STUDIES ON THE CHICK ASSAY FOR VITAMIN D AND SOME OBSERVATIONS ON CHEMICAL METHODS FOR ITS DETERMINATION

by

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A Thesis

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CLAIMS TO ORIGINAL RESEARCH

1. Comprehensive statistical comparisons of four criteria of calcification in the chick assay for vitamin D have been undertaken.
2. Comparative studies of diets, based on the A.O.A.C. diet with varying amounts of calcium and phosphorus, have been made. The curative feeding period has been compared with the preventive period using several diets. Further studies have been carried out on the effect of breed of chicks.
3. The validity and advantages of the calculation of error from the variation of replicated group means has been established.
4. The sources of variability in the response of chicks over a period of time have been studied and suggestions made for a more reproducible assay.
5. From a consideration of the above factors a modified procedure employing appropriate statistical criteria, has been proposed.
6. A comparison has been made of four methods for the chemical estimation of vitamin D. Some sources of error have been pointed out and suggestions have been made for more critical study. One method has been made much more sensitive.

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THE CHICK ASSAY FOR VITAMIN D
AND SOME OBSERVATIONS
ON THE CHEMICAL METHODS FOR ITS DETERMINATION

INTRODUCTION

Knowledge of the importance of a nutritional factor in the etiology of rickets may be said to date from the pioneer work of Mellanby in 1919. The therapeutic value of fish oils for rickets, although recognized for centuries, was shown to be due to a factor quite distinct from vitamin A. The exact identity of the factor, however, was obscured for some time when it was found that sunlight and ultraviolet irradiation also had curative effects. In 1926 accidental discovery of the role of cholesterol and related sterols made it possible to conclude that sunlight and cod liver oil cure rickets because sunlight produces in the skin the same (or a similar) vitamin which cod liver oil provides ready made.

Livestock confined under artificial conditions have a high requirement for the antirachitic factors. This is especially true for poultry and it was demonstrated as early as 1922 by the Wisconsin group that chicks could be kept healthy in confinement much longer when fed fish oils. Because of the long winters in Canada the use of vitamin D for poultry is of prime importance. It

has been responsible for changing the industry from a seasonal to an all year basis. Research has shown that various forms of vitamin D differ widely in their antirachitic potency when fed either to the same animal or to different species. Thus vitamin D_3 is approximately equal in activity to vitamin D_2 when fed to the rat, but for the chick vitamin D_3 may be 100 times as potent. This makes it necessary to employ different assay techniques for mammals and poultry. This thesis concerns only vitamin D as applied to poultry.

It is interesting to note that in spite of the volume of research which has accumulated on vitamin D, comparatively little is known regarding its fundamental action. Possibly less is known of its chemistry particularly in relation to its estimation. As the biological method is the final criterion of vitamin D activity its lack of precision and other disadvantages has delayed the development of chemical methods. The importance of biological research in this field is, therefore, twofold.

Accordingly, studies have been undertaken on the biological estimation of vitamin D with chicks with a view to developing new and simpler techniques and improving existing ones. Preliminary investigations have also been made of chemical procedures. It is the purpose of this thesis to report the results of these studies.

PART I

THE CHICK ASSAY FOR VITAMIN D

REVIEW OF LITERATURE

The vitamin D potency of a preparation is determined by comparing the calcification of animals fed this unknown preparation with those fed a known vitamin D standard. Up to 1928 the rat was used exclusively as the experimental subject. Calcification was assessed by the ash per cent of the tibia or femur, or by the width of the zone of recalcification in rachitic tibia or femur as determined by the line test or by X-ray diagnosis of these areas. About this time Dr. Carrick of Purdue University found that a solution of irradiated ergosterol (D_2) produced no healing of rickets in chicks although it was active for rats, (Bills, 1935). This observation was checked by others, e.g., Massengale and Nussmeier (1930); Hess and Supplee (1930). From the results of this work it was definitely established that activated ergosterol was comparatively inactive for the chick. It was also evident that any vitamin D supplement intended for poultry would have to be tested on poultry to determine its activity for that species. A test with chicks was the most feasible solution.

The problem of finding the best and simplest criterion of calcification then arose. In 1931 Hart, Kline and Keenan proposed

a method based on the use of the ash per cent of tibia. Subsequently various other criteria were suggested, including radiographic methods, body weight increase, and per cent ash of toes.

(a) METHODS

1. Tibia Ash

The first reported work on this method was that of Hart, Kline and Keenan (1931) who proposed a diet for the production of rickets in chicks. This ration is very similar to that recommended by the present A.O.A.C. (1940) tentative procedure. They used the ash per cent of the dry fat-free tibia as the criterion of calcification. Griem (1932), the associate referee of the A.O.A.C., proposed a preventive method for the detection of cod liver oil in feed mixtures. The unknown oil was fed at levels of 1/8, 1/4, 1/2 per cent and the calcification of the tibiae compared with that of other chicks fed a cod liver oil of known potency. The chicks were fed in groups of six or more for five weeks when they were killed and the tibiae dissected, cleaned, extracted and ashed.

Griem in 1934, after further experience with this procedure, suggested that it be officially recognized by the A.O.A.C. As a result of collaborative studies, e.g., Lachat (1935, 1936a), Griem (1938), considerable standardization of technique was effected in the various laboratories with the result that the method became more reliable. Following a detailed study of the variability of day old to four week old chicks Lachat (1936b) recommended that ten chicks

per group was sufficient and that group ashing could be practised. Slight modifications were made (Griem, 1936, 1937b) and in 1937 it was recommended (Griem, 1937a) that one group be reserved for negative control purposes and one or more additional groups for each oil to be assayed. However, judging by the variability between laboratories (Griem, 1938) and the variation between individual chicks (Griem, 1939a) it was evident that enough chicks were not being used; nevertheless, the A.O.A.C. (1940) method still specified a minimum of only one group of twenty chicks on each oil. Unless more groups and dosage levels are used it is of course impossible to predict with any accuracy the potency of the unknown.

To insure that all chicks receive the same amount of vitamin D, Grab (1936) dosed them individually daily with the vitamin contained in 0.1 ml. oil. McChesney and Homburger (1940) also dosed their chicks individually. In 1940 the A.O.A.C. procedure with the addition of several refinements was adopted as an alternative method by the British Standards Institution. At the present time its wide use seems to be its chief advantage. It is subject to many errors, particularly the human one in manipulation. It is time consuming and, therefore, costly. It is, however, as specific as any method used at present.

2. Radiographic Technique

In 1932 Schroeder reported results of studies on the vitamin D assay using an X-ray technique. The X-ray photographs of the tibia-tarso-metatarsal joint were compared with a series of standard

pictures which were coded numerically.

Grab (1936) also used X-rays to assess calcification and rated his photographs with plus signs. Halvorson and Lachat (1936) and Lachat (1937) used a somewhat similar system but based it on the density of the leg bones. All these methods have the disadvantage of the human element in rating the photographs according to the standards.

Olsson (1936) proposed a radiographic method in which the actual distance between the tarsal and metatarsal elements (t.m.t. distance) is measured in millimeters. This permits more readily the application of statistical procedures for the estimation of potency and error of the assay. Minor improvements were subsequently reported, (Olsson 1939a, b). In 1941 (but not available until September, 1945) Olsson issued a most comprehensive review of all research up to that time including also much new work concerning the estimation of vitamin D with chicks and discussed in detail all phases of the radiographic method.

Olsson's method was recommended as an alternative criterion to the tibia ash by the British Standards Institution (1940). It was compared with several other tests by McChesney et al. (1940) and found to give similar results. Jones and Elliot (1943) have studied this method in conjunction with the growth method. Baker and Wright (1940) and Olsson (1941) showed that this method yields substantially similar results to those of the tibia ash method.

However, the former authors record the results of only one test.

Where individual data are required for studying problems such as the variability of chicks, the advantage of the radiographic procedure is obvious. It eliminates much of the variation in manipulation and is more rapid.

3. Chick Growth

In many laboratories records of chick weights have been kept as a check on assays. Some laboratories have recommended the use of body-weight for the calculation of the results of an assay; e.g., Jones and Elliot (1943) and Carver et al. (1939). Jones and Elliot claimed it was equally as good as the X-ray technique and somewhat simpler but Lachat (1936b) did not support this claim. The chief criticism of the method seems to be its lack of specificity, for growth is affected by so many factors. The varying results obtained in different laboratories are undoubtedly due to the type of diet fed, for it has been shown in this laboratory (Migicovsky and Emslie, 1941a) that greater growth may or may not be obtained on an increased vitamin D intake depending on the calcium and phosphorus content of the diet.

4. Toe Ash

More recently Baird and MacMillan (1942) have suggested the use of toe ash as a criterion of calcification. It also has certain advantages over the tibia ash method and for obtaining composite group data is probably the simplest of all methods. Baird

and MacMillan suggested extracting and drying the toes as was done with tibia. Some time after the present work was begun Evans and St. John (1944) and Evans and Carver (1944) substantiated the work of Baird and MacMillan and showed that either the extracted or unextracted toes could be used. In all these papers the number of chicks used was small and no systematic attempt was made to evaluate the methods statistically. Since part of this work was completed, a more critical comparison has been made by Bliss (1945). He obtained a high correlation between tibia and toe data and found that toes were equally as reliable as tibia in the vitamin D assay.

5. Other Methods

Various attempts have been made to apply the rat line test to chicks, e.g., Carver, Heiman and Cook (1939); Lachat and Halvorson (1937); McChesney et al. (1940), but it has never met with wide acceptance. Probably the chief difficulty with this method was that there is no definite line of recalcification formed in the chick tibia as there is in the rat and consequently measurement may be difficult and unreliable.

The calcium and phosphorus content of the blood, according to Lachat and Halvorson (1936c), and Lachat (1937) were not reliable criteria of the antirachitic activity of vitamin D supplements. The range between negative and positive controls was too narrow.

The possible use of radioactive strontium in the assay has been suggested by Weissberger and Harris (1942). The amount absorbed, which was the criterion, was determined by difference between the

amount fed by stomach tube and the amount excreted. Vitamin D promoted retention of strontium in a manner similar to that of calcium. It is doubtful if this could be readily adapted to routine assays.

The role of phosphatase in calcium, phosphorus and vitamin D metabolism has been studied by Auchinachie and Emslie (1934). Its use for the estimation of vitamin D in chicks has been suggested by Motzok (1945). However, as phosphatase is affected by many factors other than vitamin D level and is more tedious than other available methods it is doubtful if the method will find wide acceptance.

(b) FACTORS AFFECTING THE RESULTS OF CHICK ASSAYS

1. Treatment of Tibiae

In the original paper by Hart et al. (1931) the chick tibia were dissected, cleaned of adhering flesh, extracted, dried and ashed at 500° C. until white. In collaborative assays (Lachat, 1937) it became evident that there was considerable difference between laboratories in their treatment of the bones and suggestions were made for standardization of technique.

It has been suggested (St. John et al., 1933; Harshaw, Fritz and Titus, 1934; Johnston, 1942 and Fritz and Halloran, 1943) that it would be simpler in cleaning tibia to remove the cartilage caps. It would also save time. Whether this procedure would greatly improve the accuracy is doubtful and has never been taken up by the A.O.A.C. The removal of the cartilage would increase the

per cent ash in the bone (Bethke and Record, 1934). Lachat (1935) observed that the removal of flesh from the bones was facilitated if they were boiled in water for a short time. In 1939 the A.O.A.C. (Griem) recommended two minutes but Motzok and Hill (1943) showed that one minute was the maximum allowable without influencing the per cent ash. Using undissected tibiae Fritz and Halloran (1943) claimed four minutes boiling had no effect. The lack of agreement was partly due to the degree of dissection used.

