THE EFFECT OF FAT ON THE VITAMIN K REQUIREMENT OF THE CHICK

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ABSTRACT

A study has been made of the relationship of dietary fat to the vitamin K requirement of the chick. In these experiments, the vitamin K status of chicks was measured by determining the blood "prothrombin time" by the method of Quick (1936) as modified by Almquist (1941).

It was possible to produce a "prothrombin deficiency" in chicks by feeding diets which consisted of unextracted natural ingredients. The extent of this deficiency was affected by the maternal supply of vitamin K. In some cases the coagulation defect was sufficiently severe to cause fairly heavy mortality from haemorrhaging.

Certain fats, when added to the diet, protected the chick from a vitamin K deficiency. Beef tallow, hydrogenated cottonseed oil, and to a lesser extent cottonseed oil and hydrogenated animal fat all shortened blood "prothrombin time" of chicks, when added to a vitamin K deficient diet. These fats prevented the haemorrhaging which sometimes resulted from vitamin K depletion.

Herring oil generally did not shorten "prothrombin time" unless the vitamin K deficient diet was also deficient in folic acid.

The effect of fats on "prothrombin time" was apparently not due to the vitamin K content of the fat, nor to an increased absorption of vitamin K resulting from increased bile secretion.

Oral administration of oils shortly before drawing blood did not affect "prothrombin time" of blood from vitamin K deficient chicks; this indicates that lipaemia per se did not cause the shortening in "prothrombin time" observed after feeding a high-fat diet. Free fatty acids added to a vitamin K deficient diet did not affect "prothrombin time" to a significant degree.

Addition of choline to a vitamin K deficient diet did not affect "prothrombin time". This observation agrees with those of Field and Dam (1945) but disagrees with those of Honorato and Moline (1942). the addition of methionine also failed to affect "prothrombin time". Herring oil also has a shortening effect when added to such a diet, but not when added to a diet containing adequate folic acid.

Cholic acid added to a vitamin K deficient diet caused a lengthened "prothrombin time." Desoxycholic acid had a similar effect in one test, but had no effect in another case. Injection of chicks with the surface active agent Triton WR-1339 likewise lengthened "prothrombin time." Triton had no effect, however, when added to blood _in vitro_.

Breast muscle and brain tissue differed in the thromboplastic properties of their extracts. The breast muscle preparation required the addition of phospholipid for maximum thromboplastic activity. Acetone partly extracted and partly destroyed the thromboplastic agent of breast muscle, but did not affect that of brain tissue.

Although bile salts may affect blood phospholipid levels, their effect on blood "prothrombin time" was not due to the sensitivity of the breast muscle thromboplastic agent to phospholipid.

The "prothrombin time" of blood, as determined with breast muscle thromboplastic agent, is not necessarily a measure of the vitamin K supplied to the chick.

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Introduction

Vitamin K, the anti-haemorrhagic vitamin, is widely distributed in vegetable feedstuffs. Moreover, it is the only vitamin of the fat-soluble group which is synthesized by intestinal bacteria. Thus the appearance within recent years of a specific deficiency of this vitamin is of considerable interest.

One clue to the appearance of such a deficiency disease at this time is the fact that it parallels the increasing use of high-energy feeds. In rations based on the Connecticut formula, fibre content is reduced to a minimum. This has resulted in a marked decrease in the use of green feed, the richest natural source of vitamin K. Concurrently, the screw-press method for extracting oil from soybeans has been replaced by solvent extraction. The oil content of soybean meal, a staple ingredient of poultry feeds, has been reduced from 5% to less than 1%, with a corresponding reduction of fat-soluble vitamins.

The increasing use of antibiotics and sulpha drugs in poultry feeds has also been implicated. Many of the sulpha drugs and broad-spectrum antibiotics have been shown to suppress the coliform organisms of the intestine. Escherichia coli and related species are the principal bacterial producers of vitamin K.

The use of high-energy, low-fibre feeds containing anti-infective sulpha drugs and antibiotics has greatly increased the growth rate of chicks. At the same time the amount of feed consumed per pound of gain has decreased.

It appears, therefore, that while the metabolic requirements for growth have been increasing, the supply of vitamin K from the diet and from intestinal synthesis has been decreasing.

At the present time, fats are being used to increase the energy content of poultry feeds. Addition of fat generally decreases the amount of feed consumed per pound of gain, and might therefore be expected to decrease the intake of dietary vitamin K per pound of gain.

It is also possible that the dietary requirement of the chick for Vitamin K may be affected by the level of fat which is fed. It has been shown that the requirement for some of the B-complex vitamins may be influenced by dietary fat.

The fact that vitamin K is fat soluble suggests that, like vitamins A and D, absorption of vitamin K from the intestine may be affected by ingested fat.

In consideration of the above observations, a study has been made of the relationship of dietary fats to the vitamin K requirement of chicks.

REVIEW OF LITERATURE

Vitamin K (Koagulations vitamine) is the name given by Dam (1935) to a dietary essential which prevented a haemorrhagic condition of chicks. The active principle was isolated from alfalfa by Dam et al (1939). Almost simultaneously McKee et al (1939) reported isolation of two different active compounds, one from alfalfa and one from fish meal. They showed that the basic structure of the two vitamers involved a naphthoquinone nucleus, with a methyl substituent at the 2- position and a hydrocarbon side-chain at the

3- position. In vitamin K_1, isolated from alfalfa, the side-chain is a phytyl group. Vitamin K_2, isolated from fish meal, contains the larger difarnesyl group. Vitamin K_2 is not a constituent of the fish meal itself, but is a bacterial product which accumulates during putrefaction (Almquist and Stokstad, 1935).

The synthetic vitamin K_3 (menadione) contains only the 2-methyl-1, 4-naphthoquinone nucleus, with no substituent at the 3 position. It appears to have the same effectiveness, on a molar basis, as the two naturally occurring vitamers, although this belief is questioned by Quick and Collentine (1951). Phthiocol (2-methyl-3-hydroxy-1, 4-naphthoquinone) was early shown to have vitamin K activity (Almquist and Klose, 1939). Thus it appeared that the 2-methyl naphthoquinone nucleus was necessary for vitamin K activity. Vitamin K_4 (2-methyl-1, 4-naphthalenediol diacetate) was believed to function by conversion to the quinoid structure _in vivo_. More recently, however, several vitamin K active compounds have been discovered which do not appear to be readily convertible to the naphthoquinone structure. Notable among these are 4-amino-2-methyl-1-naphthol hydrochloride, 2-methyl-1, 5-naphthalenediamine-2 hydrochloride, and particularly 2, 6-dimethylbenzoquinone (Hunter and Shepherd, 1955).

Quick (1935, 1936, 1937, 1938) developed a method of measuring blood prothrombin, by means of which he demonstrated that prothrombin is reduced in vitamin K deficiency. A similar reduction was demonstrated in cases of biliary dysfunction, icterus and certain types of digestive disturbances. These conditions were shown to involve impaired absorption of vitamin K.

"Sweet clover poisoning", a haemorrhagic disease of cattle which consume spoiled sweet clover, was also shown to involve reduced blood

prothrombin. The active agent in the clover is dicoumarol (3,3'-methylene-bis (4-hydroxycoumarin)) (Stahman, Heubner and Link, (1941) which bears a limited structural analogy to two naphthoquinone nuclei joined through a methyl group. For this reason it has been considered to act as a specific antimetabolite interfering with the utilization of vitamin K. Dicoumarol, and its derivative Tromexan (ethyl acetate substituted for the linking methyl) have been used therapeutically to lessen the danger of thrombosis following surgery. The mode of action of these and related drugs has been reviewed recently by Douglas (1955).

Certain observations related to the use of these anticoagulant drugs have provided indirect evidence that prothrombin is not the only coagulation factor affected by a vitamin K deficiency. Quick, Hussey and Collentine (1952) showed that the "hypoprothrombinaemia" resulting from a vitamin K deficiency does not differ from that caused by dicoumarol therapy. De Nicola (1953) stated that the principal deficiency produced in dicoumarol therapy involves the newly identified Factor VII, with a relatively unimportant change in the prothrombin level. Thus it may be a defect in the production of Factor VII, rather than of prothrombin, which produces the haemorrhagic state in vitamin K deficiency.

It is well established that the site of vitamin K action is in the liver (Brinkhouse and Warner, 1940; Carter, Chambers and Warner, 1949). Recently Witte and Dirnberger (1953) showed that several coagulation factors, including prothrombin and probably including Factor VII, are synthesized in the liver. Thus the site of vitamin K action provides no direct evidence as to which of these factors is actually dependent on the vitamin.

The chemical estimation of vitamin K activity is relatively difficult. The chemical procedures are based on the reactions of the quinone or naphthoquinone group. Thus, in the method of Scudi and Buhs (1941), the quinone is reduced by catalytic hydrogenation to the hydroquinone, which in turn is reacted with 2,6-dichlorophenol indophenol; the extent of the reaction is measured photometrically. Kofler (1945) developed a fluorometric procedure involving condensation of the naphthoquinone with o-phenylenediamine. These procedures are not sufficiently specific for assay of heterogeneous materials. As yet no microbiological method has been evolved.

The procedure most commonly used for vitamin K measurement is the biological assay developed by Almquist (1941). Chicks are depleted of prothrombin by feeding a vitamin K free diet (polished rice, ether extracted fish meal and brewer's yeast, vitamins and minerals). The test substance is administered orally in an oil solution for four days. Within 24 hours of the last dose blood is drawn by decapitation of the chick, and prothrombin level is measured by a modification of the method of Quick (1936). The values so obtained are compared with those of chicks receiving graded levels of mena-dione. Almquist (1952) has developed a formula relating vitamin K intake to prothrombin level over the range of biological activity.

In the experiments here reported this procedure was modified somewhat. The rations used consisted of unextracted natural ingredients, and the test substances were in most cases incorporated directly into the feed. The objective of the experiments was not to assess the vitamin K content of the fat, but rather to determine what effect, if any, dietary fat would have on the vitamin K requirement of the chick.

An increased riboflavin requirement in the presence of high levels of dietary fat has been reported in rats (Mannering, Orsine and Elvehjem, 1944) and dogs (Potter, Axelrod and Elvehjem, 1942). Mannering, Lipton and Elvehjem (1941) suggested that this effect might be due to a reduction in synthesis of riboflavin by the intestinal bacteria. On the other hand, Couch et al (1949) reported that high-fat diets stimulated intestinal synthesis of biotin in the fowl.

Vitamin K, like riboflavin and biotin, is synthesized in the intestinal tract of birds. In this respect it is unique among fat-soluble vitamins. The difficulties encountered in attempts to produce a vitamin K deficiency in rats (Black et al, 1942; Kornberg, Daft and Sebrell, 1944) indicate the importance of the intestinal source of this vitamin. The common coliform organisms, particularly Escherichia coli, are important vitamin K synthesizers (Orla-Jensen et al, 1942).

Any effect which fat might have on intestinal microflora, therefore, might be reflected in the vitamin K status of the chick. The report by Couch et al (1949) that fat stimulates biotin synthesis suggests a possible secondary effect. Welch and Wright (1943) have reported that biotin shortened the clotting time of blood from vitamin K deficient rats. This effect is presumably due to increased synthesis of vitamin K in the intestine.

Some reported effects of fat, on the other hand, are not due to an effect on the microflora. Several workers have reported a shortening in clotting time of blood of various species following administration of fats (Fullerton, Davie and Anastopoulos, 1953; Duncan and Waldron, 1949). Waldron, Beidelman and Duncan (1951) have reviewed effects of fat on blood coagulation; both a local haemostatic and a systemic effect are believed to be involved. The effect of fat on blood coagulation is characterized by a shortening of

coagulation time for a period beginning shortly after consumption of fat, and lasting for 4 - 6 hours. In most cases there is little apparent change in "prothrombin time". It is not likely therefore that vitamin K is affected.

The elevated blood lipid level following fat ingestion may be related to the shortened clotting time, although Boulanger (1952) did not find any change in coagulability of the blood of laying turkey hens despite the appearance of lipaemia. Pellegrino and Solinas (1952) found no change in coagulability of blood after injecting synthetic oestrogens into rabbits.

In the following experiments the whole blood clotting time of the birds was not determined. The effects of fat on coagulation time reported above may not therefore be applicable to these tests, since another part of the clotting mechanism may be involved.

EXPERIMENTAL

The general experimental procedure followed in all chick tests was as follows:

Day-old New Hampshire chicks were individually wing-banded and treated with Lederle's ocular live-virus Newcastle Disease vaccine. The chicks were distributed at random into groups in Jamesway electrically heated battery brooders. Room temperature was thermostatically controlled at 70° F. Water and experimental rations were provided ad lib. Water troughs were cleaned daily to prevent bacterial synthesis of vitamin K.

After a period of from eighteen to thirty-three days chicks from each lot were bled by venipuncture into tubes calibrated at 1.0 ml. and containing 0.1 ml. M/10 sodium oxalate. "Prothrombin time" was determined by the Quick (1936) method as modified by Almquist (1941). The "prothrombin time" of blood from a bird receiving a commercial ration was recorded for comparison of different preparations of the thromboplastic agent.

The procedure for the determination of "prothrombin time" was as follows:

Thromboplastic agent: A normal bird was killed by bleeding. Ten grams of breast muscle was freed from all visible blood vessels, minced, and macerated in a mortar with Ottawa sand and 10 ml. of 0.85% saline. The mixture was centrifuged and filtered through coarse paper (Whatman #4). The volume was made up to 200 ml. with 0.85% saline, and an equal volume of M/40 $CaCl_2$ was added. A fresh solution was prepared for each series

of determinations.

0.2 ml. of this preparation was added to 0.1 ml. of oxalated whole blood in a flat-bottomed vial 15 x 45 mm. A stopwatch was started as the agent was added. The tube was repeatedly tilted in a water bath (38.5-39.0° C.) until a gelatinous film appeared on the bottom. The time required for this film to form was recorded as the "prothrombin time".

EXPERIMENT I.

This experiment was designed to indicate whether or not light pressed herring oil would affect "prothrombin time" in chicks receiving a basal vitamin K deficient diet, with and without supplements of dried cereal grass and synthetic vitamin K (menadione). Both male and female chicks from two sources were used. The dams of one group were receiving a diet containing no green feed, while the dams of the other group were receiving a diet supplemented with 5% dehydrated cereal grass.

The rations used in this experiment are shown in table 1:1. The control ration (ration 1) was essentially a commercial type chick starter diet but contained no green feed. In rations 3 and 6, containing 5% dried cereal grass, protein level was adjusted by reduction of wheat and soybean meal levels. In rations 4, 5 and 6, 8% of commercial light pressed herring oil replaced the 8% cerelose contained in the control ration. "Prothrombin time" of five male and five female chicks from each group was measured at 26 and 27 days respectively.

Results: The mean "prothrombin time" of five birds from each ration, sex and maternal source is shown in table 1:2. A statistical analysis

of the results is shown in table 1:3.

Supplements of cereal grass and of vitamin K were equally effective in shortening "prothrombin time". Their effect was not modified by addition of herring oil to the ration or by the source of chicks used. On the other hand, both source of chicks and addition of herring oil affected the response to rations which did not include a supplementary source of vitamin K. Chicks whose dams did not receive cereal grass had significantly longer blood "prothrombin time" ($p < .05$) when fed the unsupplemented ration than did chicks from dams receiving 5% cereal grass. When herring oil was added to the unsupplemented diet, "prothrombin time" of chicks was prolonged. This effect was significant ($p < .05$) with chicks whose dams received cereal grass, and highly significant ($p < .01$) with chicks whose dams did not receive cereal grass. The response of males and females was similar in all cases, although the males tended to be more variable.

