. Modified Hassenin's medium (double strength, Veltre and Shorb, 1953)

Dipotassium phosphate	0.50
Lactose	1.40
Sodium acetate (anhy.)	1.00
Vitamin free Case-Amino acids (Difco)	1.00
Cysteine, alanine, tryptophane, each	0.04
Asparagine	0.02
Adenine, guanine, uracil, xanthine, each	0.002
	mg.
Thiamin HCl	0.04
Riboflavin	0.04
Calcium pantothenate	0.08
Pyridoxine	0.24
Nicotinic acid	0.12
p-aminobenzoic acid	0.002
Folic acid	0.002
Biotin	0.0008
Salts B*	1.0 ml
Distilled H_2O (pH adjusted to 6.8)	100 ml
Ascorbic acid (after sterilization)	1.0 mg.

*10 g magnesium sulfate. $7H_2O$, 0.5 g ferric sulfate. $7H_2O$, 0.5 g sodium chloride, 0.537 g manganese sulfate. H_2O, in 250 ml H_2O.

Composition of Medium Fermented With Aerobacter aerogenes Used in Experiment 22

B.C.F. #1*

	gms.
Biopar C	1.0
Lactose	0.25
Sodium ammonium phosphate	0.15
Monopotassium phosphate	0.10
Distilled H_2O (pH adjusted to 7.0)	100 ml

*When used, in experiment 22, at a level of 0.5 per cent in diet R-134, this material had no effect on the growth rate of the birds to 4 weeks.

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