Many different methods of extraction have been followed. Lachat (1935) found alcohol and ether extractions to be the most efficient although Bethke and Record (1934) claimed alcohol alone was as good as the two solvents. In later work Lachat (1937) confirmed his earlier conclusions. The use of alcohol and ether was also recommended by the work of Dustman (1937) and Motzok et al. (1942). Fritz and Halloran (1943) pointed out that carbon tetrachloride might break up and yield free HCl under certain conditions and consequently would be unsatisfactory.

As to whether or not bones should be crushed seemed to depend on the length and thoroughness of extraction. Crushing has been recommended particularly by Lachat (1937) and Fritz and Halloran (1943) but found unnecessary by Motzok and Hill (1943), substantiating data of this laboratory (Migicovsky and Emslie, 1941b). The storage of bones by freezing was not recommended by Lachat (1935). He suggested that, after cleaning, the bones

be held in alcohol. Motzok and Hill (1943) found that the bone ash per cent was affected by freezing.

In studying ashing technique Lachat (1937) concluded that ashing at 600° C. for 18 hours was about the same as 800° for 1 to 2 hours. Motzok and Hill (1943), found 850° for one hour to be satisfactory.

Fritz and Halloran (1943) in an attempt to speed up the tibia ash procedure suggested that extraction and drying be eliminated and that the green bones be ashed directly. This modification offered no great advantage when toes could be used with considerably less trouble.

2. Treatment of Chicks

Relatively little definite knowledge exists concerning the exact effects of such factors as temperature, lighting and moisture content of the air. Ideally temperature and moisture should be under control and particularly be uniform throughout the laboratory for all groups of an assay. It has been suggested that temperature may influence calcification (Titus 1943). If this is the case temperature variations could cause positional differences.

Frosted bulbs are probably the most satisfactory source of light for vitamin D assay, although the only indication of this is the note by Wilgeroth and Fritz (1944). Some laboratories have used low illumination or blue lights. Other laboratories, however, use ordinary unfrosted bulbs. Undoubtedly equal illumination of all

groups is important especially for feeding. A 12 hour illumination day is probably most satisfactory.

Titus (1943) has recommended that young chicks be taught to eat at once so that possible variations in feed and vitamins consumed may be eliminated as far as possible. No data are available to judge the advantage of such a procedure.

The control of feed intake in chick assays would be desirable but is rather difficult to accomplish. No data exist on the possible effect of this factor. Under present conditions of chick assays this could only be applied to groups and not to individuals. How much improvement this would effect is difficult to estimate.

3. Sex of Chicks

The effect of sex of chicks on their state of calcification was one of the first factors studied. Holmes et al. (1932) found that at six weeks of age the ash, calcium and phosphorus per cent of female chick tibiae were definitely higher than those of the tibia from male chicks. At three weeks of age this difference was only slight. Using X-ray technique on ten week old chicks Schroeder (1933) concluded that if the calcification of the female metatarsus was 100 that of the male would average 81. For bone ash the difference would not be as great, i.e., 100 to 96. The difference is explained by the existence of a different rate of calcification in the tibia and metatarsus.

Lachat (1934, 1936a, b) and Lachat and Halvorson (1936a) found differences in calcification as judged by per cent ash between male and female chicks. This effect appeared to vary with the degree of rickets, i.e., males had higher bone ash at severely rachitic levels while the reverse was true as the ration approached normality. It is doubtful if sufficient numbers of chicks were used to allow definite conclusions to be made. Lachat (1936b) concluded that in chicks up to three or four weeks of age this difference was not great and could be disregarded. This conclusion was substantiated by Olsson (1941) with X-ray data and by Migicovsky and Emslie (1941b) in this laboratory with tibia ash data.

4. Weight of Chicks

Griem (1939b), in an outline of the A.O.A.C. method, recommended that chicks below the weight of 100 gm. be eliminated from final calculations. At the time it was thought that this would reduce variability as small chicks may not reach the same degree of rickets as normal ones. Knudsen and Tolle (1940) studied this problem on collaborative data and found that in some laboratories small chicks gave a higher per cent ash while in other laboratories there was no difference. Following a statistical study by Loy et al. (1941a) it was decided to include all chicks in assays regardless of size.

Olsson (1941) also noted that the t.m.t. values varied with weight when the weights of the groups were equalized at the

beginning of the curative period. He found that with this diet the mean weight was lower than normal in groups on severe vitamin D deficiency and their t.m.t. values somewhat less than they would be if their weight were normal. He corrected for this by using the formula:

$$\frac{3Wm \cdot t.m.t.}{2Wm + Wo}$$

where Wm = mean weight for normal groups

Wo = observed weight.

5. Rachitogenic Diets

Table 1 outlines the composition of the chief rachitogenic diets used in vitamin D assays with chicks.

The diet proposed originally by Hart et al. (1931) is still used by the A.O.A.C. with only minor changes. Lachat et al. (1932) studied a series of thirteen rachitogenic diets and concluded that the Wisconsin diet was most satisfactory, although only one chick in ten was alive at the end of five weeks. Loy et al. (1941b), Baird and Barthen (1941) and very recently Bliss (1947) tested modified rations which were considered more complete than the A.O.A.C. diet, but the advantages of the new modifications were not great. The position of the response curve may have changed but the precision was not affected significantly.

Hunter et al. (1932) found that the addition of vitamin A to a rachitogenic diet had no effect on the calcification. Minor calcium and phosphorus variations of the basal diet as might occur

TABLE 1

Rachitogenic Diets

(percentage composition)

Ingredients	Hart Kline Keenan (1931)	A.O.A.C. (1940)	Massengale Bills (1936)	Olsson (1939)	B.S.I. (1940)
Corn	59	57	56	36	35
Middlings	25	25	--	--	23
Casein	12	12	--	--	--
Ground Wheat	--	--	--	30	--
Bran	--	--	10	--	16
Meat Meal	--	--	--	--	10
Linseed Oil Meal	--	--	10	--	--
Wheat Gluten	--	--	10	--	--
Skim Milk Powder	--	--	9	10	8
Soy Bean Meal	--	--	--	16	--
Grd. Alfalfa	--	--	--	6	5
Salt (Iodized)	1	1	1	1	0.5
$CaCO_3$	1	--	2	--	--
$Ca_3(PO_4)_2$	1	2	--	--	--
Dried Yeast	1	2	--	--	--
Oil	--	1	2	0.5	1
$MnSO_4$	--	0.2 g./kilo.	--	--	--
Charcoal	--	--	--	0.5 *	--
Bonemeal	--	--	--	--	--
Limestone	--	--	--	--	1.5
Calcium	0.9-1.0	0.7-0.8	1.0	0.3 *	1.6
Phosphorus	0.5-0.6	0.7-0.8	0.5	0.4	1.0

* Add 4.4% bonemeal to make Ca = 1.6%, P = 1.0%.

with different lots of feed were found (Griem et al., 1935 and Supplee 1935) to have no effect on the results of assays. It has been shown in this laboratory (Migicovsky and Emslie 1941a) that the calcium and phosphorus content of the diet and also the ratio of calcium to phosphorus may at certain levels have a considerable influence on the difference between negative and positive controls. At other levels this influence will not be nearly so marked. Within practical feeding limits the level of vitamin D in the hen's ration does not affect the per cent ash in the bones of chicks hatched from eggs produced by these hens, (Kline et al., 1935). Motzok et al. (1943) found that different types of yeast may cause wide variation in growth and calcification of chicks. Fritz and Halloran (1943) suggested that the vitamin B complex may be a factor influencing calcification.

Olsson (1941) lists a series of diets which are quite similar in calcium and phosphorus content. The composition of a typical one is given in Table 1. For the depletion period he uses a diet containing 0.3 to 0.5 per cent of calcium and phosphorus. In the curative period the calcium and phosphorus is increased to about 1.6 and 1.0 per cent, respectively, by adding mineral supplements. By the use of these diets he claims to be able to get more complete and rapid depletion than would be otherwise possible.

The question of diet becomes of considerably more importance when assaying feed mixtures for vitamin D potency. Theoretically it would be most satisfactory to extract the vitamin with a

solvent, then feed as an oil in the usual manner. Griem (1935) pointed out that this may not be feasible and recommended a dilution method. It is obvious, however, that whatever method is used the calcium and phosphorus contents of the diets for the reference and unknown oils must be the same.

6. Length of Feeding Period.

The length of feeding period has been subject to change ever since Hart et al. (1931) recommended a five week preventive period for their tibia ash method. Lachat et al. (1932) showed that the four week period was as reliable as the five week period and later (Lachat, 1936b) recommended that a three week period be used. This was adopted by the A.O.A.C. the next year (Griem, 1937b) and incorporated in the latest tentative official method (A.O.A.C., 1945). De Witt et al. (1942) have since suggested the possibility of a two week assay period. Massengale and Bills (1936) used a two week depletion and a four week curative period but this method was not put into general use. Carver et al. (1939) also used a curative method but cut the time to one and three weeks. None of this work, however, represents an intensive study of all factors concerned and the adoption of the three week period has been largely a matter of convenience.

In Europe Olsson (1936) studied the calcification of chicks and concluded that a six week period was most satisfactory. Later, Olsson (1939a, b) proposed a curative method in which the

chicks were fed a very low mineral diet (Ca and P = 0.3 - 0.5%) for the first week and then for the remaining three weeks minerals and vitamin D were added. The calcium and phosphorus content of the final diet approximates 1.6 and 1.0 per cent, respectively. In his comprehensive paper in 1941 he suggests that by using this system of feeding it is possible actually to reduce the curative period to two weeks without sacrificing the accuracy to any appreciable extent.

In adopting Olsson's procedure the B.S.I. (1940) use one feed throughout the assay, i.e., one containing about 1.6% calcium and 1.0% phosphorus. They use a three week curative period following the one week depletion. Employing their technique, Jones and Elliot (1943) compared four, five and six week old chicks and found that as judged by fiducial limits the four week chicks, i.e., three week curative period, were the least variable. The difference was not significant.

Baird and Barthen (1941), using the A.O.A.C. diet, tested the relative merits of the 3 week preventive and 2 week curative types of assay but were unable to show any difference.

7. Dose-Response Relation of Vitamin D

The relation between the dose and response has been variously interpreted by different authors. In America, chiefly because of inadequate statistical interpretation, it has been common to use the usual dose-response relation, e.g., Fritz and Halloran (1943),

Evans and St. John (1944) and Carver, Heiman and Cook (1939). However, Knudsen and Tolle (1940) using collaborative data point out that the curve is actually exponential and the log. (dose) relation should be used. This was substantiated by Homeyer (1943) using data from another collaborative assay (Baird and Barthen, 1941). The A.O.A.C. (1940) make no recommendation in this regard.

Olsson (1939a) showed that the relation between log. (dose) and response was linear in the t.m.t. method. Baker and Wright (1940) found that the use of the log. (dose)-log. (response) curve was more satisfactory. It is this system that was adopted by the B.S.I. (1940) for the radiographic data. There seems no doubt that the log. (dose) relationship should be universally used.

It has been generally understood in chick assays as in rat assays that the standard should be administered to chicks at each assay. However, Massengale and Bills (1936) used a master reference curve and more recently Bliss (1945) has shown that for his laboratory it is possibly more accurate to use the combined slope of a series of reference curves. Whether these principles will find wide support remains to be demonstrated.