EXPERIMENT II - Test 1.

Earlier work at this laboratory (Burdett, 1955) has demonstrated that herring oil will aggravate a folic acid deficiency in corn-fish meal rations. A second experiment was set up to determine whether or not the effect of herring oil on blood "prothrombin time" was modified by the level of folic acid in the diet. A simplified diet consisting mainly of wheat and herring meal was used

(table 2:1). Weights of individual chicks were recorded at 28 days. "Prothrombin time" of nine or ten birds per lot was measured at 29 days.

The results of Experiment I indicated that it was easier to produce a vitamin K deficiency in chicks when the dams were fed diets containing no green feed. For this reason, chicks used in this experiment were from dams receiving no green feed. The same source of chicks was also used in all subsequent experiments, except where otherwise stated.

Results: The growth response to folic acid (table 2:2) indicated that the basal ration was deficient in this vitamin. The effect of folic acid supplementation on "prothrombin time" is less clear (table 2:3). Statistical analysis of this data (table 2:4) indicated that while neither folic acid nor herring oil, singly, had a significant influence on "prothrombin time", a combination of the two produced a statistically significant shortening effect.

Supplementation with vitamin K shortened "prothrombin time". This effect was not modified by the addition of folic acid or of herring oil.

Test 2.

The previous test was repeated. In the second case "prothrombin time" was measured at 33 days. Individual weights of chicks were

recorded at 28 days.

Results: Growth response to folic acid supplementation was similar to that in the previous test (table 2:2). Both folic acid and herring oil significantly shortened "prothrombin time" (tables 2:3, 2:4). A combination of folic acid and herring oil produced a highly significant shortening effect. Vitamin K shortened "prothrombin time" in all cases; the effect was not modified by addition of folic acid or herring oil.

EXPERIMENT III.

The experiment reported here was part of another study being carried on at this laboratory. It was designed to investigate the effect of various fats on the folic acid requirement of chicks. During the course of the experiment, a severe haemorrhagic condition was observed in some of the chicks.

Experimental rations are shown in table 3:1. They were similar to those used in Experiment II except that corn replaced wheat. Fats used in this test were cottonseed oil, hydrogenated cottonseed oil[1] and hydrogenated animal fat[2] (m.p. 55° C.). The hydrogenated cottonseed oil was melted for addition to the feed. The hydrogenated animal fat, a dry granular product, was incorporated directly, without melting. These fats were added at the 8% level, with and without folic acid. "Prothrombin time" of ten birds from groups receiving each experimental ration was determined at 28 days.

Results: Mortality from haemorrhaging was high in groups receiving the basal ration, and the basal ration supplemented with folic acid. There

1 - Primex: a product of Procter and Gamble Ltd.

2 - Hydropoid: courtesy of Bowman Feed Products, Inc., Holland, Michigan.

was no haemorrhaging observed in any of the chicks receiving fat.
"Prothrombin time" measurements gave values which were in accord with
the above observations (table 3:2). Each of the fats produced a short-
ening in "prothrombin time" which was statistically highly significant
table (3:3). Hydrogenated cottonseed oil was more effective than the
other fats in this respect. The 28-day weights (table 3:4) indicated
that the basal ration was deficient in folic acid. Nevertheless, folic
acid alone had no effect on "prothrombin time". Folic acid plus
hydrogenated animal fat, however, produced a significantly greater effect
than did the hydrogenated animal fat alone.

EXPERIMENT IV – Test 1.

The results of the previous experiments indicated that the effect
of fat on "prothrombin time" depended on the fat used and on the composi-
tion of the ration. Lockhart, Sherman and Harris (1942) produced hypo-
prothrombinaemia in rats by feeding fats containing high levels of
dihydrosxystearic acid. Mineral oil produced a similar effect (Elliot,
Isaacs and Ivy, 1940). On the other hand, Duncan and Waldron (1949)
reported a shortening in whole blood clotting time of dogs after
administration of corn oil, olive oil or cow's cream. They considered
that the activation of prothrombin was accelerated.

Honorato and Moline (1942) found a relationship between vitamin
K and methyl donors. They reported that choline shortened "prothrombin
time" in chicks receiving a diet deficient in both vitamin K and
choline.

In the present experiment the effect on "prothrombin time" of two different fats added to an all-vegetable diet was observed. Fats tested were herring oil and stablized beef tallow[1]. The effect of supplementing the basal ration with folic acid and choline chloride was also observed. The rations used in this experiment are shown in table 4:1. "Prothrombin time" of 8-10 chicks per lot was determined at 23 days.

Results: Folic acid, choline chloride and herring oil, either alone or in any combination of the three, failed to influence "prothrombin time" of blood (table 4:2). On the other hand beef tallow, either alone or in combination with the two vitamins, decreased "prothrombin time" to a statistically significant degree (table 4:3). Neither choline nor folic acid influenced this shortening effect, which was less than that obtained by vitamin K supplementation. Microbiological assay of the feed revealed that the folic acid level of the basal diet exceeded the requirement of the chick for this vitamin (N.R.C., 1955).

Test 2.

Procedure: This test was similar to test 1, except that supplementary vitamin B_{12} and meat scrap were incorporated into the ration (table 4:1). The effect of supplementary choline and methionine in the presence and absence of beef tallow was observed by determination of average weight of all birds, and "prothrombin time" of ten birds per group, at 23 days.

1 - Sta-fat: courtesy Gordon Young, Ltd., Vancouver, B.C.

Results: The group receiving supplementary vitamin K had unexpectedly long "prothrombin times" (table 4:2). Re-examination of the ration formulae revealed that vitamin K had been inadvertently added at only one-tenth the required level (N.R.C., 1955).

Although the addition of methionine stimulated growth (table 4:4), neither methionine nor choline affected "prothrombin time." Beef tallow, either alone or in combination with choline or methionine, produced a shortening effect which was statistically highly significant (table 4:3), and which far exceeded the effect of 0.02 mg. vitamin K per pound of feed.

There appeared to be slight differences between mean "prothrombin time" of birds receiving beef tallow with and without added choline, methionine and vitamin K. These groups continued to receive their experimental diets for an additional week, to observe whether or not the differences would increase. The mean "prothrombin time" of 11-12 birds per group at 30 days was identical in all cases (table 4:2).

DISCUSSION OF RESULTS

It appears from the results of the foregoing experiments that the effect of dietary fat on "prothrombin time" in the chick is extremely variable, depending on the fat used and on the composition of the test diet. When the amount of vitamin K supplied to the chick is marginal, herring oil appears to act as a stress agent. This was demonstrated in Experiment I, where the carryover of the vitamin was apparently a critical factor even after the chicks reached four weeks of age. Under these circumstances herring oil had an unfavorable effect, lengthening

"prothrombin time" significantly. The effect was more severe in the case of chicks whose dams were depleted of vitamin K.

On the other hand, when the diet fed was actually deficient in vitamin K the effect of herring oil on "prothrombin time" varied. When the vitamin K deficient diet was also low in folic acid, herring oil shortened "prothrombin time" (Experiment II tests 1 and 2). When the folic acid level was adequate, on the other hand, herring oil appeared to have no effect (Experiment IV test 1). Supplementation of the vitamin K deficient diet with folic acid alone did not generally affect "prothrombin time", although it did produce a significant shortening effect in Experiment II test 2.

Other fats produced a dramatic shortening in "prothrombin time" and adequately protected the birds from the haemorrhagic tendency sometimes caused by the depletion diet. Cottonseed oil, hydrogenated cottonseed oil, hydrogenated animal fat and a stabilized animal fat all produced this effect. The hydrogenated cottonseed oil produced a greater shortening of "prothrombin time" than did the cottonseed oil. In the case of hydrogenated animal fat, the addition of folic acid produced a further significant shortening, although folic acid alone was without effect.

Honorato and Moline (1942) reported that when chicks fed diets deficient in vitamin K and choline received supplementary choline, the "prothrombin time" was reduced. Field and Dam (1945) were unable to duplicate this result. In the present experiments neither choline nor methionine supplementation affected "prothrombin time" (Experiment IV).

The diets used were not seriously deficient in choline, judging by the growth rates. However, methionine supplementation did produce a growth response.

EXPERIMENT V.

The general design of the first four experiments approximated that of the biological assay for vitamin K suggested by Almquist (1941). Groups of chicks were fed a basal diet deficient in vitamin K, and the basal diet was supplemented with the test substances. "Prothrombin time" of blood from these chicks was measured after 3-4 weeks. Since "prothrombin time" , under the conditions specified by Quick (1936), was considered to be a measure of the level of blood prothrombin, and since prothrombin was considered to be the coagulation factor affected by vitamin K, the test was thought to reflect the amount of vitamin K contained in the test substance. With the fats used in the above experiments, three effects were therefore possible. The fat might contain appreciable quantities of the vitamin. In the case of herring oil and beef tallow this would be surprising, since animal products generally are considered to be very poor sources of the vitamin. Alternatively, the fats might affect the intestinal absorption of the fat-soluble vitamin. A third possibility is an effect of dietary fat on microbiological synthesis of vitamin K in the digestive tract.

To test the possibility that the fats contained appreciable quantities of vitamin K a series of experiments were carried out to observe the effect of treatment of the fats with heat and alkali. Vitamin K is labile under these conditions, and therefore should be destroyed by this treatment.

Test 1.

Procedure: Herring oil was heated to 75° C. and 3% of 20% aqueous KOH was added slowly with stirring. The mixture was allowed to cool, and the oil phase removed by decanting through cheesecloth. This process removed most of the soaps formed, and left the greater part of the KOH in the aqueous phase.

The "alkali-treated" oil was incorporated into a corn-soybean meal diet (table 5:1) at 4%, 6% and 8% levels. An untreated sample of the same oil was incorporated at the same levels. A sample of herring oil which had been stored at room temperature for a year was also included at the 8% level, to test the effect of oxidation. These rations and a basal control diet were fed to eight groups of chicks as in previous tests. Individual weights were recorded at 23 days. "Prothrombin time" of five birds from each group was measured at eighteen days, and of an additional ten birds from each group at 25 days.

Results: The treated and untreated oils had similar effects on growth rate (table 5:2), producing a slight and identical depression at the 6% and 8% levels. The stored oil produced a more marked depression in growth. No significant differences in "prothrombin time" were observed at 18 days (tables 5:3, 5:4). After 25 days, however, "prothrombin time" was significantly shorter in all groups receiving untreated oil than in the comparable groups receiving treated oil, although four of these six groups did not differ significantly from the control group. The stored herring oil did not affect "prothrombin time".

<u>Test 2.</u>

Procedure: Three fats were treated with alkali as described in the previous test. The fats used in this case were herring oil, beef tallow and cottonseed oil. The treated fats and untreated samples of each were incorporated into the basal ration at 2% and 6% levels. The basal ration had the same composition as that used in the previous test (table 5:1). "Prothrombin time" of ten birds from each group was measured at 19 days.

Results: The degree of depletion of these chicks, as indicated by the "prothrombin time" (table 5:3), was less than that in previous experiments. This was presumably due to the fact that the depletion period (19 days) was shorter than usual. Few differences between groups were observed. Statistical analysis of the results (table 5:4) revealed the only significant effects to be the lengthening effect of 2% treated tallow and 6% untreated herring oil, and the shortening effect of 6% treated tallow. 2% treated tallow produced significantly longer times than 2% untreated tallow, while the 6% level of treated tallow or herring oil produced significantly shorter times than did the untreated samples.

<u>EXPERIMENT VI.</u>

Procedure: Cottonseed oil had previously produced a decreased "prothrombin time" in a corn-fish meal diet (Experiment III), but failed to do so in a corn-soya diet (Experiment V - Test 2). This experiment

was designed to observe whether or not alkali treatment would affect the response to cottonseed oil in a corn-fish meal diet, and whether or not folic acid would affect such a response. The basal ration (table 6:1) was of similar composition to that used in Experiment III. There was some loss from haemorrhaging in groups 1 and 2 (basal and basal + folic acid, respectively). "Prothrombin time" of all surviving chicks (11-20 per lot) was determined at 23 days.

Results: Cottonseed oil, treated and untreated, produced a statistically significant reduction in "prothrombin time" (tables 6:2, 6:3). Although folic acid produced a slight reduction in all cases, these differences were not statistically significant. No explanation can be offered for the prolonged "prothrombin time" of chicks receiving vitamin K, since this vitamin was included in the diet at the usual level of 0.2 mg./lb.

EXPERIMENT VII.

The previous experiments indicated that, with the possible exception of herring oil, the fats used did not shorten "prothrombin time" because of their vitamin K. content. This experiment was designed to observe whether a pure fatty acid or a mixture of fatty acids would produce a shortening effect. Oleic acid and mixed fish liver fatty acids were incorporated into a corn-fish meal diet (see table 3:1) at the $7\frac{1}{2}\%$ level.

In addition, to observe whether an oil fed just prior to drawing blood would produce a reduction in "prothrombin time",

fifteen chicks which had received the deficient basal diet were treated with 1.0 ml. of cottonseed oil by oral pipetting on the 17th day. After one hour blood was drawn from all chicks for "prothrombin time" determination.

Results: Mean "prothrombin time" values are shown in table 7:2. The mixed fatty acids and oleic acid produced a shortening effect which was not, however, statistically significant (table 7:2). Orally administered cottonseed oil produced a lengthening effect which was statistically significant. This was surprising, since other oils administered to dogs in this way had been reported to accelerate thrombin formation (Duncan and Waldron, 1949).

Two other oils were tested at different times for their effect on "prothrombin time" when administered orally. Corn oil, which had been reported by Duncan and Waldron (1949) to shorten the whole blood clotting time of dogs, was tested on two occasions by administrations to chicks which had received corn-fish meal depletion diets. Herring oil was tested on chicks receiving a wheat-fish meal diet. Blood was drawn for "prothrombin time" determination 1-2 hours after administration of the oil. The results are summarized in table 7:4. Neither oil appeared to influence "prothrombin time".

DISCUSSION OF RESULTS

It is unlikely that vitamin K would survive the treatment used in these experiments. It is unstable in the presence of heat, light or alkali (Greaves and Schmidt, 1937; McKee et al, 1939). Nevertheless,

only herring oil showed a consistent difference between treated and untreated samples (Experiment V test 1). Since a considerable quantity of soap was formed during the treatment of herring oil, the effect of removal of free fatty acids may have been an important factor in producing this difference.

The fats used in Experiment V, test 2, failed to produce the marked shortening effect noted in some previous experiments. This was also true, to a lesser extent, in experiment IV, test 1. In both of these cases the ration was a corn-soybean meal diet. In experiment IV, test 2, where meatmeal replaced part of the soybean meal, and in Experiment VI where fish meal replaced all the soybean meal, the effect of fat on "prothrombin time" was much more marked.

Oleic acid and mixed fish liver fatty acids appeared to produce some slight shortening of "prothrombin time", but this effect was not statistically significant. This might suggest that the effect of the fats was in fact due to vitamin K content, and that the treatment with alkali failed to destroy it. However, an examination of the results in Experiment IV, test 2, shows that 8% beef tallow had an effect far greater than that of 0.02 mg. menadione per pound of feed. To account for its activity in terms of vitamin K_1 or K_2 content the fat would have to contain more than 150 micrograms K_1 or 185 micrograms K_2 per 100 gm. fat. In other words, it would have to have a vitamin K content approaching that of alfalfa, the most concentrated natural source known (Wooster, 1954).