8. Biological Variation and Statistical Interpretation

Bliss (1941) has stated that a determination of potency in a biological assay should always include an estimate of its error and that no assay with an indeterminate error can be considered satisfactory. There is no question of the soundness of these statements.

As ordinarily determined and understood the error of a biological assay is generally calculated from the variance between individuals receiving the same treatment. Massengale and Bills (1936) and Oser (1939) among others have attempted to estimate the variation in this way. However, in comparing assay procedures and similar work the slope of the response line is of equal importance and should have been considered by these authors. As Tolle (1940), Knudsen and Tolle (1940) and Baird and Barthen (1941) have shown, there is also considerable variation between groups receiving the same level of vitamin D. All laboratories have experienced the inability to reproduce closely the results of assays carried out at different times. Many collaborative experiments carried out by A.O.A.C. and Animal Vitamin Research Council (A.V.R.C.), e.g., Baird and Barthen (1941) have shown that between laboratories there is a wide variation even when using feed and oils from the same lot.

In view of the importance and nature of the variability in chick assays it is not surprising that much of the work recorded above is more or less contradictory. A great deal of research has been carried out with as few as six chicks in a group and only one group on a treatment. The experiment was probably carried out only once. The hope of another laboratory duplicating such results may be rather remote and the feasibility of drawing general conclusions from such isolated experiments is extremely questionable. Data obtained by the writer have shown that by using as many as seven assays it has been possible to prove statistically that one procedure

yielded higher results than another whereas actually no difference existed on the examination of a large number of assays. The sampling error in experiments of this type is extremely large and must be reckoned with when general conclusions are to be drawn.

The sources of this variation are many and varied. Possibly the greatest source of variation is that inherent in the chicks themselves, (Baird and Barthen, 1941). O'Neil (1941) has shown a family difference in the calcification of chicks and later (O'Neil 1944) that variations in the ash content of the tibia and toes of day old chicks were not correlated. Sex is not likely an important factor in three week old chicks, (Lachat, 1934). Motzok et al. (1943) have pointed out that Leghorns may be more variable than other chicks and suggested that crosses of inbred lines be used. The lack of inbreeding in chicks as compared with rats is undoubtedly an important factor but definite conclusions cannot be drawn from these preliminary experiments. There is also the possibility of strain differences within breeds. In tibia ash there is the human element in dissection. Lachat and Halvorson (1936b) have demonstrated a seasonal variation in the response to vitamin D.

The widespread occurence of variation necessitates some expression of it in assays. In a very comprehensive study of a collaborative experiment Homeyer (1943) recommends the use of an expression of the error of each assay. In Europe (B.S.I., 1940, Olsson, 1941) somewhat wider use has been made of the estimate of error in chick assays than in America (A.O.A.C., 1940, Knudsen and

Tolle, 1940). However, since this study was completed Waddell and Kennedy (1947b) have proposed a system of statistical analysis.

Olsson in 1939 proposed a system of statistical analysis and recommended a correction for varying chick weight. In 1940 the British Standard Institution published their specification in which validity of the assay could be tested on the basis of the straightness of response lines and their parallelism. If the slope of the log. dose-response curves is not well defined a procedure for calculating the "true" fiducial limits is described. Irwin (1943) has also outlined a similar procedure for the true limits.

Bliss (1940) has published a method of factorial analysis of the rat assay for vitamin D which yields almost the same results statistically as the B.S.I. method. This method is probably simpler than the B.S.I. but all groups must have the same number of individuals. This is often difficult to obtain in chick assays unless replicated group averages are used.

As the fiducial limits are influenced to a certain extent by the difference in potency between standard and unknown they are not entirely satisfactory for the comparison of different assays and response lines. Bliss and Cattell (1943) have suggested the use of the λ ratio, i.e., $s/b = \frac{\text{standard deviation}}{\text{slope}}$. The greater this ratio is the more variable the data and the more unsatisfactory is the response line.

9. Standard of Reference

The U.S.P. reference cod liver oil No. 2 is at present used exclusively in the United States as a standard of reference for chick assay. In Canada the Canadian Standard reference oil which is bottled from a sub-lot of the U.S.P. oil is widely used. The A.O.A.C. chick unit of vitamin D (A.O.A.C., 1945) is equal in biological activity for the chick to one unit of vitamin D in the U.S.P. reference cod liver oil in this method of assay. In Great Britain, (B.S.I., 1940), and Sweden (Olsson, 1941), crystalline D_3 is used as standard. One B.S.I. unit of vitamin D_3 activity is defined as the specific activity of 1 mg. of the provisional standard preparation, i.e., 1 mg. of a solution of pure olive oil containing 0.000025 mg. of crystalline D_3. Olsson (1941) has recommended the use of the "chick unit" (Kükeneinheit) to be the antirachitic activity of 0.01 gamma of pure crystalline D_3.

The advantages of crystalline vitamin D_3 as a standard are obvious. The most important one is that its purity can be determined by exact physical and chemical analyses. Studies have been carried out in this laboratory since 1940 to determine the relative potency of the B.S.I. and A.O.A.C. standards. A summary of the data indicate that the B.S.I. preparation contains approximately 1200 A.O.A.C. units per gram. This would be equivalent to 48 million A.O.A.C. units per gram crystalline D_3. In the U.S. the Animal Nutrition Research Council (formerly Animal Vitamin

Research Council) have also studied this problem. Preliminary results were presented by Fritz (1945) in which a potency of 49 million units was assigned to the crystalline product. More recently further data by this group have been reported by Waddell and Kennedy (1947a). In separate series of assays 54.7, 53.7 and 58.4 million units per gram were assigned to their preparations. Data by Motzok et al. (1947) suggest a value of 52 million units. It seems clear, therefore, that the original value of 40 million is too low. Some of the differences in the estimates are undoubtedly due to differences in the purity of the samples as well as biological variation.

There is also the problem of a species difference in the reaction to the vitamins of fish oils and crystalline D_3. For example Boucher (1944) and Bird (1944) have shown that turkeys do not react equally to these products when fed on the same chick unit basis. Singsen and Mitchell (1945) indicate that under certain conditions chicks may not react in the same way to the two sources of vitamin.

Possible disadvantages in the use of vitamin D_3 are not great and should disappear in a relatively short time. There seems to be some question of stability. In the writer's laboratory the B.S.I. standard has shown in occasional cases a potency of only half that expected. Discussions with other workers, e.g., from Ayerst, McKenna & Harrison, have suggested similar difficulties. Furthermore, the work of Waddell & Kennedy (1947a) suggest that different

preparations of crystalline vitamin D_3 may be slightly different in potency. These difficulties should disappear, however, when more detailed studies are available on the properties of the crystalline material. In addition, the possibilities of some of the esters of vitamin D_3 must not be overlooked.

Considered as a whole, there seems no insurmountable objection, with the possible exception of precedent, for adhering further to the system of units for the expression of potency. The use of actual weights in milligrams or gamma of vitamin D_3 would put vitamin D nomenclature on the same status as the B vitamins. What is more important, it would eliminate any necessity of determining the potency of the pure material in terms of units.

This review would suggest that while there have been many variations of assay procedure proposed, none of them are eminently satisfactory. There is a lack of comparative data on the methods. There is need particularly for a thorough statistical study of the various modifications of most generally accepted methods with a view to determining the most reliable and simple one. There is also need for studying and eliminating sources of error. In view of the scarcity of information on these problems this thesis has been planned to study such factors as relative precision and accuracy of various criteria of response, system of feeding, (i.e., preventive and curative), length of feeding period, effect of diet, and breed of chicks. Particular attention has been given to the statistical interpretation of chick assays and to sources of variation.

EXPERIMENTAL

The oils used for assay in this study were samples handled in the routine work of the Vitamin Laboratory and were representative of those most commonly used in Canadian poultry feeding. In most of the assays the unknown oils were fed to three or four groups of chicks at each of two or three dosage levels while the Canadian Standard Reference oil No. 1 was always fed at three levels. The dosage levels were spaced at equal logarithmic intervals to permit coding in the calculations. Usually twenty chicks were started in each group. The diet, feeding system and preparation of tibiae recommended by the A.O.A.C. (1940) were used unless otherwise stated.

In many assays, the toes as well as the tibiae were removed and X-rays were taken of the tibia-tarso-metatarsal joint. The centre toe was removed at the third joint from the claw and either wrapped in filter paper by groups or tagged individually and put directly into the extractor. These were designated "extracted" toes. "Fresh" toes were weighed in closed weighing bottles and then dried and ashed as usual. The use of weighing bottles could be eliminated by using the dry weight of toes previous to ashing. When the variability of response of individuals was being studied the tibiae and toes were ashed individually; otherwise they were composited in groups and ashed as such.

The method of Knudsen and Tolle (1942) was used to calculate potency from composite data. For individual chick data the

B.S.I. (1940) method of analysis was used to calculate the estimate of potency and its fiducial limits. This was supplemented by the λ test ($\lambda = s/b = \frac{\text{standard deviation}}{\text{slope}}$) of Bliss and Cattell (1943) to study the precision of the assays. In the B.S.I. method where Q^2/P (i.e., regression function) exceeds 60 times s^2 (i.e., variance for error) the calculation of potency is similar to the Knudsen-Tolle method, and the "approximate" formula is used for the calculation of fiducial limits. However, where this criterion is not satisfied, i.e., $Q^2/P < 60s^2$, a correction is necessary for both the estimate of potency and fiducial limits. Under these circumstances, as Irwin (1943) has pointed out, the approximate formula not only underestimates the width of the fiducial range but also biases its position. In the comparison of the four methods described in this paper, because many of the assays using tibia and toes, were calculated from composite data where no correction was possible, the approximate formula was employed for the sake of uniformity even though the statistical criterion was not always satisfied. In the Appendix examples are worked out by the B.S.I. system for individual data and by the Bliss (1940) system for replicate data.

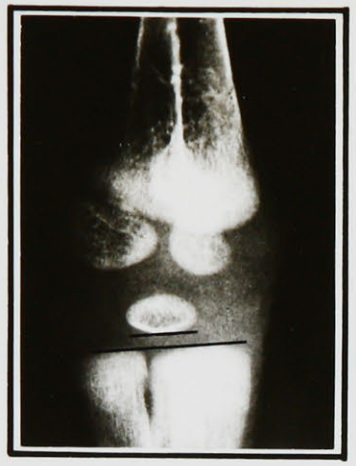
Radiographic Technique

X-ray photographs of the right tibia-tarso-metatarsal joint were taken of all chicks on the day before killing at 3 weeks of age. The radiographs were made on Dupont or Kodak, 5" x 7" no screen, fine grain film with a Victor portable model X-ray apparatus. The cone of the instrument was fitted with a lead sleeve which

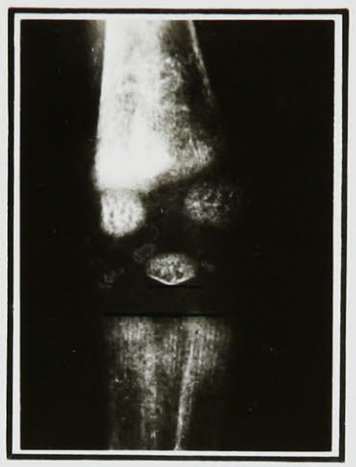
reached almost to the film and protected the operator from excessive radiation as shown in Figure 1. An exposure of one second at 12 inches, 15 m.a. and K.V. position 2 was found to be most satisfactory. The radiographs were taken through a one inch square hole cut in a lead mask under which the film holder was manipulated by means of a special frame. With this equipment it was possible to X-ray up to 300 chicks an hour. The films were examined under a microscope with a 12 x magnification (lower magnification would give equally good results) using a calibrated ocular scale with an extended zero line. This zero mark was lined up across the proximal ends of the two metatarsal bones and the distance of the tarsal bone read on the scale to the nearest 0.06 mm. Prints of radiographs of normal and rachitic chicks are shown in Figure 2, the distances between the lines being t.m.t. distances. Logarithms of the t.m.t. distance were used for all calculations unless otherwise mentioned and to avoid the difficulty of negative entries logarithms were taken of the values multiplied by 10. With practice it was found possible to read 800 radiographs a day with little effort. The suggestion of marking the end of the metatarsal bones with a scalpel under naked eye inspection was not found practicable as it could often be seen under the microscope that the line was not in the correct place.