This observation implies that the effect of dietary fat on "prothrombin time" is not related to its vitamin K content. A direct effect on intestinal absorption of the vitamin is possible. However, such an effect does not appear to be consistent with the fact that higher melting fats were generally more effective in shortening "prothrombin time." The digestibility of fats generally decreases with increasing melting point (Deuel, 1955). Alternatively, dietary fat might improve absorption of the vitamin by increasing the production of bile. Another possible explanation could be that the fat content of the diet affects microbiological synthesis of the vitamin.

None of these explanations can account for the unexpected lengthening of "prothrombin time" when cottonseed oil was administered by pipette. This effect is not likely to be mediated by vitamin K, since even if synthesis of prothrombin was completely blocked no significant drop would be expected in one hour. (Quick and Collentine, 1951, found that prothrombin is about 80% catabolized in 24 hours). Neither corn oil nor herring oil produced an effect comparable to that of cottonseed oil when administered by pipette.

EXPERIMENT VIII.

Greaves and Schmidt (1937) and Greaves (1939) showed that the haemorrhagic tendency which follows bile duct ligation in rats can be prevented by feeding bile. Bile itself has been shown to contain little vitamin K activity (Greaves, 1939). These workers therefore concluded that bile stimulated the absorption of vitamin K.

The ingestion of fats tends to stimulate the secretion of bile (Hawk, Oser and Summerson, 1947). One effect of dietary fats, therefore, might be to improve vitamin K absorption through increased bile secretion. To investigate this possibility, bile salts were fed to chicks receiving diets deficient in vitamin K. Bile salts which have been shown to be present in the chicken include cholic acid, chenodesoxycholic acid, an as yet unidentified $C_{27} H_{46} O_6$ acid, and the conjugation products of these acids with taurine (Deuel, 1955). In the present series of tests, cholic acid was the first bile salt studied.

In test 1, a wheat-fish meal diet (table 8:1) with and without the addition of 0.5% of cholic acid was fed to chicks for 21 days. At the end of this period blood "prothrombin time" was measured.

In the second test the effect of cholic acid and of beef tallow, alone and in combination, when added to a corn-fish meal diet (table 8:1) was studied. "Prothrombin time" was measured at 21 days.

Schmidt (1938) inferred from studies of other fat-soluble vitamins that desoxycholic acid was the bile salt responsible for

for vitamin K absorption. In the third test the effects of cholic acid, desoxycholic acid, and two levels of vitamin K (menadione) incorporated into a corn-fish meal diet (table 8:1) were compared. "Prothrombin time" was determined at 24 days.

Results: The mean "prothrombin time" values for these three tests are summarized in table 8:2. Statistical analyses of the results are shown in table 8:3. Cholic acid produced an unexpected lengthening in "prothrombin time" in all three cases. In the first test this effect was statistically highly significant. In the second and third tests, with fewer birds used, the effect was not significant. Beef tallow produced a marked reduction in "prothrombin time" which was not modified in any way by the addition of cholic acid. Desoxycholic acid produced a slight shortening in "prothrombin time" which was not, however, statistically significant.

DISCUSSION OF RESULTS.

Desoxycholic acid has been shown to be the bile component necessary for the absorption of other fat soluble vitamins (Greaves and Schmidt, 1935). The cholic acid of vitamin K_1 was absorbed in choledo-chocolonostomized rats (Kohrn and Schmidt, 1939), although it had been demonstrated that bile was necessary for the absorption of this vitamin (Greaves, 1939). Fantl, Nelson and Lincoln (1951) showed that water-insoluble diacetyl 2-methylnaphthoquinone was absorbed in the absence of bile, but explained this on the basis of the relatively small size of the molecule.

If the effect of fat had been to increase the production of bile and thus to facilitate absorption of vitamin K, a similar effect might be expected of bile acids added to the ration. Even if the specific bile acids studied in this experiment were not directly involved in vitamin K absorption, the administration of these acids would increase the secretion of other bile acids. "Bile salts absorbed from the intestine have a marked power to stimulate bile formation " (Hawk, Oser and Summerson, 1947). Since the addition of bile acids to the diet failed to shorten "prothrombin time", it does not appear that increased bile secretion can explain the effect of fats on "prothrombin time".

The effect of cholic acid in lengthening "prothrombin time" might be due to supression of bacteria synthesizing vitamin K. However, Best and Taylor (1945) state that "bile will serve as a suitable culture medium for microorganisms."

Cholic acid and desoxycholic acid have been shown to differ in their effects on blood lipid level. In a series of studies, Byers and Friedman showed that cholic acid is the bile salt responsible for the hypercholesteraemia and hyperlipaemia which follows bile-duct ligation, and that other bile salts do not produce this effect (Byers, Friedman and Michaelis, 1950; Friedman, Byers and Michaelis, 1951; Friedman and Byers, 1951, 1952; Byers and Friedman, 1952). Swell et al (1953) showed that dietary cholic or taurocholic acids produced hypercholesteraemia in rats receiving high-fat, high-cholesterol diets, while desoxycholic acid had considerably less effect. To determine whether

a direct effect on blood fat level could be responsible for the effect of cholic acid on "prothrombin time", a surface active agent known to induce hyperlipaemia in chicks was studied.

EXPERIMENT IX.

The surface active agent Triton WR-1339[1] has been shown to produce lipaemia in chicks (March and Biely, 1955). Three tests were conducted to determine whether this effect is reflected in the "prothrombin time".

In the first test, five chicks which had been fed a depletion diet for 24 days received 1.0 ml. of the surface active agent by subcutaneous injection. After 31 hours blood was drawn from these chicks and from five untreated chicks for "prothrombin time" determination.

Similarly, in the second test "prothrombin time" of five birds injected with 1.0 ml. Triton and of four control birds was determined 40 hours after injection of Triton. The birds were treated at 22 days.

In the third test, 0.5 ml. Triton was injected into each of five birds at 21 days. "Prothrombin time" of these and of five untreated birds was determined after 42 hours.

A wheat-fish meal ration was used to deplete the birds of vitamin K in the first test. In the second and third tests a corn-fish meal diet was used.

Results: "Prothrombin time" was prolonged in the chicks which had received Triton injections (table 9:1). In the first test this effect was statistically highly significant (table 9:2). In the second and third tests, however, due to the small numbers of birds used and to the extreme variability within groups, the differences were not statistically significant.

1 - A polymeric p-isooctyl polyoxyethylene. Courtesy of Rohm
and Haas Company, Philadelphia.

Test 4.

To observe whether or not its effect on "prothrombin time" was
a direct result of its presence in the blood, Triton was added to blood
and to the thromboplastic agent in vitro. A solution of 2.5% Triton in
0.85% saline ("Triton-saline") was added to blood or to the clotting
agent at graded levels previous to the determination of "prothrombin time".
Comparable dilutions were made with saline. 0.1 ml., 0.2 ml., 0.4 ml.,
and 1.0 ml. of saline or of Triton-saline were added to 1.0 ml. samples of
normal blood. Similar additions of Triton-saline were made to 1.0 ml.
samples of the clotting agent; these diluted agents were tested with
undiluted blood.

Results: The final concentration of the added saline or Triton-saline
in the combined solution of blood+clotting agent, together with the
"prothrombin time" of each solution, is shown in table 9:3. There is no
apparent difference between the effect of Triton added to the blood or to
the clotting agent, and that of saline alone.

DISCUSSION OF RESULTS

The effect of Triton WR-1339 in raising blood lipid level has been
demonstrated with ruminants (Chung and Shaw, 1951), mice (Cornforth et al,
1951), rats (Friedman and Byers, 1953) rabbits (Kellner, Correll and
Ladd, 1951) and chicks (March and Biely, 1955). Several of these workers
demonstrated an elevated blood cholesterol level; Friedman and Byers
stated that the effect of Triton was similar to, but greater than that of
cholic acid. The effect on "prothrombin time" observed in the present
experiments was also similar to that of cholic acid.

Thus cholic acid and Triton, both of which have been shown to cause elevation of blood lipid level, caused a lengthening of "prothrombin time". Oral administration of cottonseed oil, which would be expected to produce alimentary lipaemia, likewise lengthened "prothrombin time". On the other hand, oral administration of corn oil or herring oil had no effect. Oral administration of corn oil, olive oil or cream accelerated prothrombin activation in dogs (Duncan and Waldron, 1949). The administration of oestrogens, which would also be expected to elevate blood lipid, did not affect blood coagulation time of rabbits (Pellegrino and Solinas, 1952). Boulanger (1952) found that the lipaemia of turkey hens during the laying season did not influence blood coagulation. The elevation of blood lipid level _per se_ cannot adequately explain the effects of Triton or of cholic acid in the present experiments.

From the results of the chick tests it is apparent that dietary fat has a complex effect on the one-stage "prothrombin time" of chicks fed vitamin K deficient diets. The effect is dependent on the fat, and on the composition of the diet in which it is used. It does not appear that any of the fats, with the possible exception of herring oil, contained appreciable quantities of vitamin K. The possibility of increased microbiological synthesis of the vitamin cannot be dismissed, since herring oil and corn oil failed to affect "prothrombin time" when administered shortly before blood was drawn. On the other hand, oral administration of cottonseed oil and injection of a surface active agent both caused a lengthening of "prothrombin time". These latter effects, at least, cannot be accounted for by an effect on the microflora.

Moreover, if the fat produced conditions which were favorable for active growth of bacteria, it is difficult to understand why the addition of 0.5% cholic acid failed to influence in any way the effect of 8% beef tallow. Cholic acid, with its strong surface action, could reasonably be expected to modify conditions in the intestine. An effect on intestinal absorption of the vitamin through increased bile secretion appears unlikely, since a high level of desoxycholic acid produced little if any effect on "prothrombin time."

EXPERIMENT X.

The variations in "prothrombin time" observed in the previous experiments were not a reflection of the vitamin K content of the diet. The shortened "prothrombin time" which resulted when fat was included in the diet was not caused by increased bile secretion with resultant improved vitamin K absorption. The effect of Triton on "prothrombin time" probably could not have resulted from decreased intestinal synthesis of vitamin K. In the case of Triton, at least, it appeared that some in vivo change was involved.

The "prothrombin time" method used in these experiments was studied with a view to determining whether variables other than vitamin K could be expected to affect "prothrombin time". The recent literature pertaining to blood coagulation suggested that several variables might be involved. Since much of this work deals with the coagulation of human blood, the possibility of species differences must be recognized.

The Quick one-stage prothrombin test is based on the following theory of coagulation:

$$\text{Prothrombin} \xrightarrow{\text{Thromboplastin} + Ca^{++}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$
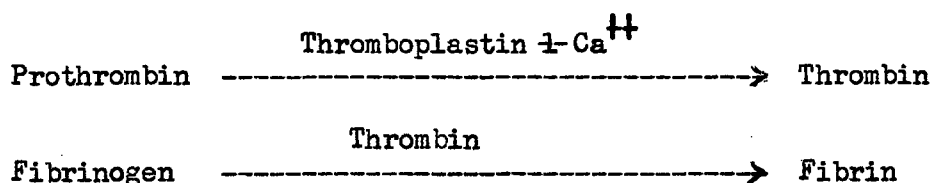
It is assumed that if "thromboplastin" (tissue extract from brain, lung or thymus) and fibrinogen are present in excess, and Ca^{++} in optimum concentration (0.008 M), the clotting time is directly proportional to

the level of prothrombin present (Quick 1935, 1936, 1937).

This concept of blood coagulation has received much criticism for more than a decade. Certain unexplained characteristics of the coagulation mechanism in this test led to the postulation of another component of the reaction. This component has been variously named Thrombogen (Nolf, 1945), prothrombin accelerator (Fantl and Nance, 1946), accelerator globulin (or Ac globulin) (Ware, Guest and Seegers, 1947), prothrombokinase (Milstone, 1948), plasma prothrombin conversion factor (PPCF) (Stefanini, 1951), labile factor (Quick, 1943), plasmatic co-factor of thromboplastin (PCT) (Honorato, 1947), pro-accelerin and accelerin (Owren, 1951), and factors V and VI (Owren, 1947). Though there is considerable confusion in the use of nomenclature by different authors, the above-named compounds have certain characteristics in common. All are relatively labile, especially in the presence of oxalate. They are adsorbed on aluminum hydroxide, are present in plasma and absent from serum. They are therefore generally classified as one compound (Biggs, 1951; De Nicola, 1953). In this report the nomenclature adopted by both Biggs and De Nicola, who refer to this compound or group of compounds as Factor V, will be followed.

Another factor or group of factors involved in the Quick "prothrombin time" test includes co-thromboplastin (Mann, 1949), proconvertin-convertin (Owren, 1952), prothrombin conversion factor (PCF) (Jacox, 1949), prothrombinogen (Quick, 1949), serum prothrombin conversion accelerator (SPCA) (Alexander et al, 1949), and factor VII (Koller, Loeliger and Duckert, 1951). Once again following the

pattern of De Nicola (1953) and Macfarlane and Biggs (1955) this group will be referred to as Factor VII in this report. Factor VII is relatively stable in fresh and stored blood, is not adsorbed by aluminum hydroxide, and is present in both plasma and serum.

It is again emphasized that the factors referred to above are directly involved in the Quick "prothrombin time" test. Other newly-identified factors such as anti-hemophillic globulin (AHG), Christmas factor, platelet factor (serotonin), and the postulated plasma thromboplastin antecedent (PTA) will be referred to in connection with the newer theories of blood coagulation, but are not directly involved in the one-stage prothrombin assay method under study.

The classical theory of blood coagulation (Schmidt, 1895; Morowitz, 1905) has already been mentioned. The steps involved are still accepted as being a part of the true coagulation mechanism. However, the single term "thromboplastin" in the earlier theory has since been shown to include a number of important steps not previously recognized and not yet fully understood.

If blood is drawn from a vein with precautions to avoid contamination from damaged tissue, coagulation will occur normally in from five to ten minutes. Further precautions (silicone-treated or collodion-lined glassware) to avoid the presence of a "wettable" surface will delay this coagulation indefinitely. Normal coagulation is believed to be due to agglutination and rupture of the blood platelets on a wettable surface, with the release of a thromboplastic substance (Ware, Fahey and Seegers, 1948), or a substance involved

in thromboplastin formation (Biggs and Macfarlane, 1953). The
addition of extracts of brain tissue, lung, thymus (Quick, 1936), or
muscle (Almquist, 1941) will accelerate the reaction, presumably due
to their thromboplastic action. Other compounds having such an
effect include the venoms of certain snakes, particularly Russel's
viper (Lamb, 1903).

Seegers (1949) reported some activation of purified prothrom-
bin by 25% sodium citrate, and an almost quantitative conversion by
the use of certain substituted diphenyl sulfones. This report is
particularly interesting since it shows the possibility of activation
of prothrombin to thrombin in the absence of ionic calcium. The
removal of calcium from blood by precipitation as the citrate or
oxalate is a common method of preventing in vitro coagulation of
blood. In the absence of calcium, thrombin formation is blocked.

It has been shown that snake venom requires the presence of
lipid to act as a thromboplastic agent (Macfarlane, 1938). Quick
(1936) found that extraction with organic solvents reduced the
activity of brain thromboplastin, and suggested the rupture of a
lipoprotein. However Lein and Lein (1948) reported two thromboplastic
agents in tissue, one protein and one lipid in nature. Moreover the
brain thromboplastic agent used by Biggs (1951) is extracted
repeatedly with acetone; the action of this agent is not dependent
on the amount of lipid present.

The steps preceding the activation of prothrombin are not
yet understood. It is clear that the reactions in whole blood

coagulation in vitro are not identical with those of the Quick

"prothrombin time" test, since "'Thromboplastin' formation occupies

most of the 'clotting time'" (Macfarlane, 1955). Macfarlane and

Biggs (1955) postulate the following series of reactions for coagula-

tion of mammalian blood: on contact with a foreign surface an

interaction is initiated which involves the platelets, antihaemophilic

globulin (AHG) and the Christmas factor. Schematically,

$$AHG + platelets \xrightarrow[Ca^{++}]{Christmas\ factor} Intermediate\ product$$

$$Intermediate\ product + Factor\ V \xrightarrow[Ca^{++}]{Factor\ VII} Thromboplastin$$

$$Prothrombin \xrightarrow{Thromboplastin,\ Ca^{++}} Thrombin$$

$$Fibrinogen \xrightarrow{Thrombin} Fibrin$$
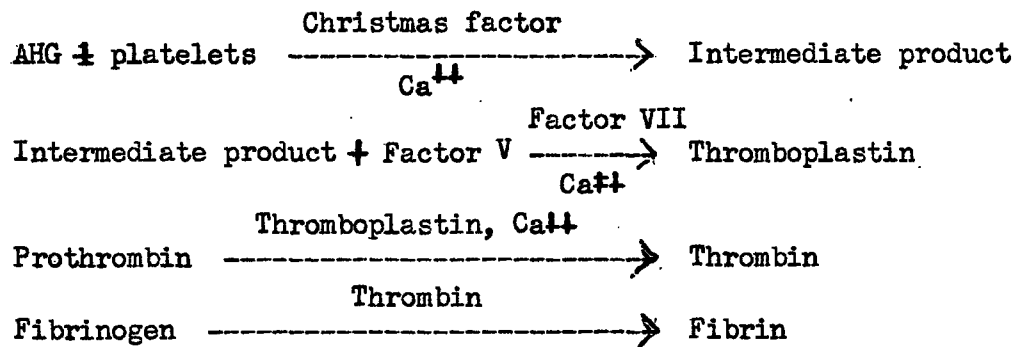
(Composite scheme from Macfarlane and Biggs (1955),

Biggs (1955), and Macfarlane (1955)).

Marbet and Winterstein (1954) suggest that clotting involves

five steps or phases:

Prephase - agglutination and vasoconstriction by serotonin from

platelets.

First principal phase - thromboplastin formation.

Second principal phase - thrombin formation.

Third principal phase - fibrin formation.

Postphase - retraction and fibrinolysis.

It is important to note that both these groups use the term

"thromboplastin" in a new sense. It is now considered to be a

"potent but transitory" agent produced by a series of reactions, and not a constituent of tissues or of platelets, as earlier believed. "Tissue extract" is believed to be similar to the hypothetical "intermediate product", in its thromboplastic action (Biggs, Douglas and Macfarlane, 1953). Andreenko and Kudryashov (1955) have recently reported that the thromboplastic activity of the blood is increased when vitamin B_{12} is injected into white rats. Graham et al (1954) have suggested that platelets and antihaemophilic globulin are also involved in the "prothrombin time" test.

If the above scheme is accepted as a working hypothesis for the coagulation of avian blood it is readily apparent that the Quick "prothrombin" assay includes steps two to four, with the substitution of tissue extract for the "intermediate product". Thus the test will measure variations in any or all of Factor V, Factor VII, prothrombin, Ca^{++} or fibrinogen. Deficiencies of Factor V or Factor VII may be hereditary or acquired. They may also be affected by development of inhibitors of thromboplastin formation (Macfarlane and Biggs, 1955). The measurement of prothrombin is further complicated by the indication that "prothrombin deficiency may not cause much lengthening of the one-stage method" (Biggs and Douglas, 1953). De Nicola (1953) states that variations in prothrombin time are correlated with variations in Factor VII.

Several other assay methods for prothrombin have been developed in an attempt to overcome these difficulties. Most are classified as "two-stage" methods as distinct from Quick's "one-stage" assay. Of

this type are the procedures of Warner, Brinkhous and Smith (1936),
Herbert (1940), Ware and Seegers (1949), Sternberger (1947), and
Biggs (1951). In these methods diluted plasma is incubated with
tissue extract and samples are removed periodically for addition
to a fibrinogen solution. When the clotting time of the fibrinogen
reaches a minimum the conversion of prothrombin to thrombin is
considered to be complete. These methods separate steps two and three from
step four in the postulated scheme. They have the advantage of
measuring the rate of prothrombin activation and of assaying maximum
thrombin level, neither of which is accomplished by the one-stage
technique. Unfortunately the maximum thrombin level is not an accurate
assay of prothrombin concentration, particularly in cases of delayed
activation, due to the presence of an antithrombic agent in plasma
(Quick, 1944). Thus the thrombin present at any given time is a
measure of thrombin produced minus that which has been inactivated by
anthrombin (Biggs, 1951). When a deficiency of either Factor V or
Factor VII exists, the rate of activation will be reduced. Anti-
thrombin will then have a greater opportunity to inactivate the
thrombin produced, resulting in a lower final concentration than if
the activation rate were normal.

This delay can be eliminated by adding concentrates of the
deficient factors, and at the same time a distinction may be made
between the two. Factor V is present in aluminum hydroxide-treated
normal plasma, while prothrombin and Factor VII are adsorbed and

removed. Factor VII, which is not consumed in coagulation, is present in normal serum, while Factor V and prothrombin are not. Thus the addition of either or both, serum or $Al(OH)_3$ adsorbed plasma will provide the missing activators (Biggs and Macfarlane, 1953).

From the foregoing it is apparent that a quantitative measure of prothrombin level cannot be made by the one-stage test. An assessment of the level of this constituent requires that a two-stage test be performed in the presence of an excess of Factors V and VII. If thrombin level (as measured by the time required to clot fibrinogen) is plotted against time, the area under the curve gives a reasonable estimate of the prothrombin originally present (Biggs and Macfarlane, 1953). However, in most cases it is the presence or absence of a coagulation defect which is to be determined. For this purpose the simple one-stage test is of great value provided it is regarded as an empirical measure of several factors, rather than as a quantitative measure of prothrombin.

A possible explanation for the results obtained in the present series of experiments is suggested by Macfarlane's (1938) observation that a thromboplastic agent prepared from the venom of Russel's viper is influenced by blood lipid level. This effect, which can be overcome by addition of lecithin to the agent, is not evident in the thromboplastic preparation from brain tissue.

A series of tests was performed to determine whether or not the breast muscle preparation used in the previous tests differed significantly from brain extract in thromboplastic properties.

EXPERIMENTAL

"Prothrombin-deficient" blood was obtained from chicks 20-35 days of age which had been depleted of vitamin K by the method previously described. A corn-fish meal diet was used in all cases (see table 3:1).

Normal blood was obtained from chicks which had been receiving a commercial diet containing green feed.

Test 1.

The breast muscle clotting agent described previously was diluted with 0.85% saline and with a 1% emulsion of soybean lecithin (mixed soybean phospholipids) in 0.85% saline. 10%, 20%, 30%, 40%, 50% and 67% of lecithin-saline and of saline was added to the normal preparation. An equal volume of $M/40$ $CaCl_2$ was also added to provide the same calcium ion concentration in each solution. "Prothrombin time" of blood from a normal and a vitamin K deficient bird was measured with the normal agent and with each dilution.

Results: The composition of each clotting agent, together with observed "prothrombin time", is shown in table 10:2. The results are illustrated graphically in figure 1. Addition of saline alone produced progressive increases in "prothrombin time", with only minor irregularities. Addition of lecithin-saline, on the other hand, caused a decreased "prothrombin time", at the lower concentrations, in both normal and "prothrombin-deficient" blood. As the amount of added lecithin was increased, the "prothrombin time" also increased, but at

no time was it as long as that of the comparable saline dilution. In the case of the blood from the vitamin K deficient chick, the clotting agent diluted with twice its volume of lecithin-saline (ignoring the M/40 $CaCl_2$ content) produced a "prothrombin time" shorter than that produced by the undiluted agent.

Test 2.

Procedure: Brain tissue thromboplastic agent prepared by the method of Herbert (1940) is not sensitive to blood lipid level, according to Biggs (1951). A preparation of this type was subjected to saline and to lechithin-saline dilution. The procedure followed for preparation of the agent was as follows: Fresh chicken brain was macerated in four times its volume of acetone in a mortar. The acetone was filtered off and discarded. This procedure was repeated four times. The final granular powder was allowed to dry. For use, 0.5 gm. of the powder was suspended in 5.0 ml. of 0.85% saline and incubated, with occasional shaking, for 15 minutes at 38° C. The mixture was centrifuged briefly, and the supernatant diluted to five times its volume with 0.85% saline.

The determination of "prothrombin time" as described by Biggs and Macfarlane (1953) differed from the method used with breast muscle thromboplastic agent in that the agent was added to the blood sample separately rather than with M/40 $CaCl_2$. That is, 0.1 ml. of clotting agent was added to 0.1 ml. of blood in a vial. The mixture was warmed

briefly in a water bath at 38.5-39.0°C. 0.1 ml. M/40 CaCl$_2$ was then added and a stopwatch was started at the same time. As in the previously described method, the time required for formation of a definite fibrin film was recorded as the "prothrombin time".

The clotting agent described above was diluted by the addition of saline or of lecithin-saline to make up 10%, 20%, 40%, 50% and 67% of the final solutions. These dilutions were then used to measure "prothrombin time" of blood from a normal and a vitamin K deficient chick.

Results: "Prothrombin times" are shown in table 10:3, and are illustrated graphically in figure 2. The results differ considerably from those obtained with breast muscle thromboplastic agent (test 2). In the case of the brain tissue preparation, the addition of increasing quantities of lecithin-saline produced a small but steady increase in "prothrombin time" in blood from both the normal and the vitamin K deficient chick. On the other hand, saline dilution up to one-third the original concentration failed to affect "prothrombin time".

Test 3.

In tests 1 and 2 the methods of "prothrombin time" determination differed. In the first test, Ca^{++} and thromboplastic agent were added to the blood as one solution. In the second test the Ca^{++} was added to a mixture of blood and thromboplastic agent. In test 3 the effect of the method of adding Ca^{++} on "prothrombin time" was studied.

(a)

A solution of breast muscle thromboplastic agent, prepared as described previously, was diluted with the same quantities of saline and of lecithin-saline as in the previous two tests. To an aliquot from each dilution was added an equal volume of $M/40$ $CaCl_2$. "Prothrombin time" was determined by (i) adding 0.2 ml. of agent + $CaCl_2$ solution to 0.1 ml. of blood, and (ii) adding 0.1 ml. of $M/40$ $CaCl_2$ to 0.1 ml. of blood $\pm$ 0.1 ml. of agent.

Results: "Prothrombin time" values are shown in table 10:3, and are illustrated in figure 3. The results are similar to those in test 2, except that addition of Ca^{++} to the thromboplastic agent appeared to increase the effect of lecithin in lengthening "prothrombin time".

(b)

Breast muscle thromboplastic agent was prepared in the manner previously described except that $M/40$ $CaCl_2$ was not added. Saline and lecithin-saline dilutions with and without added $CaCl_2$ were prepared and "prothrombin time" determinations carried out as described in test 3 (a).

"Calcium time" of blood samples was also measured, by adding 0.1 ml. saline or lecithin-saline and 0.1 ml. $M/40$ $CaCl_2$ to 0.1 ml. blood.

Results: "Prothrombin time" values are shown in table 10:3, and are illustrated in figure 4. The results were somewhat more variable than in previous tests, but the pattern was similar to that of test 2. Addition

of $CaCl_2$ to the clotting agent rather than to the blood appeared to increase the effect of dilution in all cases; under these conditions lecithin had much less effect in shortening "prothrombin time". In the determination of "calcium time", when no thromboplastic agent was added, blood samples containing lecithin-saline had shorter clotting times than those containing saline only.

Test 4.

In order to obtain a high concentration of lecithin in an undiluted thromboplastic agent, the 1% lecithin-saline emulsion was used in place of saline for the extraction of tissue thromboplastic agents. These extracts were compared to saline extracts from the same tissue.

The thromboplastic agents used in this test were breast muscle extract and dried brain tissue extract, prepared as described previously, and the same agents prepared by using lecithin-saline in place of saline throughout. The same weight of tissue was used for preparations with and without lecithin. These extracts were used to measure "prothrombin time" of blood samples from five normal and five vitamin K deficient chicks. $CaCl_2$ was added separately to the blood + clotting agent.

Results: The individual and mean "prothrombin time" values, as determined using each agent, are shown in table 10:4. It is apparent that lecithin affected the response to the breast muscle preparations, particularly in testing the blood of vitamin K deficient chicks. On the other hand, little if any effect of lecithin on the brain tissue

preparation was observed. The "sensitivity" of the breast muscle

preparation appeared to be reduced by lecithin. That is, mean

"prothrombin time" of the normal birds was 21 seconds when measured

with saline extract of breast muscle; for the vitamin K deficient

birds the mean value was 61 seconds. When lecithin-saline was used

as solvent, the value for normal birds was 17 seconds, and for

vitamin K deficient birds, only 36 seconds.

The brain powder extract was much less affected. Mean

"prothrombin time" figures with saline and lecithin-saline prepara-

tions were 14 and 16 seconds, respectively, for normal blood, and

53 and 50 seconds respectively for "prothrombin-deficient" blood.

It is possible, of course, that the use of a phospholipid

solution for extraction of breast muscle produced a more powerful

thromboplastic solution than did the normal saline extraction.

Test 5.

It was desired to determine whether or not the methods of

preparation of tissue thromboplastic agents affected the response of

the agents to lecithin. Fresh breast muscle was therefore dried with

acetone according to the procedure described previously for brain

tissue. The powder thus obtained was prepared for use as follows:

0.5 gm. powder was extracted with 5 ml. or 0.85% saline, as for

dried brain tissue. As a preliminary test to determine the optimum

concentration of the agent, the resultant solution, and dilutions of

the extract at 50%, 20%, 10% and 5% of its original concentration

(saline used as diluent) were used to determine the "prothrombin time" of normal blood. The "calcium time" (0.1 ml. saline $+$ 0.1 ml. M/40 $CaCl_2$ added to 0.1 ml. blood) was also determined.

The following thromboplastic agents were used to determine "prothrombin time" of blood samples from five normal birds; saline extract of fresh breast muscle; saline extract of fresh brain tissue; saline extract of dried breast muscle powder; saline extract of dried brain tissue powder; and the corresponding lecithin-saline extracts in each case. The extracts of dried breast muscle powder were diluted 1:5 as in the case of extracts of dried brain tissue. 0.1 ml. M/40 $CaCl_2$ was added separately in making the determinations.

Results: The preliminary assay of the dried breast muscle powder indicated no thromboplastic activity (table 10:5). Individual and mean "prothrombin time" values of blood from five normal birds confirmed this observation (table 10:6). The decrease in mean "prothrombin time" when breast muscle powder in lecithin-saline was used may be a function of lecithin alone or it may be an effect of the solvent action of lecithin. Lecithin again shortened the "prothrombin time" values obtained with the fresh breast muscle preparation. A slight prolongation of "prothrombin time" was noted on addition of lecithin to either brain preparation.

Test 6.

Procedure: The thromboplastic solutions prepared from fresh and from dried tissue represented different tissue content on a dry-weight basis.