It was found in preliminary tests that the t.m.t. measurements made by this method were quite reproducible. The results of

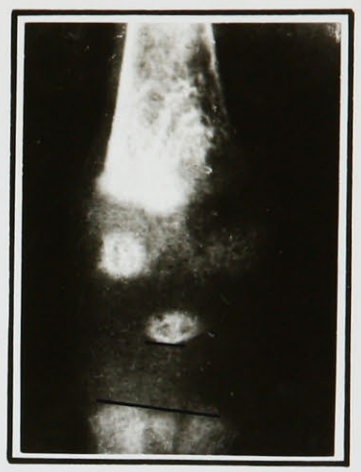
FIGURE 1. Method of X-raying chicks. The lead sleeve is raised to show position of the chick leg.



NORMAL GROWTH



RICKETS



SEVERE RICKETS

RADIOGRAPHIC DEMONSTRATION OF RICKETS

FIGURE 2. Prints of radiographs of tarso-metatarsal (t.m.t.) distance (between lines) from normal and rachitic chicks.

some of these tests are reported in Table 2. It is evident from this table that, considering data from inexperienced readers, there is a maximum difference of about 10 per cent between the results of different readers. There is a difference of 6 per cent between readings of the same person before and after becoming experienced. These differences would be considerably reduced with experience. Data reported in this paper for complete experiments or series of experiments were read by the same person. It was found after much of this work was completed, that reading the X-rays with a microfilm viewer was more satisfactory and much easier than with the microscope.

TABLE 2

Reproducibility of Results by the Radiographic Technique

(Assay 104)

ASSAY PERIOD (WEEKS)	GROUP	INEXPERIENCED				EXPERIENCED	
		Observer R		Observer C		Observer R	
		Potency	Limits*	Potency	Limits*	Potency	Limits*
			Percent		Percent		Percent
2	1-20	117	79-127	106	80-125	113	80-125
2	21-40	88	78-128	--	--	94	75-133
3	1-20	115	83-120	105	82-122	112	83-121

* Approximate limits at P = 0.05.

TABLE 3

A Comparison of Four Criteria of Calcification

ASSAY NO.	EXPECTED POTENCY	POTENCY (A.O.A.C. UNITS) AS CALCULATED FROM				DIFFERENCE AS PERCENT OF TIBIA VALUES		
		Tibia Ash	Toe Ash		Log. t.m.t.	Toe ash		Log. t.m.t.
			Extracted	Fresh		Extracted	Fresh	
84	1200	1835	1957	2066	2689	+ 7	+13	+47
86	2000	3432	4056	4280	3772	+18	+25	+10
87	200	258	281	--	267	+ 9	--	+ 3
88	200	294	379	--	289	+29	--	- 2
89	200	380	424	--	383	+12	--	+ 1
90	400	498	551	540	560	+10	+ 8	+12
91	400	422	481	566	408	+13	+34	- 3
92	400	434	507	536	456	+17	+24	+ 5
93	400	475	478	--	561	+ 1	--	+18
94	400	519	--	--	516	--	--	- 1
95	400	378	--	--	372	--	--	- 2
96	400	248	265	248	242	+ 7	0	- 2
97	400	324	244	232	252	-25	-28	-22
98	4000	3664	2962	2727	3228	-19	-26	-12
99	200	377	377	324	360	0	-14	- 5
100	200	233	211	219	199	- 9	- 6	-15
101	200	214	210	189	202	- 2	-12	- 6
102	200	238	226	211	244	- 5	-11	+ 3
103	200	401	361	318	386	-10	-21	- 4
104	100	102	108	110	112	+ 6	+ 8	+10
105	400	538	536	582	421	0	+ 8	-22
106	200	447	225	342	269	-50	-23	-40
107	400	864	446	587	522	-48	-32	-40
108	200	321	246	308	223	-23	- 4	-31
111	2000	3122	2828	2874	3090	- 9	- 8	- 1
112	1000	1380	1758	1443	1157	+27	+ 5	-16
114	400	373	570	666	444	+53	+52	+19
115	200	224	280	272	247	+25	+21	+10
Means						+ 1.3	+ 0.5	- 3.1

methods, the chief variation being in the estimates of the individual oils. If a mean of all four assays can be considered the most probable estimate of the true potency of an oil, then the most accurate method is the one which will most closely approximate that value, i.e., have the smallest standard deviation. To compare the methods on this basis, means of the estimates of potency by all methods for each oil and the standard deviations of the differences of the four methods from these means were calculated. The standard deviations were 15.0, 11.4, 10.6, and 10.0 per cent for tibia, extracted toes, fresh toes and t.m.t., respectively, while the mean differences were 2.4, 0.2, 0.7 and -3.3. In general then, there seems to be no doubt that the toe ash and X-ray methods yield estimates of potency equally as reliable as those by the tibia ash method. This conclusion substantiates the work of Baird and MacMillan (1942) using a smaller number of chicks.

Data from assay 104 were plotted on semi-log. paper and reproduced in Figure 3 to illustrate the ranges of response of the four criteria of calcification and the similarity of their log. (dose)-response curves. Approximately 45 chicks (3 groups of 15 each) were used to determine each of the points on the graph. To facilitate comparison the scale for the radiographic method has been inverted. The regression lines were calculated from the combined data for reference and unknown oil and the combined decoded slopes are entered in the charts. It is evident that the response curves for all four methods are quite similar and although in this parti-

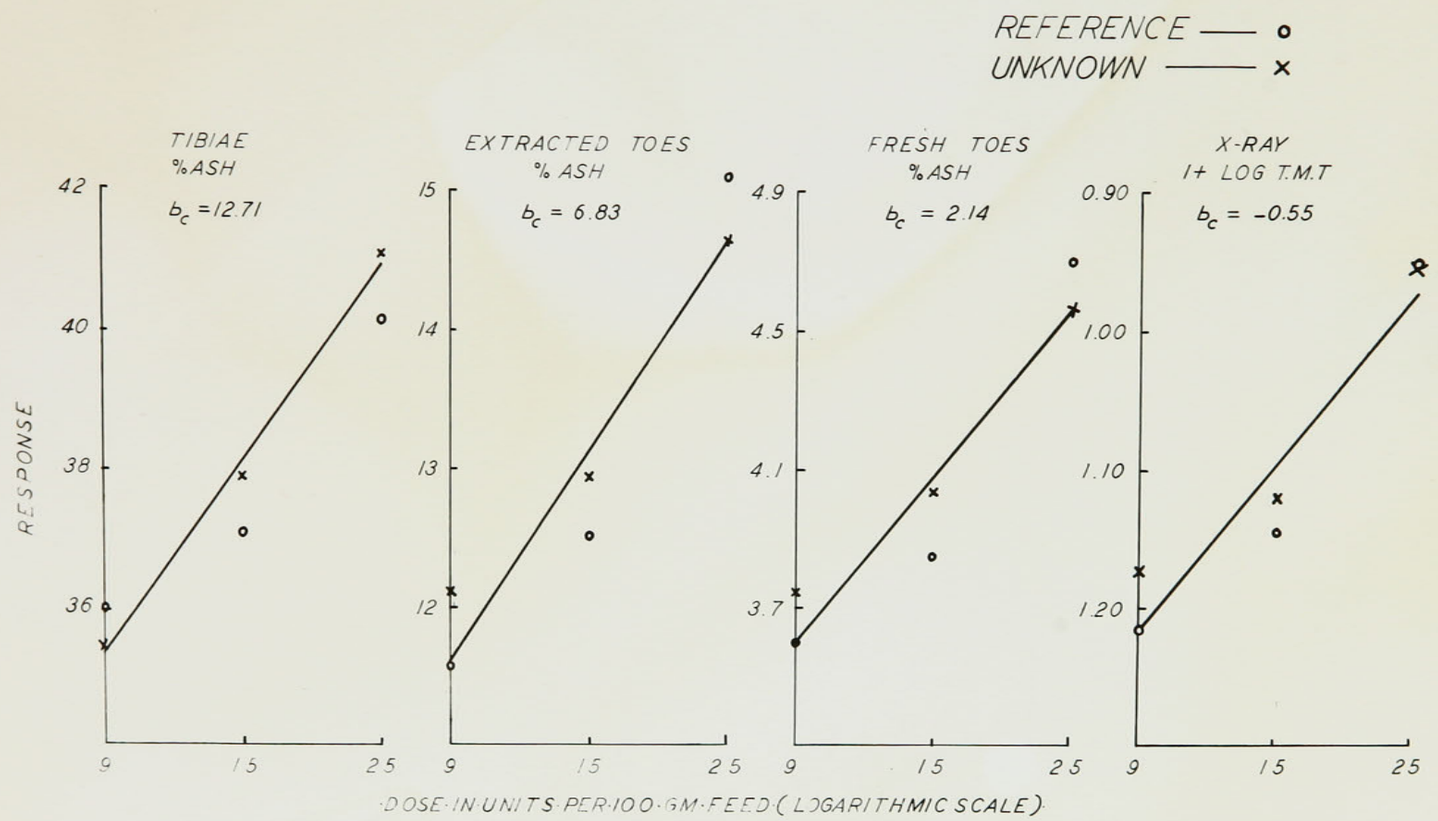


FIGURE 3. Response of chicks as measured by four criteria of calcification.

cular assay some of the curves may not be strictly linear, they show good agreement.

In general, it may be stated that the chief differences between the methods lie in the time and labour involved and not in the estimate obtained.

2. Relative Precision of Methods

Individual data were collected for comparison of the precision of the different methods. Table 4 summarizes the results of a series of 7 assays involving 1740 chicks in which the data for tibiae and toes are paired. It can be seen, according to the fiducial limits, that the toe ash method gives approximately the same precision as does the tibia ash method. Individual assays may favour either tibia or toe in precision.