In other words, the extract from 10 gm. fresh breast muscle, which contained approximately 3 gm. dry matter, was made up to 200 cc. of solution, representing 1.5 gm. dry matter/100 ml. In the case of dried brain tissue the extract from 0.5 gm. was made up to 25 ml., representing 2.0 gm. dry matter /100 ml. To determine whether or not the difference in the amount of dry matter extracted would account for the difference in unit thromboplastic activity of the extract, extracts of breast muscle were prepared at 4/3 the usual concentration. Saline and lecithin-saline extracts of breast muscle and of dried brain powder were diluted 1:10, 1:100, 1:1000 and 1:10000 with the appropriate diluent. "Prothrombin time" of blood from a normal and from a vitamin K deficient chick was measured using each dilution of each preparation. "Calcium time" was also measured.

Results: The more concentrated breast muscle extract failed to show thromboplastic activity equal to that of dried brain tissue extract (table 10:8). Serial dilution of the clotting agents showed that brain extract retained some activity when diluted 1:1000, while breast muscle extract did not have any effect at that dilution. Addition of lecithin reduced the effectiveness of both agents. Lecithin-saline extract of breast muscle had no effect at dilutions greater than 1:10, while the lecithin-saline extract of dried brain powder appeared to retain slight activity at the 1:100 dilution.

Test 7.

Quick (1936) reported that tissue thromboplastic agents were destroyed by extraction with organic solvents. In the present

experiments breast muscle failed to show thromboplastic activity
after extraction with acetone. The activity of brain tissue, on the
other hand, was not affected by this treatment.

In order to determine whether the thromboplastic component
of breast muscle was destroyed or was extracted by acetone, the
activity of the acetone extract was tested. Five grams of fresh
breast muscle was dried with five 20 ml. portions of acetone. The
dried extracted powder weighed 1.5 gm. The acetone washings were
evaporated under reduced pressure at room temperature until the odour
of acetone could no longer be detected. The volume of the acetone
extract was 3.3 ml.

In the method of Biggs and Macfarlane (1953) the thrombo-
plastic extract is prepared from 0.5 gm. of dried tissue. Thus one-
third of the powder prepared from breast muscle in the present test
was sufficient to prepare a thromboplastic solution by that method.
If all activity had been extracted by the acetone, therefore, one-
third of the concentrated acetone extract should have contained
sufficient activity to prepare a thromboplastic solution. Following
this reasoning, two thromboplastic solutions were prepared. For
the first of these, 0.5 gm. of dried breast muscle was extracted with
5 ml. saline, as described previously. The second solution was pre-
pared by diluting one-third of the concentrated acetone extract
(1.1 ml.) to 5 ml. with saline. The thromboplastic activity of these
two solutions was studied by using each of them to measure "prothrom-
bin time" of a sample of normal blood. The "calcium time" of the

blood sample was measured for comparison.

Results: As in the previous test, the extract of acetone-dried breast muscle had no thromboplastic activity. "Prothrombin time" using this solution was 76 seconds, compared to a "calcium time" of 75 seconds (averages of duplicate determinations). The agent prepared from the acetone extract, on the other hand, showed some activity, producing a "prothrombin time" measurement of 43 seconds. A third thromboplastic agent was prepared by combining equal volumes of each of the previous two agents. "Prothrombin time" using this solution was 60 seconds.

Since "prothrombin time" increases as the concentration of the thromboplastic agent exceeds the optimum level (Biggs and Macfarlane, 1953), the solution prepared from the acetone extract was diluted 1:5 with saline. "Prothrombin time" using the diluted agent was 48 seconds.

DISCUSSION OF RESULTS

The breast muscle thromboplastic agent differed from the agent in brain tissue in several respects. The brain tissue preparation was not generally affected by dilution to one-third its normal concentration. This dilution gave an extract equivalent to 1 gm. dry matter extracted by 150 ml. saline. Breast muscle thromboplastic agent, on the other hand, was appreciably weakened by the addition of as little as 20% saline, equivalent to 1 gm. dry matter extracted by 100 ml. Thus the concentration of thromboplastic activity per unit dry matter was apparently greater in brain tissue than in breast muscle.

A second difference between the two agents was evident in their reaction to the addition of lecithin. An extract of acetone-dried brain tissue suffered a gradual loss of activity as increasing concentrations of lecithin were added. That this was not a direct effect of dilution is shown by the fact that equal additions of saline produced no effect. On the other hand, the activity of the extract from fresh breast muscle was increased by lecithin. This latter effect was particularly noticeable in blood with a prolonged "prothrombin time" from vitamin K deficient chicks. Macfarlane (1938) showed that the thromboplastic activity of the venom of Russel's viper was dependent upon blood lipid level unless lecithin was added. Biggs, Douglas and Macfarlane (1953) showed that lipoid level is a factor in the thromboplastic activity of trypsin.

The difference in the response of brain tissue extract and of breast muscle extract to lecithin is probably due to the fact that brain contains considerably more phospholipid than does skeletal muscle. In the rat phospholipid makes up 21-27% of the dry matter of brain, and only 6-9% of the dry matter of skeletal muscle (Deuel, 1955). Since phospholipids are insoluble in acetone they would not be removed by the drying process in the present tests. Presumably there is an optimum phospholipid level for thromboplastic activity, since the addition of lecithin solution to the brain extract reduced its activity.

Quick (1936) reported that the effectiveness of tissue thromboplastic agents was reduced by extraction with organic solvents, possibly due to the rupture of a lipoprotein. In the present series of tests it

was found that extraction of the tissue with acetone extracted and partially destroyed the agent present in breast muscle, but did not affect that present in brain tissue. On a dry-weight basis the unit activity of acetone-dried brain tissue appeared to be at least equal to that of fresh brain tissue. This is surprising, since Quick found the agent in brain tissue to be more labile in the presence of organic solvents than was the agent in other tissues. The difference may be due to the fact that Quick used solvents which would extract phospholipids. The possibility of differences between mammalian and avian tissues must also be considered.

The addition of lecithin appeared to reduce the sensitivity of breast muscle thromboplastic agent in the test for vitamin K deficiency. The addition of lecithin shortened average "prothrombin time" by 15 seconds (25%) with breast muscle thromboplastic agent, and by only 3 seconds (6%) with brain tissue thromboplastic agent. It was not possible to judge which method of assessment gave a more accurate indication of a haemorrhagic tendency.

The addition of Ca^{++} to the thromboplastic agent rather than to a mixture of blood and thromboplastic agent generally resulted in prolonged "prothrombin time" measurements. This treatment reduced the ability of lecithin to shorten "prothrombin time" measurements with the breast muscle agent.

Dilution of the two agents indicated that the brain preparation had about ten times the thromboplastic activity of the breast muscle

preparation. In the presence of lecithin, the minimum concentration of either agent which showed thromboplastic activity was about 10 times as great as when lecithin was not added.

It appeared that some of the thromboplastic activity of breast muscle was extracted by acetone. The greater part, however, was destroyed during the acetone treatment. The thromboplastic solution prepared from the acetone extract of breast muscle represented a concentration of 10 gm. dry matter extracted per 100 ml. agent, compared to the usual saline extract of fresh muscle which represents a concentration of 1.5 gm. dry matter per 100 ml. Nevertheless, this highly concentrated acetone extract showed less activity than the saline extract. That this low potency was not the result of too high a concentration of thromboplastic agent is shown by the fact that dilution of the solution decreased its activity.

"Reconstituting" the breast muscle agent, by adding the saline extract to the acetone extract, failed to restore normal activity. The mixed solution, in fact, had less activity than did the acetone extract alone. This loss of activity was not the result of the 1:2 dilution of the acetone extract, since 1:5 dilution of this extract with saline gave a smaller loss of activity. No explanation is offered for the decreased activity when the two extracts were combined.

EXPERIMENTAL

There appeared to be a possibility that some of the effects on "prothrombin time" observed in the present series of chick tests were the result of sensitivity of the thromboplastic agent to the blood lipid level. An experiment was designed to examine this possibility.

EXPERIMENT XI.

The effect on "prothrombin time" of two bile salts added to a vitamin K deficient ration was measured using two different tissue thromboplastic agents. Cholic acid and desoxycholic acid were added to a corn-fish meal ration (table 11:1) and fed to chicks as in previous tests. Blood for "prothrombin time" determinations was drawn at 24 days. The two thromboplastic agents used were the fresh breast muscle extract used in the previous series of chick tests, and the extract of dried brain tissue described above.

Results: "Prothrombin time" of 7-9 chicks from each group, measured with the two thromboplastic agents, is shown in table 11:2. It is not known why the experimental diet failed to produce a satisfactory depletion of vitamin K in this test. As far as is known, conditions were the same as those prevailing in previous tests, in which a marked depletion was generally reached within 3 weeks.

The brain tissue thromboplastic preparation appeared to have lost some of its activity. The dry powder had been prepared two months previously, and had not showed any decrease in activity during the intervening period. Biggs and Macfarlane (1953) state that the activity of the powder is not lost in three months at room temperature.

Both cholic and desoxycholic acids produced lengthening effects as measured by both agents. The effect of cholic acid was statistically significant with brain tissue, but not significant with breast muscle; that of desoxycholic acid was significant in each case (table 11:3).

DISCUSSION OF RESULTS

The effect of the bile acids on "prothrombin time" was not dependent on the thromboplastic agent used. It appears unlikely, therefore, that their effect can be ascribed to a change in blood lipid level to which the breast muscle was sensitive.

The effect of bile salts could be due to some adverse effect on microbiological synthesis of vitamin K, although it is not apparent from this explanation why cholic acid should have failed to modify the effect of beef tallow on "prothrombin time" (table 8:2).

CONCLUSIONS

A series of experiments was conducted to observe the effect of dietary fat on the vitamin K requirement of the chick. The criterion of vitamin K status used in these experiments was the "prothrombin time" of the blood.

It was possible to produce a "prothrombin deficiency" in chicks by feeding diets which consisted of unextracted natural ingredients. The extent of this deficiency was affected by the maternal supply of vitamin K. In some cases the coagulation defect was sufficiently severe to cause fairly heavy mortality from haemorrhaging.

Certain fats, when added to the diet, protected the chick from a vitamin K deficiency. Beef tallow, hydrogenated cottonseed oil, and to a lesser extent cottonseed oil and hydrogenated animal fat all shortened blood "prothrombin time" of chicks, when added to a vitamin K deficient diet. These fats prevented the haemorrhaging which sometimes resulted from vitamin K depletion.

Herring oil generally did not shorten "prothrombin time" unless the vitamin K deficient diet was also deficient in folic acid.

The effect of fats on "prothrombin time" was apparently not due to the vitamin K content of the fat, nor to an increased absorption of vitamin K resulting from increased bile secretion.

Oral administration of oils shortly before drawing blood did not affect "prothrombin time" of blood from vitamin K deficient chicks; this indicates that lipaemia per se did not cause the shortening in

"prothrombin time" observed after feeding a high-fat diet. Free fatty acids added to a vitamin K deficient diet did not affect "prothrombin time" to a significant degree.

Addition of choline to a vitamin K deficient diet did not affect "prothrombin time". This observation agrees with those of Field and Dam (1945) but disagrees with those of Honorato and Moline (1942). The addition of methionine also failed to affect "prothrombin time". Addition of folic acid to a diet deficient in both vitamin K and folic acid sometimes shortened "prothrombin time". Herring oil also had a shortening effect when added to such a diet, but not when added to a diet containing adequate folic acid.

Cholic acid added to a vitamin K deficient diet caused a lengthened "prothrombin time". Desoxycholic acid had a similar effect in one test, but had no effect in another case. Injection of chicks with the surface active agent Triton WR-1339 likewise lengthened "prothrombin time". Triton had no effect, however, when added to blood _in vitro_.

Breast muscle and brain tissue differed in the thromboplastic properties of their extracts. The breast muscle preparation required the addition of phospholipid for maximum thromboplastic activity. Acetone partly extracted and partly destroyed the thromboplastic agent of breast muscle, but did not affect that of brain tissue.

Although bile salts may affect blood phospholipid levels, their effect on blood "prothrombin time" was not due to the sensitivity of the breast muscle extract to phospholipid.

The "prothrombin time" of blood, as determined with breast muscle thromboplastic agent, is not necessarily a measure of the vitamin K supplied to the chick.

A P P E N D I X

TABLE 1:1.

Experimental Diets - Experiment I.

Ingredients common to all Rations

Ingredient	%	
Ground Wheat	42.25	
Ground Yellow Corn	5.0	
Pulverized Oats	5.0	
Soybean Oil Meal (44%)	22.0	
Herring Meal	5.0	
Meat Meal	5.0	
Bone Meal	1.0	
Limestone	1.0	
Salt (Iodized)	0.5	
Feeding Oil (2250A, 300 D_3)	0.25	
$MnSO_4$	0.0125	
Riboflavin		0.2 gm.
Niacin		1.0 gm.
Calcium Pantothenate		0.5 gm.
Folic Acid		0.035 gm.
	87.0	

Composition of Individual Rations.

Ingredient	Pounds per Ration					
	1	2	3	4	5	6
Premix (as above)	52.2	52.2	52.2	52.2	52.2	52.2
Cerelose	4.8	4.8	4.8	----	----	----
Soybean Oil Meal	0.9	0.9	----	0.9	0.9	----
Ground Wheat	2.1	2.1	----	2.1	2.1	----
Menadione	----	0.05gm.	----	----	0.05gm.	----
Dehydrated Cereal Grass	----	----	3.0	----	----	3.0
Herring Oil	----	----	----	4.8	4.8	4.8
	60.0	60.0	60.0	60.0	60.0	60.0

Mean "Prothrombin Time" – Experiment I

(Figures in parentheses represent number of birds tested)

Group	Supplement to Basal Ration	Mean "Prothrombin Time" in Seconds.
1. Males	None	
Depleted Stock		39 (5)
Undepleted Stock		31 (5)
Females		
Depleted Stock		35 (5)
Undepleted Stock		30 (5)
2. Males	Menadione	
Depleted Stock		28 (5)
Undepleted Stock		31 (5)
Females		
Depleted Stock		31 (5)
Undepleted Stock		29 (5)
3. Males	Dehydrated Cereal Grass	
Depleted Stock		30 (5)
Undepleted Stock		27 (5)
Females		
Depleted Stock		28 (5)
Undepleted Stock		30 (5)
4. Males	Herring Oil	
Depleted Stock		49 (5)
Undepleted Stock		37 (5)
Females		
Depleted Stock		40 (5)
Undepleted Stock		35 (5)
5. Males	Herring Oil Menadione	
Depleted Stock		28 (5)
Undepleted Stock		30 (5)
Females		
Depleted Stock		31 (5)
Undepleted Stock		29 (5)
6. Males	Herring Oil Dehydrated Cereal Grass.	
Depleted Stock		30 (5)
Undepleted Stock		28 (5)
Females		
Depleted Stock		29 (5)
Undepleted Stock		29 (5)
Control Bird		25

TABLE 1:3

Analysis of Variance – Experiment I

Males.

Source of Variation	SS	df	Variance	"f"
Total	3424	59		
Rations	1897	5	379	22.9 **
Source of Chicks	216	1	216	12.5 **
Rations X Source	465	5	93	5.4 **
Error	846	48	17.6	

Least Difference required for Significance =

$$\sqrt{\frac{2 \text{ X Error Variance}}{\text{Individuals / Group}}} \quad \text{X "t" for group d.f.}$$

	5%	1%
L.S.D. rations	4	6
L.S.D. sources	2	3
L.S.D. rations X sources	7	12

Females.