Table 5 presents a more detailed study of the variability within the assays. In this table the standard errors in logarithms, from which the fiducial limits of the previous table were calculated, have been listed along with the corresponding standard deviations (s) and coefficients of variation. The combined slopes (b) of the log. (dose)-response curves have been decoded to permit the calculation of the statistic λ (= s/b) with its standard error for each assay. In order to remove any bias due to the fact that all four series of assays do not contain the same number of oils and that oils of any one series tend to show the same type of slope and degree of variability, means of the four series rather than of the 7 individual values were used in the final comparison. It is evident, over the period of time during which these assays were carried out, that the variance for error and slope change to a certain extent. Their relationship to each other is also subject to change though of a lesser degree. In general, the standard errors of the assays in logarithms show the same interpretation of variability as does

TABLE 4

Summary of Assays Calculated from Individual Tibia and Toe Ash Data

SERIES No.	ASSAY No.	TYPE OF OIL	EXPECTED POTENCY (A.O.A.C. UNITS)	ASSAYED POTENCY				NUMBER OF CHICKS
				Tibiae		Toes		
				Potency	Limits[1]	Potency	Limits[1]	
1	62	D_3 Preparation	1000	1533	1323-1777 (86-116%)	1443	1219-1707 (85-118%)	340
2	77	D_3 Preparation	1000	990	898-1092 (91-110%)	1272	1159-1395 (91-110%)	400
3	78	Feeding Oil	400	343	292-405 (85-118%)	356	310-409 (87-115%)	394
	79	Feeding Oil	400	305	256-363 (84-119%)	376	320-440 (85-117%)	
4	87	Feeding Oil	200	258	219-304 (85-118%)	281	225-352 (80-125%)	606
	88	Feeding Oil	200	294	249-348 (85-118%)	379	300-478 (79-126%)	
	89	Feeding Oil	200	380	320-451 (84-119%)	424	330-544 (78-128%)	
Total Means					87-116[2]		86-117[2]	1740

[1] Approximate fiducial limits calculated for 5% points. (According to B.S.I. criterion approximate limits are valid for these assays.)

[2] Means of the four series of assays.

TABLE 5

Precision of Tibia and Toe Ash Methods as Expressed by Their S.E., λ , and C.V.

ASSAY	TIBIA ASH					TOE ASH					DIFFERENCE (Toe λ - Tibia λ)	NECESSARY[1] DIFFERENCE
	S.E. (in logs.)	C.V. %	S.D. s	Slope (decoded) b	λ (s/b)	S.E. (in logs.)	C.V. %	S.D. s	Slope (decoded) b	λ (s/b)		
62	0.0326	8.61	3.504	16.201	0.216 ± 0.022	0.0370	14.42	2.067	8.120	0.255 ± 0.030	+0.039	0.073
77	0.0215	7.72	2.850	13.249	0.215 ± 0.016	0.0204	12.57	1.714	8.765	0.196 ± 0.013	-0.019	0.040
78	0.0364	6.63	2.717	11.433	0.238 ± 0.022	0.0320	11.04	1.607	7.688	0.209 ± 0.018	-0.029	0.056
79	0.0377	6.18	2.558	10.802	0.237 ± 0.022	0.0353	9.91	1.476	7.048	0.209 ± 0.018	-0.028	0.056
Mean	0.0371	6.41	2.638	11.118	0.238 ± 0.022	0.0337	10.48	1.542	7.368	0.209 ± 0.018	-0.029	0.056
87	0.0364	7.47	3.038	9.806	0.310 ± 0.032	0.0497	9.91	1.359	3.330	0.408 ± 0.053	+0.098	0.121
88	0.0368	7.15	2.925	10.117	0.289 ± 0.028	0.0513	9.40	1.309	3.731	0.351 ± 0.040	+0.062	0.096
89	0.0377	6.98	2.896	10.626	0.273 ± 0.025	0.0550	9.57	1.341	3.605	0.372 ± 0.045	+0.099	0.101
Mean	0.0370	7.20	2.953	10.183	0.290 ± 0.028	0.0520	9.63	1.336	3.555	0.376 ± 0.046	+0.086	0.104
Means of 4 series	0.0321	7.49	2.986	12.688	0.235 ± 0.019	0.0358	11.78	1.665	6.952	0.239	+0.019	0.053

[1] Necessary difference for significance = t (at P = 0.05) X standard error of difference between λ for tibiae, λ for toes.

the λ test both between the various assays and between toes and tibiae. The coefficients of variability (C.V.), on the other hand, because they do not take into account the slope of the log. (dose)-response lines do not reflect the same degree of variability. The fallacy of their use in studying assay procedures is clearly shown by the data. For example, as the coefficients of variation for each of the 7 comparisons are greater for toes than for tibiae, toes would be considered more variable, but this does not necessarily mean that the same relationship holds for precision.

It is evident, in comparing toes and tibiae by the λ test, that for the 6 pairs of values none of the differences between toes and tibiae are significant at the 5 per cent point, nor are they all of the same sign. The means of the four series are practically identical for toes and tibiae and the mean difference is negligible. It may, therefore, be concluded that there is no demonstrable difference between the precision of assays calculated from toe ash and those calculated from tibia ash.

In order to illustrate further the similarity of the log. (dose)-response curves for toe and tibia ash, data from Assay 62 are presented in Table 6. As originally set up this assay consisted of four levels each of reference and assay oils. It was found that both toes and tibiae showed a curved response line for the D_3 preparation and, therefore, the upper level was omitted. Using only the three lower levels, rendering the log. (dose)-response lines for

TABLE 6

Characteristics of Tibia Ash and Toe Ash Response Curves in Assay 62

	TIBIA ASH			TOE ASH		
	C.S.R.O.	D_3 preparation	Combined	C.S.R.O.	D_3 preparation	Combined
For 4 levels						
Error Variance	12.823	6.830		4.309	2.918	
F for deviation from st. line	1.99	4.72		1.23	7.50	
F at 5% point	3.04	3.07		3.04	3.07	
For 3 levels						
Mean ash %	39.57	42.37	40.71	13.85	15.04	14.33
No. of chicks	154	106	260	154	106	260
F for deviation from st. line	2.14	2.86		1.25	0.003	
F at 5% point	3.91	3.94		3.91	3.94	
F for non-parallel lines			0.697			1.354
F at 5% point			3.88			3.88
Error Variance	15.034	8.239	12.276	5.211	2.900	4.273
S.D.	3.877	2.870	3.504	2.283	1.703	2.067
Coded slope (b)	3.034	2.589	2.853	1.281	1.647	1.430

each rectilinear and parallel, toes again agree well with tibiae.

Differences in the responses and estimates of potency obtained in five assays with each of three different toes (inner, middle and outer) are illustrated in Table 7. The data indicate that there are constant differences between the ash content of the 3 toes and, therefore, if toes are used to determine the response, the same toe must be used throughout any one assay. Approximately the same variation may be expected between the potencies determined from different toes as between the other methods. The use of two toes in an assay readily furnishes duplicate results, an average of which would yield a considerably more reliable estimate of potency than either one alone.

Another series of assays, namely, assays 87 to 95, involving 1693 chicks, was carried out using both individual tibia ash and radiographic methods. As in the tibia-toe comparison, the data used here were comparable, each value for tibia ash having a corresponding value for the radiographic method. In Table 8 the fiducial limits in per cent, the standard deviations (s), the decoded slopes (b) and the s/b ratio of the log. (dose)-response curves have been tabulated. It can be seen that, although s/b for the radiographic method is greater in every case than that for the tibia ash method, yet only in the case of assay 94 does the difference approach significance at the 5 per cent point. By combining the 9 comparisons by the χ^2 method as outlined by Fisher (1934) an overall measure of the difference was obtained. The χ^2 value was found to be 26.036 which, with n = 18, yielded a P value of

TABLE 7

Comparison of the Ash Content and Potency Calculated from Different Extracted Toes

ASSAY No.	TOE ASH (Percent)			No. OF GROUPS AVERAGED	ESTIMATION OF POTENCY (A.O.A.C. UNITS)		
	Inside Toes	Middle Toes	Outside Toes		Inside Toes	Middle Toes	Outside Toes
93	12.58	13.39	13.74	20	483	478	481
111	13.90	14.56	15.04	38	2868	2828	3888
122A*	14.06	14.72	15.01	15	1390	1972	1888
112B*	13.57	14.40	14.97	16	1191	1544	1719
114	14.07	14.78	15.41	16	476	570	508

* A = A.O.A.C. method.

B = B.S.I. method.

TABLE 8

The precision of the Tibia Ash and Radiographic Procedures

ASSAY	TIBIA ASH METHOD				RADIOGRAPHIC METHOD (log t.m.t.)				DIFFERENCE t.m.t. λ - tibia λ	NUMBER OF CHICKS
	Fiducial* Limits Percent	Standard Deviation s	Decoded Slope b	$\lambda = (s/b) \pm$ S.E.	Fiducial* Limits Percent	Standard Deviation s	Decoded Slope b	$\lambda = (s/b) \pm$ S.E.		
87	85-118	3.0382	9.8075	0.310 ± 0.031	83-121	0.1854	-0.5223	0.355 ± 0.041	+0.045	
88	85-118	2.9260	10.1166	0.289 ± 0.028	82-123	0.1774	-0.5034	0.352 ± 0.041	+0.063	606
89	84-119	2.8962	10.6268	0.273 ± 0.025	82-121	0.1775	-0.5584	0.318 ± 0.033	+0.045	
90	87-115	3.5029	13.8683	0.253 ± 0.023	85-118	0.1982	-0.6963	0.285 ± 0.028	+0.032	
91	85-118	3.6033	13.2644	0.272 ± 0.028	84-119	0.2066	-0.7156	0.289 ± 0.031	+0.017	509
92	86-117	3.6302	14.1148	0.257 ± 0.025	85-118	0.2093	-0.7643	0.274 ± 0.028	+0.017	
93	86-116	4.0631	13.7714	0.295 ± 0.029	82-122	0.2479	-0.6719	0.369 ± 0.044	+0.074	
94	88-114	3.7767	15.5736	0.243 ± 0.020	84-119	0.2339	-0.7161	0.372 ± 0.035	+0.084	578
95	87-115	3.9353	16.2649	0.242 ± 0.021	85-118	0.2200	-0.7089	0.310 ± 0.034	+0.068	
Means	86-117	3.4858	13.0454	0.270 ± 0.023	84-120	0.2062	-0.6508	0.320 ± 0.033	+0.050	

* Approximate fiducial limits at P = 0.05. According to the criterion of the B.S.I. method, approximate limits are valid for the assays reported in this table.

approximately 0.09. Although approaching the 5 per cent point, the differences are not considered to be significant with these data. This conclusion substantiates the work of Baker and Wright (1940) although in their data the bone ash tends to be the more variable. This slight but constant difference is also reflected in the fiducial limits. It is pointed out again that the radiographic technique of Olsson as used in this paper involved only the actual principle of X-raying the chicks and was not used as a complete method.

Considering the results of these comparisons as a whole, it is evident that there is no statistically significant difference between the precision of the three criteria of calcification.

3. Two Week Assay Period

As it was possible to X-ray the same chicks at both two and three weeks, the radiographic procedure offered the most valid basis for a comparison of the two feeding periods. Accordingly, the potency, approximate fiducial limits and λ ratios were calculated from a series of 8 assays for both feeding periods. The results of these comparisons are presented in Table 9. It should be pointed out that for this particular comparison of λ's the data were not strictly paired, that is if a chick died after being X-rayed at 2 weeks, its 2 week value was not removed. However, as mortality was low in this period (less than 3 per 100), the data are comparable.

It can be seen, from Table 9, that in all but 2 of the assays the 2 week estimate is higher than that at 3 weeks. The mean difference between estimates of potency at 2 and 3 weeks is 11 per cent. The combined probability of the significance of differences between estimates, using the χ^2 method of Fisher (1934) was found to be greater than 0.80. It may, therefore, be concluded that there is no significant difference between the estimates of potency determined at 2 and at 3 weeks.

Considering the precision of the method, it is evident from the λ ratios that in every case the 2 week assay period yields more variable results than does the 3 week period. The differences, although large, are not significant when each assay is viewed by itself or when combined, as the standard errors of the 2 week data

TABLE 9

Comparison of two and three week data

CRITERION OF CALCIFICATION	ASSAY NO.	TWO WEEK DATA					THREE WEEK DATA					DIFFERENCE BETWEEN λ's
		Potency	Limits* Percent	Standard Deviation s	Slope b	$\lambda = (s/b)$	Potency	Limits* Percent	Standard Deviation s	Slope b	$\lambda = (s/b)$	
Radiographs	104	113	80-125	0.1819	0.4561	0.399 ± 0.057	112	83-121	0.1905	0.5507	0.346 ± 0.044	0.053
	105	396	72-139	0.1586	0.3028	0.524 ± 0.110	421	79-127	0.1981	0.5390	0.368 ± 0.056	0.156
	106	275	64-157	0.1653	0.2726	0.606 ± 0.146	269	73-138	0.2025	0.4669	0.434 ± 0.077	0.172
	107	584	66-152	0.1613	0.3015	0.535 ± 0.114	522	76-132	0.1920	0.5020	0.382 ± 0.060	0.153
	108	249	69-146	0.1624	0.3042	0.534 ± 0.114	223	74-135	0.2050	0.4678	0.438 ± 0.079	0.096
	111	4432	76-132	0.1463	0.2402	0.610 ± 0.102	3090	83-121	0.1679	0.4375	0.384 ± 0.042	0.226
	112	1533	59-171	0.1385	0.2285	0.606 ± 0.168	1124	80-126	0.1492	0.4777	0.312 ± 0.047	0.294
	114	402	63-158	0.1367	0.1825	0.749 ± 0.222	444	70-144	0.1959	0.3538	0.554 ± 0.125	0.195
Means			69-148	0.1564	0.2861	0.570		77-131	0.1876	0.4744	0.402	0.168

* Approximate limits at P = 0.05.

are also relatively large. However, in view of the fact that the ratios are uniformly less for the 3 week data, on the average 42 per cent less, it would seem to indicate that the 2 week data are definitely less precise than the 3 week data. The chief reason for this seems to be that the lower slope (b) of the log. (dose)-response curves at 2 weeks is not sufficiently balanced by a lesser variability (s). Substantially similar observations were made by De Witt et al. (1942). Although for some of the assays reported in Table 9 the approximate fiducial limits, particularly at 2 weeks, underestimate the true width of the fiducial range, yet they show the same trend as the λ ratios. Whatever limits are calculated, of course, do not affect the comparison by the λ method.

To furnish some idea as to how the other 3 criteria would react, one of the assays (No. 104) was carried out entirely in duplicate under the same conditions and at the same time. One set of chicks was killed at two weeks and the other was killed at 3 weeks. The estimates of potency were as follows:

	At 2 weeks	At 3 weeks
Radiographic	94	112
Extracted toe	87	108
Fresh toe	104	110
Tibia	98	102

The results of these comparisons, when considered in conjunction with those of Table 9 substantiate the conclusion that there is no significant difference between estimates of potency determined at

2 and at 3 weeks.

If it may be considered as established that two week data may be used for assay purposes, the method offers a certain advantage as regards time required. In view of the relatively poorer definition of the slope of the response lines in such tests, the question of the use of true or approximate fiducial limits assumes greater importance. Ordinarily, with three week data the slope is sufficiently well defined that the approximate formula gives the same results as the true one. As Irwin (1943) has pointed out, if the slope is not well defined the approximate formula not only underestimates the fiducial range, but also biases its position. In view of these facts it seemed desirable to ascertain the relative merits of the two formulae for two and three week chick data, and also determine whether the correction should be applied to the potency as well as to the fiducial range. The data, presented in Table 10, indicate that the approximate formula gives a fair estimate of the potency at two weeks. The correction factor used in the true formula tends to spread the potency from the expected value, e.g., in those cases where the potencies are above the expected potency the true formula gives still higher estimates.

It is suggested, then, that for a preliminary test, two week data, in which the slope is not well defined, can furnish indications of the potency of unknown samples without the use of the correction factor and may serve a useful purpose in preliminary surveys of oil potency.

TABLE 10

True and Approximate Fiducial Limits
Calculated for Two and Three Week Radiographic Data

ASSAY No.	TWO WEEK DATA				THREE WEEK DATA			
	True		Approximate		True		Approximate	
	Potency	Limits %	Potency	Limits %	Potency	Limits %	Potency	Limits %
1	114	79-126	113	80-125	112	83-121	112	83-121
2	416	70-143	404	72-139	434	78-128	421	79-127
3	323	59-171	275	64-157	288	71-142	269	73-138
4	666	62-161	584	66-152	550	74-135	522	76-132
5	273	66-153	249	69-146	234	72-138	223	74-135
6	4870	67-150	4432	76-132	3090	83-121	3090	83-121
7	2005	50-199	1533	59-171	1156	79-127	1124	80-126
8	468	56-179	402	63-158	484	66-151	444	70-144

4. Reproducibility of Methods

Besides giving a similar estimate of potency and degree of precision in assaying unknown materials, a method, to be entirely satisfactory, must also yield reproducible results when the same material is assayed a number of times. In vitamin D assays the cost of such a repetition of tests would be prohibitive if carried out for that purpose alone. As it has been the custom in this laboratory to use four groups of chicks at each of three dosage levels of the reference oil for most of the assays reported above, an opportunity was afforded of considering two groups at each level as "standard" and two as "unknown," and thus calculating the potency of the "unknown." A mean of several assays for each method would furnish an estimate of the precision or reproducibility (Knudsen, 1944).

Four groups of chicks were fed at each dosage level of standard reference oil in most of these assays. In a few cases only three groups were fed at each dose. In order to obtain further comparisons of the five criteria, data were also taken from four other test oils which had been fed to three or four groups of chicks at each of three levels. For each assay there were nine to twelve groups available for partition into "standard" and "unknown" categories. To accomplish this separation most efficiently, a series of arrangements was made out. These arrangements were then applied to the assays in two ways, namely, (1) arbitrary and systematic allocation of the arrangements, and (2) randomization of

the same arrangements between assays. Randomization was carried out according to the method outlined by Goulden (1939). This permitted two separate estimations to be made of the data. The potency of the "standard" was considered as 100 for convenience and the "unknown" defined in relation to that value. Analysis of variance was carried out on the data to determine the relative importance of the variability between methods and assays.

The results of the assays are presented graphically in Figures 4 and 5, and a summary of the data according to criterion of calcification is given in Table 11. The figures show no definite difference between methods and the data of Table 11 substantiate this observation. As there are a few rather discordant values, the data have been considered in two ways: (1) using all values and (2) omitting those values lying outside $\pm$ 3 S.D. In eliminating these questionable results it has been assumed that values lying outside $\pm$ 3 S.D. (odds 370:1) have been affected by factors other than those ordinarily encountered in tests of this type. From this point of view, it is evident that all five criteria are approximately equally accurate as judged by the means and equally precise as judged by the standard deviations. If anything, the tibiae are the most variable. In the systematic partition of groups the means for all methods were higher.

The partition of the total variability between methods and error in Table 12 shows that the variance between methods was in all cases considerably less than that for error. In view of pre-

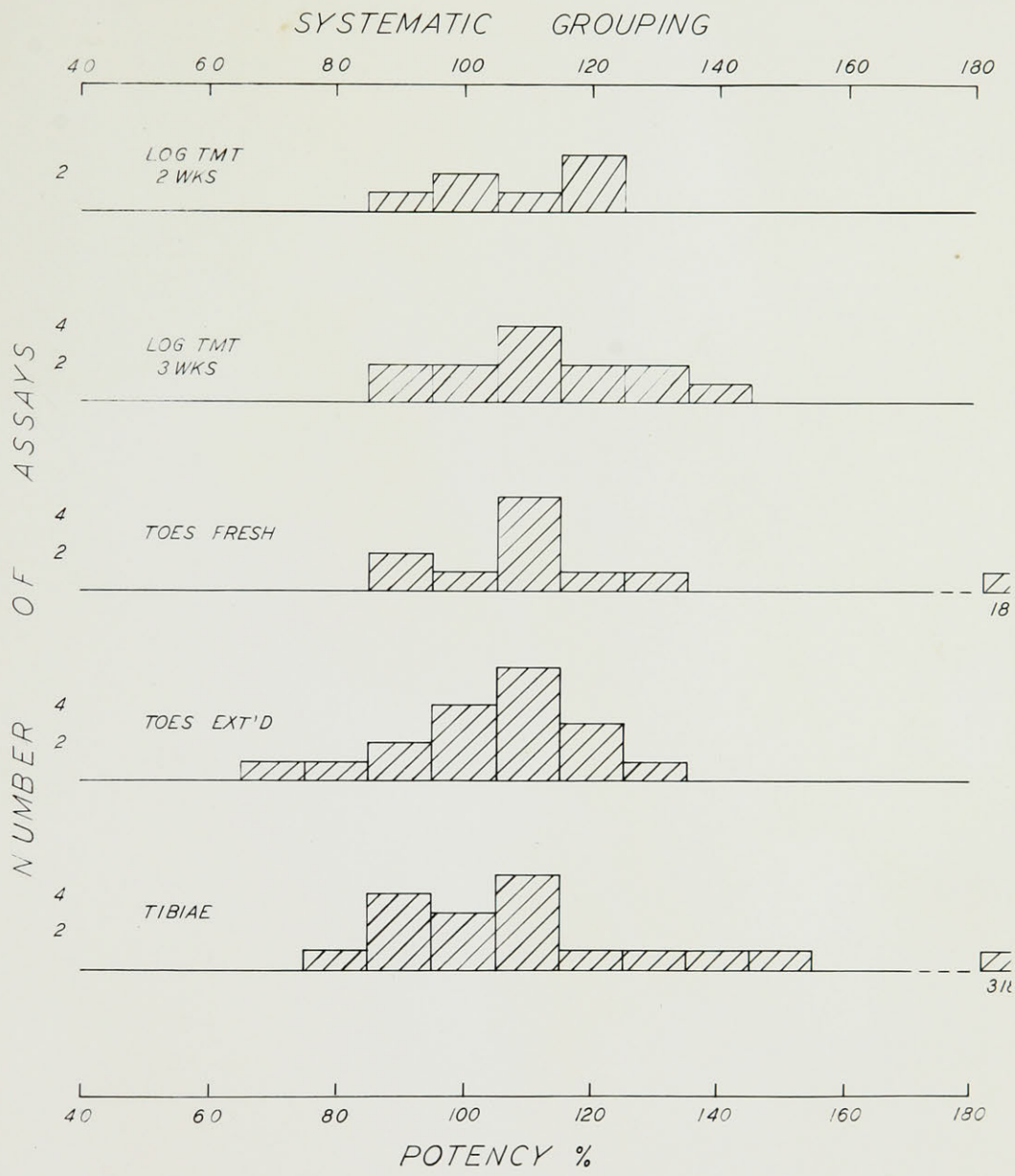


FIGURE 4. Potency of "unknown" in terms of "standard" by five criteria of response. Groups separated arbitrarily.