Source of Variation	SS	df	Variance	"f"
Total	934	59		
Rations	547	5	109	20.8 **
Source of Chicks	52	1	52	9.9 **
Rations X Source	82	5	16	3.0 *
Error	253	49	5.25	

	5%	1%
L.S.D. Rations	2	3
L.S.D. sources	1	2
L.S.D. rations X sources	4	7

TABLE 2:1.

Experimental Diets - Experiment II

Ingredients common to all rations:

Ingredient	%
Ground Wheat	70.75
Herring Meal	19.0
Salt (Iodized)	0.5
Limestone	1.25
Choline Chloride (25%)	0.25
Feeding Oil (2250 A, 300 D)	0.25
MnSO$_4$	10.0 gm.
Riboflavin	0.16 gm.
Calcium Pantothenate	0.50 gm.
Niacin	0.80 gm.
	92.0

Composition of Individual Rations:

Ration #	Premix (lbs) (as above)	Cerelose (lbs)	Amount per Ration Herring Oil (lbs)	Folic Acid (gms.)	Menadione (gms.)
1.	46.0	4.0	----	----	----
2.	46.0	4.0	----	0.018	----
3.	46.0	4.0	----	----	0.045
4.	46.0	4.0	----	0.018	0.045
5.	46.0	----	4.0	----	----
6.	46.0	----	4.0	0.018	----
7.	46.0	----	4.0	----	0.045
8.	46.0	----	4.0	0.018	0.045

TABLE 2:2

Mean Weights of Chicks – Experiment.II.

(Figures in parentheses indicate number of birds per group)

Group #	Supplement to Basal Ration	Weight in grams	
		Test 1 (28 days)	Test 2 (28 days)
1	None	262 (15)	207 (18)
2	Folic acid	306 (14)	257 (18)
3	Menadione	217 (9)	238 (17)
4	Folic acid + Menadione	281 (15)	257 (18)
5	Herring oil	215 (11)	214 (16)
6	Herring oil + Folic acid	273 (15)	242 (17)
7	Herring oil + Menadione	233 (15)	206 (14)
8	Herring oil + Folic acid + Menadione	323 (16)	257 (17)

TABLE 2:3

Mean "Prothrombin Time" – Experiment II

(Figures in parentheses indicate number of birds per group)

Group #	Supplement to Basal Ration	Test 1 (29 days)	Test 2 (33 days)
		"Prothrombin Time" in seconds.	
1	None	62 (10)	92 (10)
2	Folic Acid	68 (10)	72 (10)
3	Menadione	38 (9)	38 (6)
4	Folic Acid + Menadione	34 (10)	44 (9)
5	Herring oil	52 (10)	72 (10)
6	Herring oil + Folic acid	44 (10)	64 (9)
7	Herring oil + Menadione	36 (10)	40 (10)
8	Herring oil + Folic acid + Menadione	34 (10)	36 (9)
	Control Bird	30	30

TABLE 2:4

Analysis of "Prothrombin Time" Variance – Experiment II

Test 1.

Source of Variation	SS	df	Variance	"f"
Total	25242	78		
Treatment	10245	7	1463	6.93 **
Error	14997	71	211	

L.S.D. treatments			5% 15	1% 21

Test 2.

Source of Variation	SS	df	variance	"f"
Total	41625	72		
Treatment	28055	7	4007	19.2 **
Error	13570	65	208	

L.S.D. treatments			5% 16	1% 23

TABLE 2:5

Effect of Herring Oil on "Prothrombin Time" – Experiment II

(Figures in Parentheses indicate number of birds treated)

	"Prothrombin Time" in Seconds	
	Basal Diet	Basal Diet + 8% Herring Oil
1	45 (14)	44 (15)
2	36 (10)	40 (10)

TABLE 3:1

Experimental Diets - Experiment III.

Ingredients common to all rations:

Ingredient	%
Ground Yellow Corn	71.5
Herring Meal	18.75
Iodized Salt	0.5
Limestone	1.25
Choline Chloride (25%)	0.25
Feeding Oil (2250 A, 300 D)	0.25

MnSO$_4$	10.0 gm.
Riboflavin	0.16 gm.
Calcium Pantothenate	0.50 gm.
Niacin	0.80 gm.
	92.5

Composition of Individual Rations in Pounds:

Ration	Premix (as above)	Cerelose	Cottonseed Oil	Hydrogenated Cottonseed Oil	Hydrogenated Animal fat	Folic Acid (gms.)
1.	55.5	4.5	----	----	----	----
2.	55.5	4.5	----	----	----	0.021
3.	55.5	----	4.5	----	----	----
4.	55.5	----	4.5	----	----	0.021
5.	55.5	----	----	4.5	----	----
6.	55.5	----	----	4.5	----	0.021
7.	55.5	----	----	----	4.5	----
8.	55.5	----	----	----	4.5	0.021

TABLE 3:2.

Mean "Prothrombin Time" - Experiment III.

(Figures in parentheses indicate number of birds tested)

Group	Supplement to Basal Diet	"Prothrombin Time" Seconds.
1.	None	50 (10)
2.	Folic Acid	50 (10)
3.	Cottonseed Oil	34 (10)
4.	Cottonseed Oil + Folic Acid	29 (10)
5.	Hydrogenated Cottonseed Oil	23 (10)
6.	Hydrogenated Cottonseed Oil + Folic Acid	21 (10)
7.	Hydrogenated Animal Fat	31 (10)
8.	Hydrogenated Animal Fat + Folic Acid	24 (10)
	Control Bird	24

TABLE 3:3

Analysis of "Prothrombin Time" Variance - Experiment III.

Source of Variation	SS	df	Variance	"f"
Total	12085	79		
Treatment	9215	7	1316	33.7 **
Error	2870	72	39	

	5%	1%
L.S.D. Treatments	6	9

TABLE 3:4

Mean 28-day Weights of Chicks - Experiment III

(Figures in Parentheses indicate number of birds per lot)

Group	Supplement to Basal Diet	Mean Body Weight (grams)	
1	None	239	(14)
1A		240	(15)
		240	
2	Folic Acid	324	(18)
2A		313	(16)
		318	
3	Cottonseed Oil	256	(19)
3A		258	(21)
		257	
4	Cottonseed Oil + Folic Acid	330	(19)
4A		324	(21)
		327	
5	Hydrogenated Cottonseed Oil	241	(20)
5A		277	(22)
		259	
6	Hydrogenated Cottonseed Oil + Folic Acid	335	(22)
6A		331	(21)
		333	
7	Hydrogenated Animal Fat	233	(20)
7A		244	(19)
		238	
8	Hydrogenated Animal Fat + Folic Acid	310	(21)
8A		318	(22)
		314	

TABLE 4:1

Experimental Diets - Experiment IV.

Ingredients Common to all Rations:

Ingredient	Test 1 %	Test 2 %
Ground Yellow Corn	51.5	46.25
Soybean Oil Meal (44%)	37.0	30.0
Meat Meal	----	10.0
Limestone	1.0	1.0
Bone Meal	2.0	----
Salt (Iodized)	0.5	0.5
Feeding Oil (2250 A, 300 D)	----	0.25
MnSO$_4$	10.0 gm.	6.0 gm.
Riboflavin	0.16 gm.	0.16 gm.
Calcium Pantothenate	0.50 gm.	0.50 gm.
Niacin	0.80 gm.	1.00 gm.
Folic acid	----	0.035 gm.
B$_{12}$	----	0.0004 gm.
Vitamin A (10,000 IU/gm.)	20.0 gm.	----
Vitamin D (1650 ICU/gm.)	81.8 gm.	----
	92.0	88.0

Composition of Individual Diets in Pounds:

Ration	Premix (see above)	Cerelose	Beef Tallow	Herring Oil	Choline Chloride (25% gms)	Folic Acid (gms)	Methionine (gms)	Menadione (gms)
Test 1								
1	36.8	3.2	----	----	----	----	----	----
2	36.8	----	----	3.2	----	----	----	----
3	36.8	----	3.2	----	----	----	----	----
4	36.8	3.2	----	----	72.64	----	----	----
5	36.8	----	----	3.2	72.64	----	----	----
6	36.8	----	3.2	----	72.64	----	----	----
7	36.8	3.2	----	----	----	0.014	----	----
8	36.8	----	----	3.2	----	0.014	----	----
9	36.8	----	3.2	----	----	0.014	----	----
10	36.8	3.2	----	----	72.64	0.014	----	----
11	36.8	----	----	3.2	72.64	0.014	----	----
12	36.8	----	3.2	----	72.64	0.014	----	----
13	36.8	3.2	----	----	----	----	----	0.036
Test 2.								
1	35.2	4.8	----	----	----	----	----	----
2	35.2	4.8	----	----	----	----	----	0.0014
3	35.2	4.8	----	----	24.00	----	----	----
4	35.2	4.8	----	----	----	----	45.4	----
5	35.2	----	4.8	----	----	----	----	----
6	35.2	----	4.8	----	----	----	----	0.0014
7	35.2	----	4.8	----	24.0	----	----	----
8	35.2	----	4.8	----	----	----	45.4	----

Mean "Prothrombin Time" Values - Experiment IV

(Figures in Parentheses indicates number of birds treated)

Supplement to Basal Diet.	Mean "Prothrombin Time" in Seconds		
	Test 1 23 days	Test 2. 23 days	30 days
None	67 (10)	134 (10)	----
Herring Oil	78 (9)	----	----
Beef Tallow	45 (9)	33 (10)	24 (12)
Choline Chloride	64 (9)	141 (10)	----
Herring Oil + Choline	62 (10)	----	----
Beef Tallow + Choline	47 (9)	30 (10)	24 (12)
Folic Acid	73 (9)	----	----
Herring Oil + Folic	80 (10)	----	----
Beef Tallow + Folic	43 (10)	----	----
Choline + Folic Acid	68 (9)	----	----
Choline + Folic + Herring Oil	76 (9)	----	----
Choline + Folic + Beef Tallow	45 (8)	----	----
Menadione	30 (8)	103 (10)	----
Menadione + Beef Tallow	----	27 (10)	23 (12)
Methionine	----	135 (10)	----
Methionine + Beef Tallow	----	28 (10)	23 (11)
Control Bird	31	28	24

TABLE 4:3

Analysis of "Prothrombin Time" Variance - Experiment IV.

Test 1.

Source of Variation	SS	df	Variance	"f"
Total	51304	118		
Treatment	27270	8.15	3346	15.28 **
Error	24034	109.85	219	

			5%	1%
L.S.D. Treatments			16	23

Test 2.

Source of Variation	SS	df	Variance	"f"
Total	250439	79		
Treatment	203925	7	29132	45.1 **
Error	46514	72	646	

			5%	1%
L.S.D. Treatments			26	37

TABLE 4:4

Mean Weight at 23 days - Experiment IV Test 2.

Group	Supplement to Basal Diet	Mean Body Weight in Grams.
1	None	220 (21)
2	Menadione	205 (22)
3	Choline chloride	221 (19)
4	Methionine	243 (20)
5	Beef Tallow	214 (22)
6	Tallow + Menadione	205 (22)
7	Tallow + Choline	222 (22)
8	Tallow + Methionine	244 (21)

Experimental Diets - Experiment V.

Ingredients Common to All Rations:

Ingredient	%
Ground Yellow Corn	51.5
Soybean Oil Meal	37.0
Salt (Iodized)	0.5
Limestone	1.0
Bone Meal	2.0

MnSO$_4$	10.0 gm.
Riboflavin	0.16 gm.
Calcium Pantothenate	0.50 gm.
Niacin	0.80 gm.
Vitamin A (10,000 IU/gm)	20.0 gm.
Vitamin D (1650 ICU/gm)	81.8 gm.
Choline Chloride (25%)	181.6 gm.
Folic Acid	0.035 gm.

Composition of Individual rations in Pounds:

Ration #.	Premix (as above)	Cerelose	Herring Oil alkali treated	Herring Oil not treat.	Cottonseed Oil alkali old treated	Cottonseed Oil not treated	Beef Tallow alkali treat'd	Beef Tallow not treat'd.
Test 1.								
1	27.6	2.4	----	---	---	----	----	----
2	27.6	1.2	1.2	----	---	----	-----	----
3	27.6	0.6	1.8	----	---	----	----	----
4	27.6	----	2.4	----	---	----	----	----
5	27.6	1.2	----	1.2	----	----	----	----
6	27.6	0.6	----	1.8	---	----	----	----
7	27.6	----	----	2.4	---	----	----	----
8	27.6	----	----	----	2.4	----	----	----
Test 2.								
1	36.8	3.2	----	----	---	----	----	----
2	36.8	2.4	----	0.8	---	----	----	----
3	36.8	0.8	----	2.4	---	----	----	----
4	36.8	2.4	----	----	---	0.8	----	----
5	36.8	0.8	----	----	---	2.4	----	----
6	36.8	2.4	----	----	---	----	----	0.8
7	36.8	0.8	----	----	---	----	---	2.4
8	36.8	2.4	0.8	----	---	----	----	----
9	36.8	0.8	2.4	----	---	----	----	----
10	36.8	2.4	----	----	0.8	----	----	----
11	36.8	0.8	----	----	2.4	----	----	----
12	36.8	2.4	----	----	---	----	0.8	----
13	36.8	0.8	----	----	---	----	2.4	----
14	36.8	3.2	plus 0.036 gm. menadione.					

Mean Body Weight at 23 days – Experiment V Test 1.

(Figures in Parentheses indicate number of birds per group)

Group	Supplement to Basal Ration	Mean Body Weight.(Gms.)
1	None	210 (19)
2	4% Alkali-treated Herring Oil	214 (17)
3	6% Alkali-treated Herring Oil	194 (18)
4	8% Alkali-treated Herring Oil	195 (18)
5	4% Untreated Herring Oil	217 (18)
6	6% Untreated Herring Oil	199 (20)
7	8% Untreated Herring Oil	199 (19)
8	8% Stored Herring Oil	182 (17)

TABLE 5:3

Mean "Prothrombin Time" Values – Experiment V

(Figures in Parentheses indicate number of birds tested)

Supplement to Basal Ration	Test 1 18 days	Test 1 25 days	Test 2 19 days
None	65 (5)	77 (10)	38 (9)
2% Treated Herring Oil	----	----	50 (9)
4% Treated Herring Oil	71 (5)	87 (10)	----
6% Treated Herring Oil	67 (5)	97 (10)	39 (9)
8% Treated Herring Oil	71 (5)	80 (10)	----
2% Untreated Herring Oil	----	----	47 (10)
4% Untreated Herring Oil	71 (5)	65 (10)	----
6% Untreated Herring Oil	76 (5)	66 (10)	49 (10)
8% Untreated Herring Oil	56 (5)	60 (10)	----
8% Stored Herring Oil	60 (5)	72 (10)	----
2% Treated Beef Tallow	----	----	41 (9)
6% Treated Beef Tallow	----	----	26 (8)
2% Untreated Beef Tallow	----	----	30 (10)
6% Untreated Beef Tallow	----	----	36 (10)
2% Treated Cottonseed Oil	----	----	35 (10)
6% Treated Cottonseed Oil	----	----	30 (10)
2% Untreated Cottonseed Oil	----	----	38 (10)
6% Untreated Cottonseed Oil	----	----	36 (9)
Menadione	----	----	21 (7)
Control Bird	30	36	23

5

TABLE 5:4

Analysis of "Prothrombin Time" Variance - Experiment V

Test 1 - 18 days.

Source of Variation	SS	df	Variance	"f"
Total	9010	39		
Treatment	1462	7	209	0.88
Error	7548	32	236	

Test 1 - 25 days.

Source of Variation	SS	df	Variance	"f"
Total	26622	79		
Treatment	10843	7	1549	7.07 **
Error	15779	72	219	

			5%	1%
L.S.D. treatments			15	22

Test 2 - 19 days.