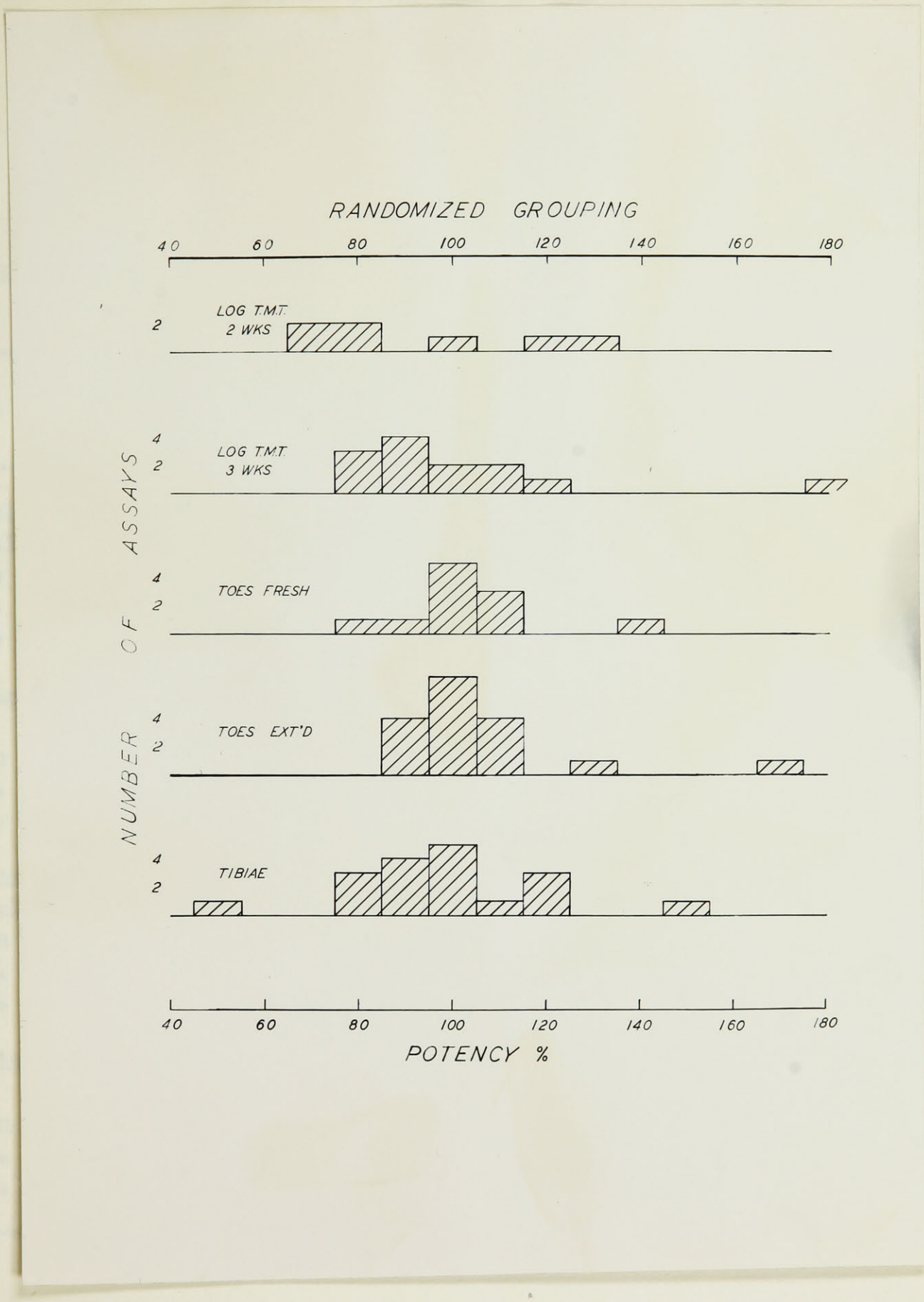


FIGURE 5. Potency of "unknown" in terms of "standard" by five criteria of response. Groups separated by randomization.

TABLE 11

Comparison of Mean Estimates of Potency by Five Criteria of Calcification
(expected potency = 100)

	Tibia Ash	Toe Ash (extracted)	Toe Ash (fresh)	Log t.m.t. 3 Weeks	Log t.m.t. 2 Weeks	All Methods
Systematic Grouping						
All values						
Mean	121	105	116	112	109	113
S.D.	52	15	26	16	13	31
S.E.	12	4	8	4	5	4
Omitting values outside ± 3 S.D.						
Mean	109	--	--	--	--	110
S.D.	18	--	--	--	--	18
S.E.	4	--	--	--	--	2
Random Grouping						
All values						
Mean	98	107	103	103	94	102
S.D.	22	20	15	26	25	22
S.E.	5	5	5	7	10	3
Omitting values outside ± 3 S.D.						
Mean	--	103	--	97	--	100
S.D.	--	11	--	12	--	18
S.E.	--	3	--	3	--	2

TABLE 12

Summary of Analysis of Variance of Estimates of Potency

GROUPING	VARIANCE DUE TO	FOR ALL VALUES		OMITTING VALUES OUTSIDE ± S.D.	
		D.F.	Mean Squares	D.F.	Mean Squares
Systematic	Methods	4	565.8	4	202.3
	Error	62	987.3	61	326.4
Random	Methods	4	290.0	4	176.3
	Error	62	471.1	60	304.6

vious results, this observation was to be expected.

These results furnish further evidence in support of conclusions reached above that there is no essential difference in accuracy or precision between the tibia ash, toe ash, and radiographic (t.m.t. distance) methods for vitamin D assay with chicks at three weeks of age.

5. A Comparison of Diets and Feeding Periods

It has been observed in this laboratory that chick assays for vitamin D in which the British Standards Institution (1940) diet and feeding period were used, often show less variability as measured by fiducial limits than is commonly encountered in the A.O.A.C. (1940) procedure. These procedures differ chiefly in two ways. The A.O.A.C. method consists of the three week preventive period using a diet with a calcium and phosphorus content of approximately 0.8 and 0.7 per cent, respectively. On the other hand, the B.S.I. recommends for the X-ray method a one week depletion and three week curative period with a diet having a calcium and phosphorus content of about 1.5 and 1.0 per cent, respectively. (For bone ash method the A.O.A.C. diet is used.) The observed difference in variability could be caused by either one or both of these factors or the difference in age of the chicks. Although the two diets differ in their constituents and source of calcium and phosphorus, the content and ratio of these elements are possibly the most important factors.

Four experiments were carried out at different times over

a period of more than a year. Precision was judged by the s/b ratio of the response lines of X-ray and bone ash data. Analysis of variance was carried out on all data and the distribution of variance between replicates and individuals was determined.

The calcium and phosphorus content of the diets used are given in Table 13. Each value is the mean of 3 to 5 samples. The variation in composition is about what would be expected when mixed from different lots of feed over a period of time.

Experiment 1. A preliminary experiment was carried out in which both the A.O.A.C. and B.S.I. procedures were used to test the potency of an oil. Both procedures were adhered to as regards diet and feeding period. The Canadian Standard Reference oil was fed to three groups of 20 chicks at each of three dosage levels and the assay oil at two levels. The pooled values of this preliminary test are presented.

Experiment 2. In a more comprehensive experiment, only the Canadian Standard Reference oil was used. The study involved a comparison of the response curves of the A.O.A.C. and B.S.I. diets, each fed according to both the A.O.A.C. and B.S.I. procedures. The standard oil was fed at three levels, 6.67, 15, 33.75 units as representing the lower, middle, and upper dosage range. Four groups of 20 chicks each were started at each level with one other group serving as negative control for each comparison. The whole experiment involved the use of approximately 1000 chicks.

TABLE 13

Calcium and Phosphorus Content of Diets
(as percent of air-dry sample)

EXPERIMENT	A.O.A.C. DIET		B.S.I. DIET	
	Calcium	Phosphorus	Calcium	Phosphorus
1	0.91	0.87	1.59	1.01
2	0.82	0.74	1.36	0.80
3	0.90	0.82	--	--
4	0.77	0.78	1.46	0.88
Means	0.85	0.80	1.47	0.90

Experiment 3. This was a repetition of half of Experiment 2, using only the A.O.A.C. diet.

Experiment 4. In the previous tests only three levels of oil were fed. It was thought desirable to determine the position of the curves in somewhat more detail. Accordingly another experiment was set up in which the reference oil was fed to 3 groups of 20 chicks at each of 6 dosage levels equally spaced on the logarithmic scale and ranging from 4.45 to 33.75 units per 100 grams feed. The steepest straight line 3 point portion of each curve was used for comparison.

The radiographic data are summarized in Table 14. It is evident that as the age of the chicks increases up to 3 or 4 weeks the precision as measured by the s/b ratio also increases. This increase is caused chiefly by the greater slope of the log. (dose)-response curve. The B.S.I. assay period (i.e., 1 week depletion + 3 week curative) is in 4 cases more precise than the 3 week A.O.A.C. period and in one test equally precise. The differences are not always significant, however. There is no difference between the 1 + 2 week and the 3 week periods.

As the slope of the log. (dose)-response curve is the chief factor determining the magnitude of the λ ratio, the response curves in Figure 6 plotted from the mean level response for X-ray and toe ash data show the same trends as the ratios. It should be pointed out in this connection that, although the response lines were not always linear for the first three experiments, it was felt

TABLE 14

Precision Ratios s/b for A.O.A.C. and B.S.I. Feeds and Procedures Using Radiographic Criterion

Experiment	Diet	Feeding Procedure	Age of Chicks (Weeks)	Standard Deviation s	Slope b	Ratio s/b
1	A.O.A.C.	A.O.A.C.	2	0.139	-0.228	0.61 ± 0.17
			3	0.149	-0.478	0.31 ± 0.05
	B.S.I.	B.S.I.	1 + 1	0.124	-0.191	0.65 ± 0.21
			1 + 2	0.112	-0.328	0.34 ± 0.06
			1 + 3	0.153	-0.653	0.23 ± 0.03
2	A.O.A.C.	A.O.A.C.	2	0.147	-0.211	0.70 ± 0.13
			3	0.188	-0.436	0.43 ± 0.05
		B.S.I.	1 + 1	0.194	-0.256	0.76 ± 0.15
			1 + 2	0.183	-0.514	0.56 ± 0.04
			1 + 3	0.181	-0.631	0.29 ± 0.03
	B.S.I.	A.O.A.C.	2	0.123	-0.145	0.85 ± 0.18
			3	0.152	-0.300	0.51 ± 0.07
		B.S.I.	1 + 1	0.159	-0.138	1.16 ± 0.33
			1 + 2	0.156	-0.267	0.55 ± 0.08
			1 + 3	0.175	-0.363	0.48 ± 0.06
3	A.O.A.C.	A.O.A.C.	2	0.167	-0.228	0.73 ± 0.24
			3	0.214	-0.672	0.32 ± 0.05
		B.S.I.	1 + 1	0.198	-0.272	0.73 ± 0.23
			1 + 2	0.202	-0.711	0.28 ± 0.04
			1 + 3	0.196	-0.817	0.24 ± 0.03
4	A.O.A.C.	A.O.A.C.	3	0.145	-0.857	0.17 ± 0.02
		B.S.I.	1 + 2	0.179	-0.885	0.20 ± 0.03
			1 + 3	0.182	-1.038	0.18 ± 0.02
	B.S.I.	B.S.I.	1 + 2	0.135	-0.277	0.49 ± 0.14
			1 + 3	0.161	-0.394	0.41 ± 0.10