Source of Variation	SS	df	Variance	"f"
Total	17570	129		
Rations	8032	13	618	7.44 **
Error	9538	116	83	

			5%	1%
L.S.D. treatments			10	14

Experimental Diets – Experiment VI

Ingredients Common to All Rations:

Ingredient	%
Ground Yellow Corn	71.5
Herring Meal	18.75
Salt (Iodized)	0.5
Limestone	1.25
Choline Chloride (25%)	0.25
Feeding Oil (2250 A, 300 D)	0.25

MnSO$_4$	10.0 gm.
Riboflavin	0.16 gm.
Calcium Pantothenate	0.50 gm.
Niacin	0.80 gm.
	92.5

Composition of Individual Rations:

Ration #	Premix – lbs. (as above)	Cerelose lbs.	Cottonseed Oil–treated lbs.	Cottonseed Oil–untreated lbs.	Folic Acid (gms)
1.	37.0	3.0	----	----	----
2.	37.0	3.0	----	----	0.014
3.	37.0	----	----	3.0	----
4.	37.0	----	----	3.0	0.014
5.	37.0	----	3.0	----	----
6.	37.0	----	3.0	----	0.014
7.	37.0	3.0 +	0.008 gm. Menadione		----

TABLE 6:2

Mean "Prothrombin Time" Values - Experiment VI.

(Figures in parentheses indicate number of birds tested)

Group	Supplement to Basal Ration	Mean "Prothrombin Time" in Seconds.
1	None	131 (11)
2	Folic Acid	114 (14)
3	Cottonseed Oil	80 (19)
4	Cottonseed Oil + Folic Acid	65 (18)
5	Alkali Treated Cottonseed Oil	71 (19)
6	Alkali Treated CSO + Folic Acid	64 (18)
7	Menadione	58 (20)
	Control Bird	22

TABLE 6:3

Analysis of "Prothrombin Time" Variance - Experiment VI.

Source of Variation	SS	df	Variance	"f"
Total	182995	137		
Treatment	76728	7	10961	13.4 **
Error	106267	130	817	

		5%	1%
L.S.D. treatments		37	56

TABLE 7:1

Experimental Diets - Experiment VII

Ingredients Common to all Rations:

Ingredient	%
Ground Yellow Corn	71.50
Herring Meal	18.75
Salt (Iodized)	0.5
Limestone	1.25
Choline Chloride (25%)	0.25
Feeding Oil (2250 A, 300 D)	0.25
MnSO$_4$	10.0 gms.
Riboflavin	0.16 gm.
Calcium Pantothenate	0.50 gm.
Niacin	0.80 gm.
	92.5

Composition of Individual Rations in Pounds:

Ration #	Premix (as above)	Cerelose	Oleic Acid	Mixed Fish Liver Fatty Acids
1.	27.75	2.25	----	----
2.	18.5	----	1.50	----
3.	18.5	----	----	1.50

TABLE 7:2

Mean "Prothrombin Time" Values - Experiment VII
(Figures in parentheses indicate number of birds tested)

Group	Supplement to Basal Ration	Mean "Prothrombin Time" in Seconds
1.	None	80 (15)
2.	Oleic Acid	72 (15)
3.	Mixed Fatty Acids	63 (15)
4.	Cottonseed Oil administered 1 hour before Blood was Drawn	99 (15)
	Control Bird	24

TABLE 7:3

Analysis of "Prothrombin Time" Variation – Experiment VII

Source of Variation	SS	Df	Variance	"f"
Total	39078	59		
Treatment	10981	3	3660	7.29 **
Error	28097	56	502	

	5%	1%
L.S.D. treatments	24	18

TABLE 7:4

Effect of Orally Administered Oil on "Prothrombin Time"

(Figures in parentheses indicate number of chicks tested)

Test	Basal Ration	Treatment	"Prothrombin Time" (Seconds) Untreated Chicks	Treated Chicks	Control Bird
1	Wheat-Fishmeal	Herring Oil	48 (4)	44 (4)	26
2	Corn-Fishmeal	Corn Oil	399 (4)	383 (5)	31
3	Corn-Fishmeal	Corn Oil	104 (5)	94 (5)	22

Experimental Diets - Experiment VIII

Ingredients Common to All Rations

Test 1 ---------- As in Table 2:1, 0.35 mg./lb. folic acid added.

Test 2 ---------- As in Table 3:1, 0.5% ground corn subtracted.

Test 3 ---------- As in Table 3:1, 7.5% ground corn added.

Composition of Individual Rations

	Ration	Premix (as above) (pounds)	Cerelose (pounds)	Beef Tallow (pounds)	Cholic Acid (grams)	Desoxy-cholic Acid (grams)	Menadione (grams)
Test 1	1	18.4	1.6	----	----	----	----
	2	18.4	1.6	----	45.4	----	----
Test 2	1	18.4	1.6	----	----	----	----
	2	18.4	1.6	----	45.4	----	----
	3	18.4	----	1.6	----	----	----
	4	18.4	----	1.6	45.4	----	----
Test 3	1	20.0	----	----	----	----	----
	2	20.0	----	----	45.4	----	----
	3	20.0	----	----	----	45.4	----
	4	20.0	----	----	----	----	0.00036
	5	20.0	----	----	----	----	0.0072

TABLE 8:2

Mean "Prothrombin Time" Values – Experiment VIII
(Figures in parentheses indicate number of birds tested)

Supplement to Basal Ration	Mean "Prothrombin Time" in Seconds		
	Test 1	Test 2	Test 3
None	45 (14)	127 (8)	78 (10)
Cholic Acid	67 (15)	166 (8)	109 (10)
Desoxycholic Acid	----	----	58 (10)
Beef Tallow	----	35 (10)	----
Beef Tallow + Cholic Acid	----	35 (10)	-----
Menadione (1/10 requirement)	----	----	43 (10)
Menadione (2 X requirement)	----	----	25 (9)
Control Bird	26	26	28

TABLE 8:3

Analysis of "Prothrombin Time" Variance – Experiment VIII

Test 1. Source of Variation	SS	df	Variance	"f"
Total	9659	27		
Treatment	3366	1	3366	13.9 **
Error	6293	26	242	

			5%	1%
L.S.D. treatments			13	18

Test 2. Source of Variation	SS	df	Variance	"f"
Total	39987	15		
Treatment	6202	1	6202	2.6
Error	33785	14	2413	

Test 3. Source of Variation	SS	df	Variance	"f"
Total	93802	48		
Treatment	40591	4	10148	8.4 **
Error	53211	44	1209	

			5%	1%
L.S.D. treatments			36	51

TABLE 9:1

Mean "Prothrombin Time" Values - Experiment IX
(Figures in parentheses indicate number of birds tested)

	Ration Fed	Mean "Prothrombin Time - Seconds Triton-treated	Untreated	Control Bird.
Test 1	Wheat-fishmeal	50 (5)	37 (5)	24
Test 2	Corn-fishmeal	1182 (5)	399 (4)	31
Test 3	Corn-fishmeal	211 (5)	104 (5)	22

TABLE 9:2

Analysis of "Prothrombin Time" Variance - Experiment IX

Test 1	Source of Variation	SS	df	Variance	"f"
	Total	589	9		
	Treatment	423	1	423	20.1 **
	Error	166	8	21	

		5%	1%		
	L.S.D. treatment	8	13		

Test 2	Source of Variation	SS	df	Variance	"f"
	Total	3536201	8		
	Treatment	1361550	1	1361550	4.4
	Error	2174651	7	310644	

Test 3	Source of Variation	SS	df	Variance	"f"
	Total	116800	9		
	Treatment	28515	1	28515	2.58
	Error	88285	8	11036	

TABLE 9:3

Effect of Triton WR-1339 _in vitro_ on Blood "Prothrombin Time"

0.85% Saline or 2.5% Triton in 0.85% Saline Added to Blood or Clotting Agent.

Concentration of Added Saline or Triton-saline as % of Final Solution	"Prothrombin Time" in Seconds
Triton-saline Added to Blood	
3.0%	37
5.6%	38
9.5%	36
16.7%	45
Saline Added to Blood	
3.0%	40
5.6%	36
9.5%	38
16.7%	44
Triton-saline Added to Clotting Agent	
6.1%	38
11.1%	40
19.1%	43
33.3%	46
Nothing Added	32

TABLE 10:1

Effect of Lecithin on Breast Muscle Thromboplastic Agent

0.85% Saline or 1% Lecithin in 0.85% Saline plus an Equal Volume of M/40 $CaCl_2$ Added to Tissue Extract.

% of Saline or of Lecithin-saline added Ignoring $CaCl_2$ solution	"Prothrombin Time" in Seconds	
	Normal	Vitamin K Deficient
Saline Added to Make		
20%	31	82
30%	32	87
40%	35	100
50%	36	94
67%	38	118
Lecithin-saline Added to Make		
10%	23	49
20%	22	43
30%	22	44
40%	24	48
50%	30	51
67%	33	56
Undiluted Agent	24	62

TABLE 10:2

Effect of Lecithin on Dried Brain Thromboplastic Agent

0.85% Saline or 1% Lecithin in 0.85% Saline added to Tissue Extract.

% of Saline or of Lecithin-saline added	"Prothrombin Time" in Seconds.	
	Normal	Vitamin K Deficient
Saline Added to Make		
10%	16	38
20%	16	38
30%	15	38
40%	16	38
50%	16	38
67%	16	39
Lecithin-saline Added to Make		
10%	16	42
20%	17	42
30%	18	46
40%	18	47
50%	20	51
67%	21	57
Undiluted Tissue Extract	16	38

TABLE 10:3

Effect of Preincubation with $CaCl_2$ on Response of Tissue Extracts to Lecithin

"Prothrombin Time" in Seconds

| | Breast Muscle Extract | | | | Dried Brain Extract | | | |
| | $CaCl_2$ Added to Blood | | $CaCl_2$ Added to Agent | | $CaCl_2$ Added to Blood | | $CaCl_2$ Added to Agent | |
	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood
Saline Added to Make								
10%	34	50	32	68	16	40	19	40
20%	27	54	34	78	16	42	19	42
30%	29	56	32	86	16	40	19	38
40%	34	64	39	105	18	41	19	38
50%	31	63	34	89	17	41	20	39
67%	32	72	39	122	18	43	21	42
Lecithin-saline Added to Make								
10%	19	34	25	44	17	43	19	42
20%	19	35	27	49	16	44	20	44
30%	20	38	27	58	19	46	21	50
40%	20	39	30	61	22	48	24	57
50%	22	48	30	67	23	50	28	63
67%	27	65	34	82	23	54	27	75
Undiluted Agent	26	50	26	54	17	42	18	38
"Calcium Time" with Saline	96	10'	105	10'				
"Calcium Time" with Lecithin-Saline	93	9'13"	88	8'46"		Not Tested		

TABLE 10:4

Comparison of Activity of Four Thromboplastic Agents

	Breast Muscle Thromboplastic Agent				Dried Brain Tissue Thromboplastic Agent			
	Prepared with Saline		Prepared with Lecithin–Saline		Prepared with Saline		Prepared with Lecithin–Saline	
Blood Sample #	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood	Normal Blood	Vitamin K Deficient Blood
1	20		18		14		15	
2	22		16		14		17	
3	21		16		13		16	
4	22		18		15		16	
5	22		17		14		15	
6		57		33		50		47
7		47		30		38		33
8		77		47		76		73
9		60		35		46		45
10		64		36		54		51
Mean Value	21	61	17	36	14	53	16	50

TABLE 10:5

<u>Thromboplastic Activity of Acetone-dried Breast Muscle</u>

Clotting Agent Diluted in Saline
Tested with Oxalated Normal Blood

Concentration of Original Extract - %	"Prothrombin Time" Seconds
100	145
50	167
20	138
10	217
5	138
"Calcium Time"	130

TABLE 10:6

Comparison of Thromboplastic Properties of Extracts of Fresh and of Acetone
Dried Breast Muscle Tissue and Brain Tissue
Tested with Normal Blood

Bird #	"Calcium" Time	Fresh Breast Muscle		Fresh Brain Tissue		Dried Breast Muscle		Dried Brain Tissue	
		Saline Extract	Lecithin-Saline Extract	Saline Extract	Lecithin-Saline Extract	Saline Extract	Lecithin-Saline Extract	Saline Extract	Lecithin-Saline Extract
1	122	25	18	20	24	126	80	13	18
2	145	26	23	23	26	139	70	13	18
3	146	30	23	20	27	194	193	14	16
4	200	35	27	23	29	173	136	15	21
5	140	28	26	27	31	141	166	16	18
Mean	151	29	23	23	27	155	129	14	18

Relative Thromboplastic Activity of Fresh Breast Muscle and Dried Brain
Tissue on a Dry-weight Basis

"Prothrombin Time" in Seconds

Final Concentration of Agent	Dried Brain Thromboplastic Agent		Breast Muscle Thromboplastic Agent (1 1/3 X Normal Strength)	
	Normal Blood	Vitakin K Deficient Blood	Normal Blood	Vitakin K Deficient Blood
Agent Diluted with Saline				
100%	17	32	27	66
10%	21	48	40	123
1%	40	177	62	246
0.1%	74	328	87	6'
0.01%	86	6'	91	6'
"Calcium Time" with Saline	87	6'	92	6'
Agent Diluted with Lecithin-Saline				
100%	22	81	37	85
10%	51	202	81	240
1%	90	326	108	318
0.1%	98	345	112	328
0.01%	104	350	112	338
"Calcium Time" with Lecithin-Saline	103	340	108	324

TABLE 11:1

Experimental Diets - Experiment XI

Ingredients Common to All Rations

As in Table 3:1, 0.5% ground corn subtracted.

Composition of Individual Rations in Pounds:

Ration	Premix (as above)	Supplement
1	23	2# Cerelose
2	23	2# Cerelose + 57 gm. Cholic Acid.
3	23	2# Cerelose + 57 gm. Desoxycholic Acid.

TABLE 11:2

Mean "Prothrombin Time" - Experiment XI

(Figures in parentheses indicate number of birds tested)

Group	Supplement to Basal Ration	"Prothrombin Time" (Seconds	
		Breast Muscle Agent	Brain Tissue Agent
1	None	24 (8)	32 (8)
2	Cholic Acid	29 (9)	42 (9)
3	Desoxycholic Acid	32 (8)	45 (8)
	Control Bird	20	24

TABLE 11:3

Analysis of "Prothrombin Time" Variance - Experiment XI

Brain Tissue Thromboplastic Agent

Source of Variation	SS	df	Variance	"f"
Total	2413	24		
Treatments	809	2	404	5.53 *
Error	1604	22	73	
		5%	1%	
L.S.D. Treatments		10	15	

Breast Muscle Thromboplastic Agent

Source of Variation	SS	df	Variance	"f"
Total	842	24		
Treatments	275	2	138	5.31 *
Error	567	22	26	
		5%	1%	
L.S.D. Treatments		6	9	

% of Phospholipid Solution or of Saline Added to Thromboplastic Agent
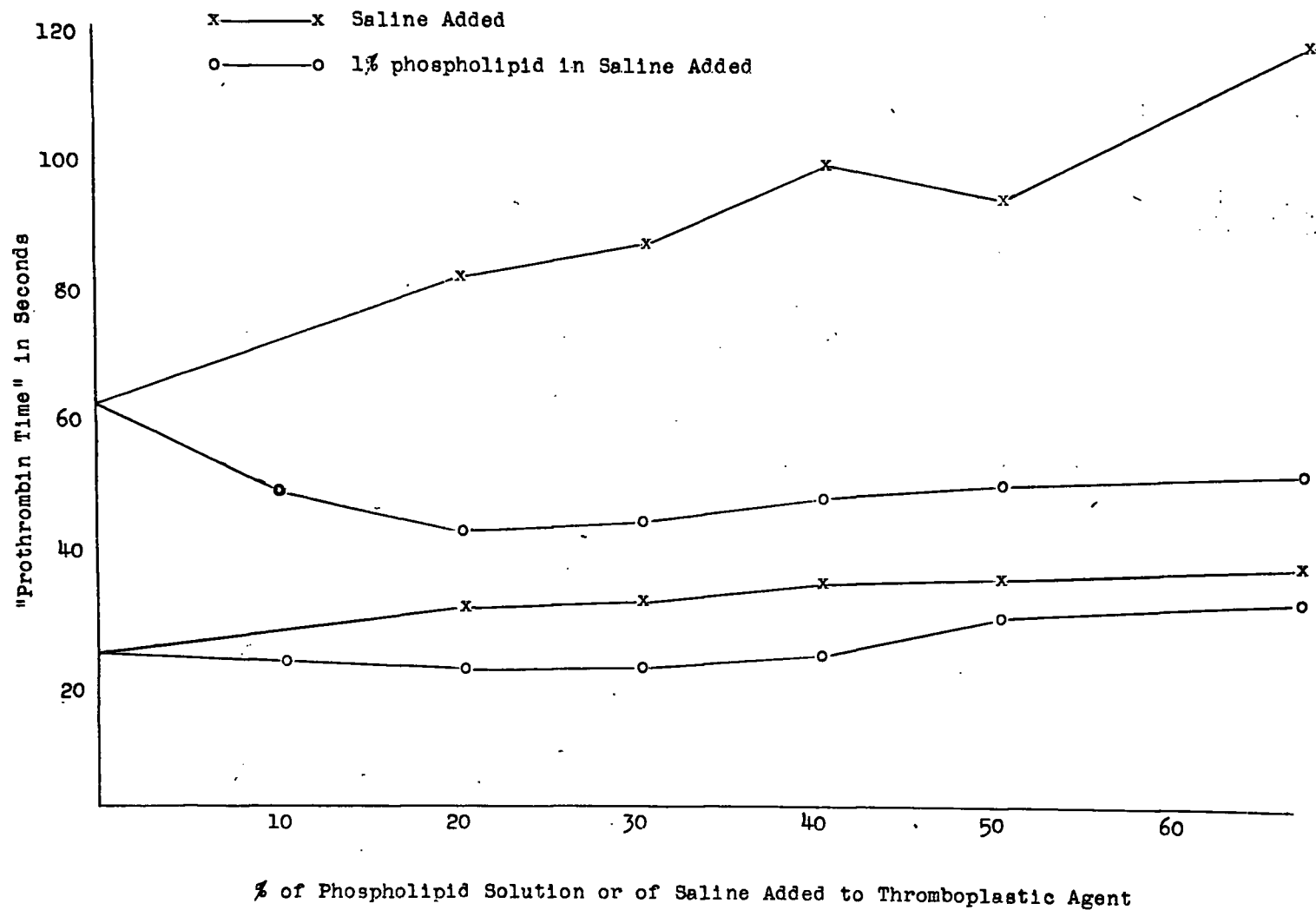
FIGURE 1. Effect of phospholipid on breast muscle thromboplastic agent.

"Prothrombin time" of blood from normal bird (lower curve)
and vitamin K deficient bird (upper curve).
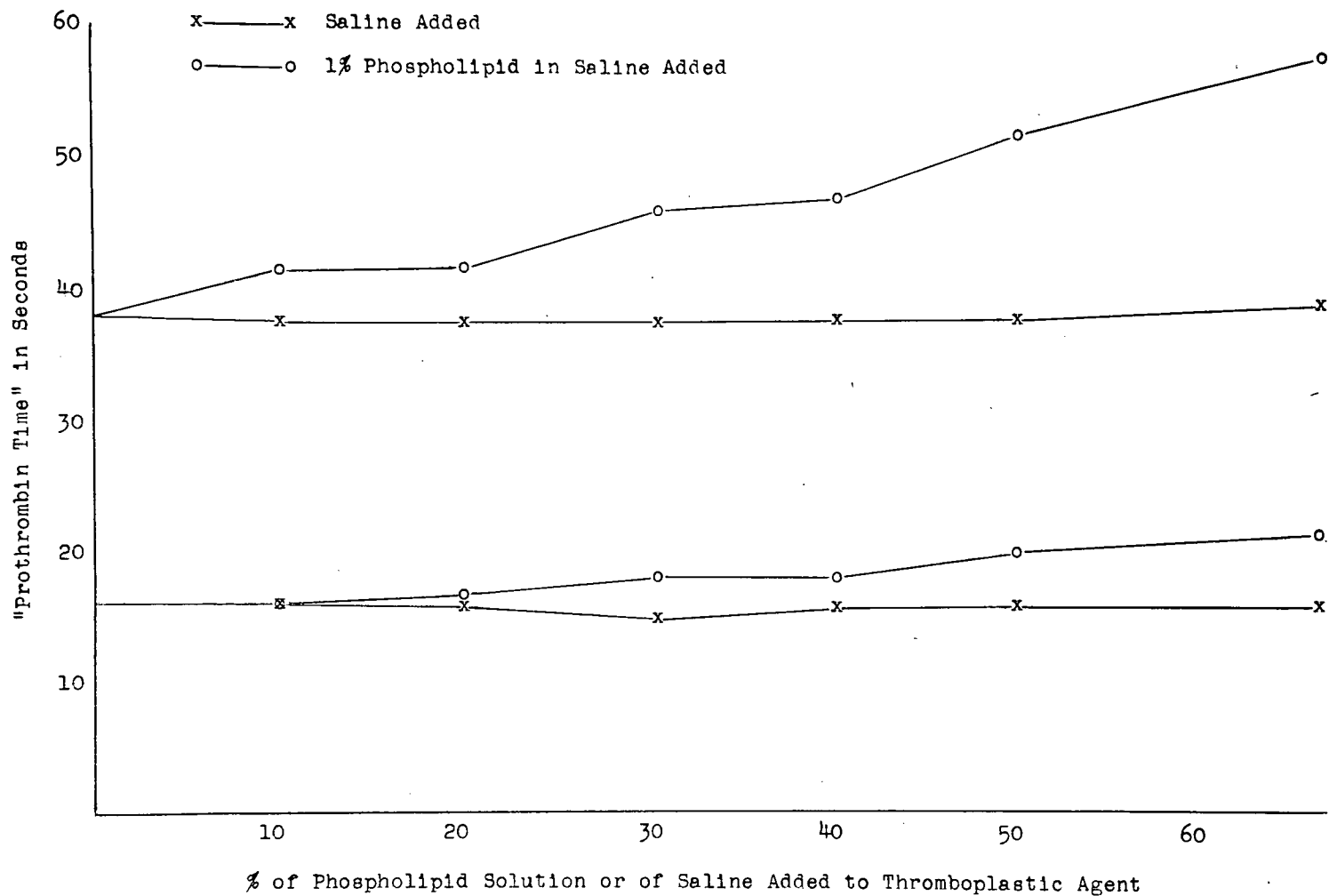
FIGURE 2. Effect of phospholipid on brain tissue thromboplastic agent.

"Prothrombin time" of blood from normal bird (lower curve)
and Vitamin K deficient bird (upper curve).
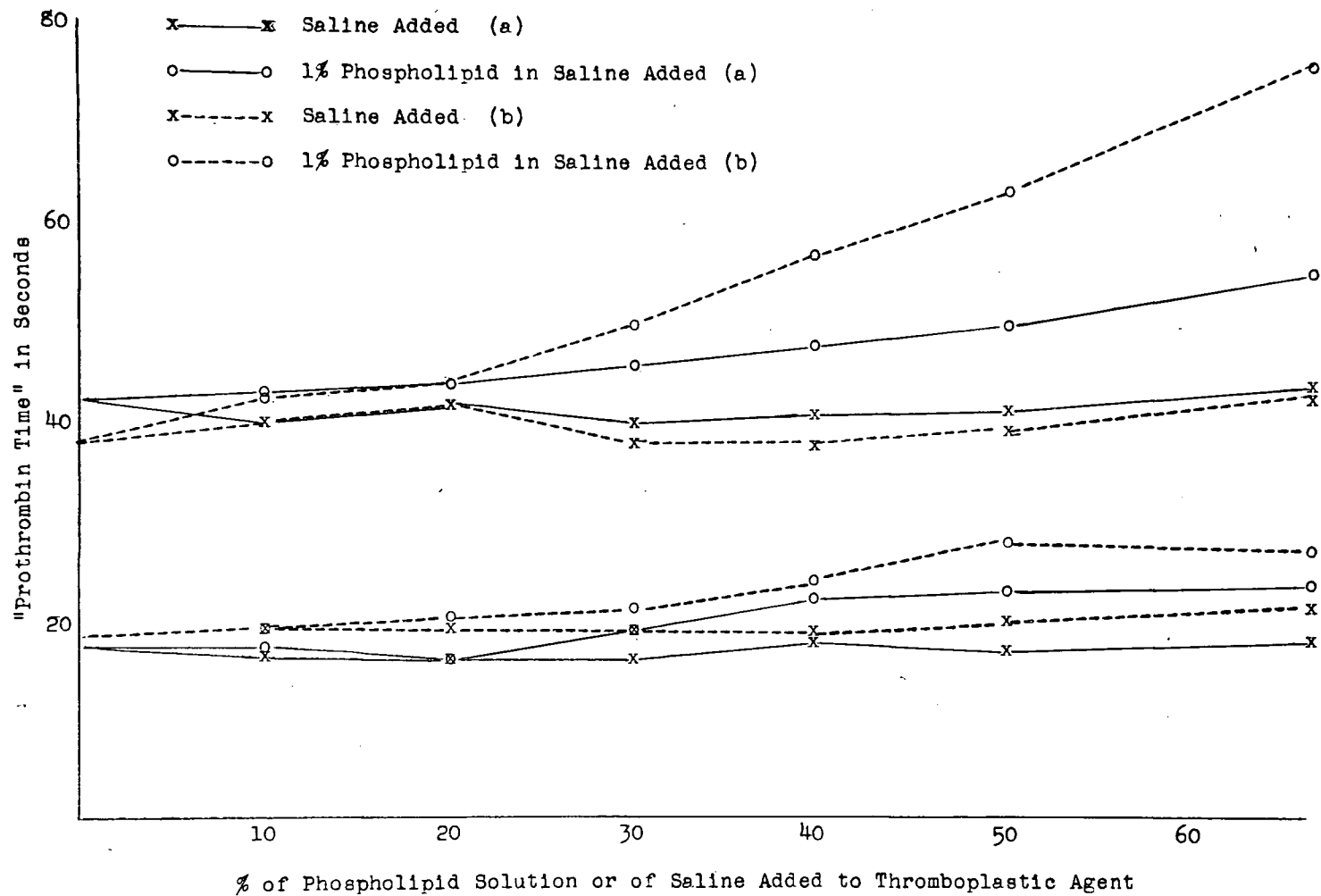
FIGURE 3. Effect of method of calcium addition on response
of brain tissue thromboplastic agent to phospholipid.

"Prothrombin time" of blood from normal bird (lower curves)
and vitamin K deficient bird (upper curves). (a) Calcium
added to blood. (b) Calcium added to thromboplastic agent
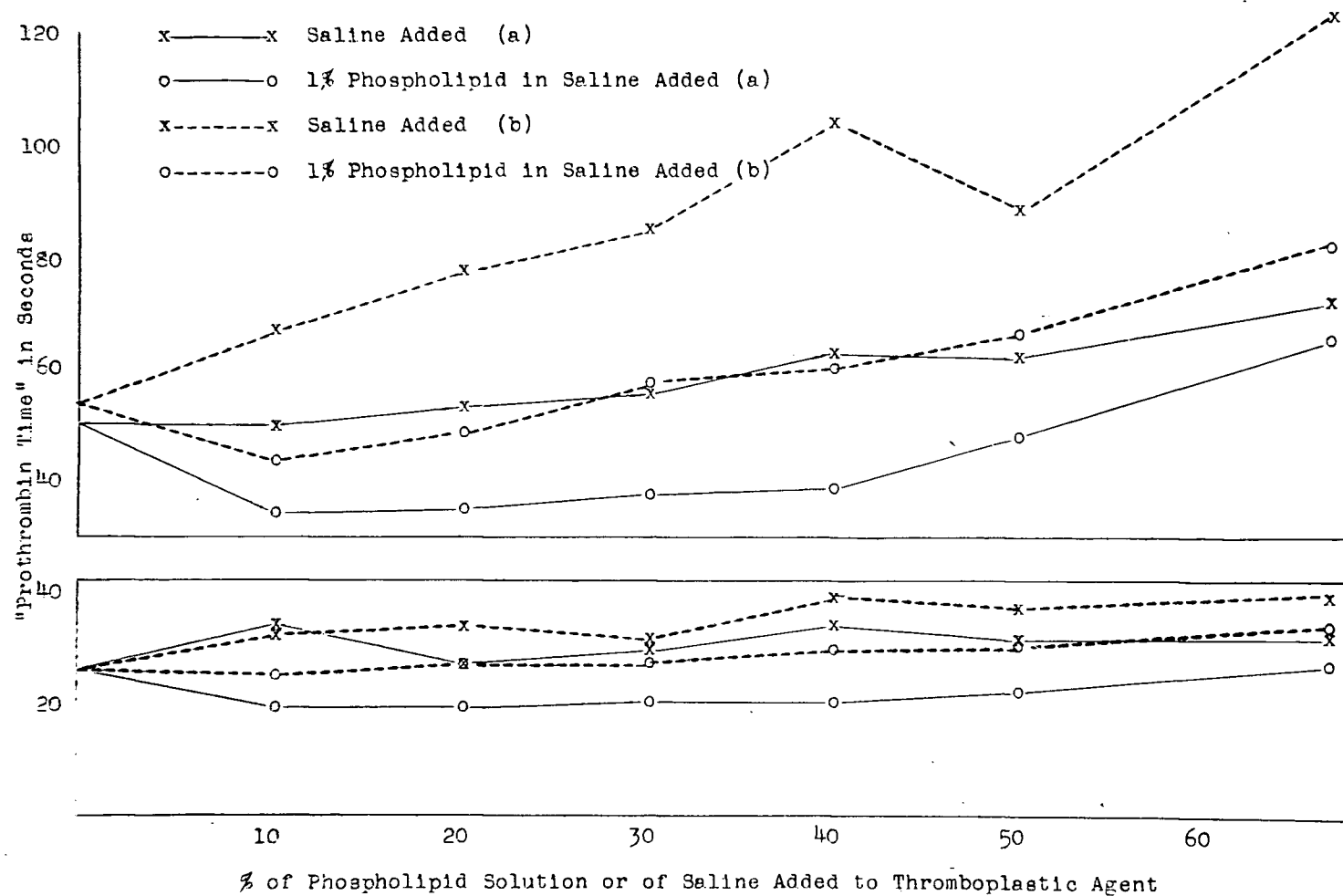
FIGURE 4. Effect of method of calcium addition on response
    of breast muscle thromboplastic agent to phospholipid.

"Prothrombin time" of blood from normal bird (lower curves)
and vitamin K deficient bird (upper curves). (a) Calcium
added to blood. (b) Calcium added to thromboplastic agent.

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