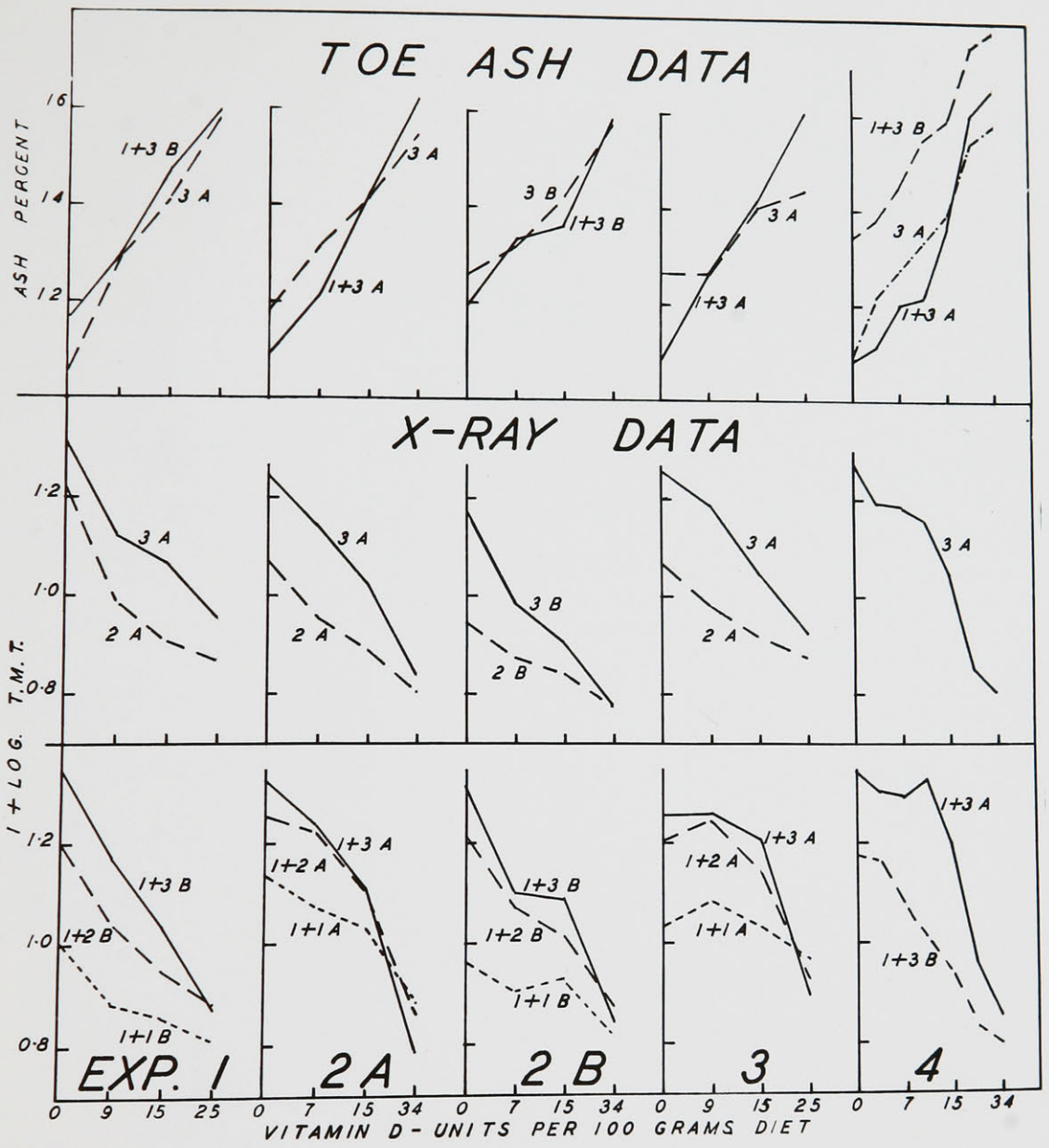


FIGURE 6. Log. (dose)-response curves for two criteria in four experiments. A = A.O.A.C. diet, B = B.S.I. diet. Numbers indicate type and length of feeding period. For convenience the 0 dosage level was plotted at log. interval equal to that between other levels.

that the overall slope for the s/b ratio gave a fairer comparison of the procedures than if the steepest two dose portion had been used. If only the two higher levels had been considered the differences in slope and ratio would have been magnified particularly in comparisons involving the 1 and 3 week period. In each experiment the root mean square (s) for replicates has been calculated for both criteria and from this the s/b ratio has been obtained. As interest lies chiefly in the comparison of the 3 week and the 1 + 3 week assay period, replicate and individual data have been assembled in Table 15. In the replicate data the same trends are evident and emphasized, but as the error becomes larger with increasing ratios, the significance of the differences is in some cases actually less than when using the error (s) from individuals. Bone ash data are not as favorable to the 1 + 3 week assay as are the radiographic data.

For the A.O.A.C. diet alone the average percentage decrease in the s/b ratios (i.e., s/b at 3 weeks - s/b at 1 + 3 weeks) has been calculated to be approximately 43 per cent for the ratios using replicate error and 34 per cent for the ratios using individual error. This represents an important increase in precision which, to yield the same standard error of log-ratio of potencies, would require the use of more than twice as many chicks. Using the B.S.I. diet the difference is not as marked.

Another estimation of the merits of assay procedures is the relation between the variances for doses and for replicates.

TABLE 15

Ratios s/b for 3 Week and 1 + 3 Week Periods when s is Calculated from Replicate and Error Variance

Criterion	Experiment	Diet	s Calculated From	Feeding Period: 3 Week	Feeding Period: 1 + 3 Week	Necessary Difference (P=0.05)
X-ray	1	A.O.A.C.	Replicate	0.38 ± 0.09	--	--
			Error	0.31 ± 0.05	--	--
		B.S.I.	Replicate	--	0.22 ± 0.05	--
			Error	--	0.23 ± 0.03	--
	2	A.O.A.C.	Replicate	0.67 ± 0.18	0.29 ± 0.06	0.43
			Error	0.43 ± 0.05	0.29 ± 0.02	0.11
		B.S.I.	Replicate	0.95 ± 0.30	0.82 ± 0.24	0.86
			Error	0.51 ± 0.07	0.48 ± 0.06	0.18
	3	A.O.A.C.	Replicate	0.83 ± 0.37	0.29 ± 0.08	0.93
			Error	0.32 ± 0.05	0.24 ± 0.03	0.11
	4	A.O.A.C.	Replicate	0.25 ± 0.07	0.18 ± 0.05	0.20
			Error	0.17 ± 0.02	0.17 ± 0.02	0.06
		B.S.I.	Replicate	--	0.42 ± 0.14	--
			Error	--	0.41 ± 0.10	--
Bone Ash[1]	1	A.O.A.C.	Replicate	0.51 ± 0.15	--	--
			Error	--	--	--
		B.S.I.	Replicate	--	0.28 ± 0.06	--
			Error	--	--	--
	2	A.O.A.C.	Replicate	1.00 ± 0.32	0.44 ± 0.10	0.77
			Error	0.52 ± 0.07	0.26 ± 0.02	0.15

In an ideal assay the variance between doses should be a maximum and that between replicates a minimum. This is actually a different way of expressing the relation between slope and error. The variance ratios are given in Table 16 and lead to the same conclusions as the s/b ratios. If replicate variance is taken as error then almost all the ratios are significant and differ only in degree of significance.

The difference in the 3 and 1 + 3 week periods may be due to either one or both of two factors, i.e., the depletion period and the age of the chicks. If only the two higher levels in the assays are considered, it is evident that, with time, chicks on the preventive assay are becoming more rachitic at both levels while those on the curative assay are becoming more rachitic on the lower level and less rachitic on the upper level. That is, in the curative assay the response lines tend to cross while in the preventive assay the change of slope is relatively less and there is not the same tendency for crossing. For assay purposes the cross-over point could be taken as the mid-point of the response line for a three point assay. In view of the fact that there is no difference between the 3 and 1 + 2 week periods it is suggested that the chief factor may be the age of the chicks. It is obvious then that if facilities are available for depletion of chicks there is an advantage in using week-old rather than day-old chicks to start the assay. This problem is discussed at greater length later in this section.

TABLE 16

Variance Ratios for Levels/Replicates for
3 Week and 1 + 3 Week Periods

EXPERIMENT	DIET	X-RAY DATA		BONE ASH DATA[1]	
		3 Week	1 + 3 Week	3 Week	1 + 3 Week
1	A.O.A.C.	11.94**	---	---	---
	B.S.I.	---	21.16**	---	---
2	A.O.A.C.	17.68**	110.02**	8.33**	44.19**
	B.S.I.	9.77**	15.91**	31.72**	16.64**
3	A.O.A.C.	3.93	38.17**	24.67**	40.56**
4	A.O.A.C.	26.74**	44.10**	10.55*	12.42**
	B.S.I.	---	9.15*	---	19.30**

* Significant at P = 0.05

** " " P = 0.01

1 Toe ash data except for Experiment 3 which is tibia ash.

In comparing the two diets it will be noticed that there is not a great deal of difference between them. The X-ray data (Table 13 and 14) show the A.O.A.C. diet to be better in all cases and in most cases significantly. However, the bone ash data show no difference. In this connection it might be mentioned that preliminary evidence obtained in this laboratory indicates that X-ray and bone ash criteria may not always show the same degree of calcification when different diets are compared. It has already been shown that with the same diet both criteria give the same response. From the curves of Figure 6 it will be noted that the B.S.I. diet at the lower levels of vitamin D produces a lesser degree of rickets than the A.O.A.C. diet. It may, therefore, be assumed that, in general, the A.O.A.C. diet under conditions of this laboratory is somewhat more satisfactory.

Olsson (1941) has claimed certain advantages for a low mineral depletion period and a curative period where the diet is supplemented with both calcium and phosphorus as well as varying levels of vitamin D. In view of these considerations and the data reported earlier in this section, an experiment was set up to test three diets in different combinations. The basis of all diets was the A.O.A.C. diet. The low mineral diet was the A.O.A.C. without mineral. The percentage composition from analysis of the diets was as follows:

	Calcium	Phosphorus
Low mineral	0.10	0.25
A.O.A.C.	1.01	0.79
A.O.A.C. + Mineral	1.82	0.84

Three combinations were made of these diets for a curative type of assay. In the first one the A.O.A.C. diet was used in both the depletion and curative periods. In the second the low mineral diet was used as depletion for the A.O.A.C. in the curative period, and in the third the low mineral diet was used as a depletion for the A.O.A.C. diet supplemented with mineral. The latter diet approximated that of Olsson in content of calcium and phosphorus although the depletion was probably somewhat more severe. The diets were judged by the ratios of the variance between doses to that within doses for the steepest two dose portion of the response curve in each case. The chicks were X-rayed at two, three and four weeks of age, and the magnified radiographs read in millimeters.

The data are presented in Table 17 and the curves are plotted in two ways in Figure 7. It may be noted that the low mineral diet produces more rachitic chicks at one week of age than does the A.O.A.C. diet. This allows the higher levels to become more normal while the lower levels become more rachitic. The remainder of the data, however, do not indicate that this makes for a great difference in the curves. There is a difference in the location of the most sensitive part of the curve, e.g., the steepest part

TABLE 17

Variation Within and Between Doses for Three Diet Combinations Using a Depletion Period

PERIOD (WKS.)	VARIANCES	DIET: AOAC - AOAC D.F.	AOAC - AOAC Mean Squares	AOAC - AOAC F.	Low Min. - AOAC D.F.	Low Min. - AOAC Mean Squares	Low Min. - AOAC F	Low Min. - AOAC + Min. D.F.	Low Min. - AOAC + Min. Mean Squares	Low Min. - AOAC + Min. F
1 + 1	Doses	1	452	12.2	2	1340	12.6	2	573	6.2
	Error*	103	37	--	101	106	--	100	92	--
1 + 2	Doses	1	2850	44.5	1	3444	32.5	--	--	--
	Error*	70	64	--	68	106	--	--	--	--
1 + 3	Doses	1	4371	60.7	1	7541	69.8	1	5510	41.7
	Error*	70	72	--	66	108	--	66	132	--

* Combination of variance between replicate groups and individuals.

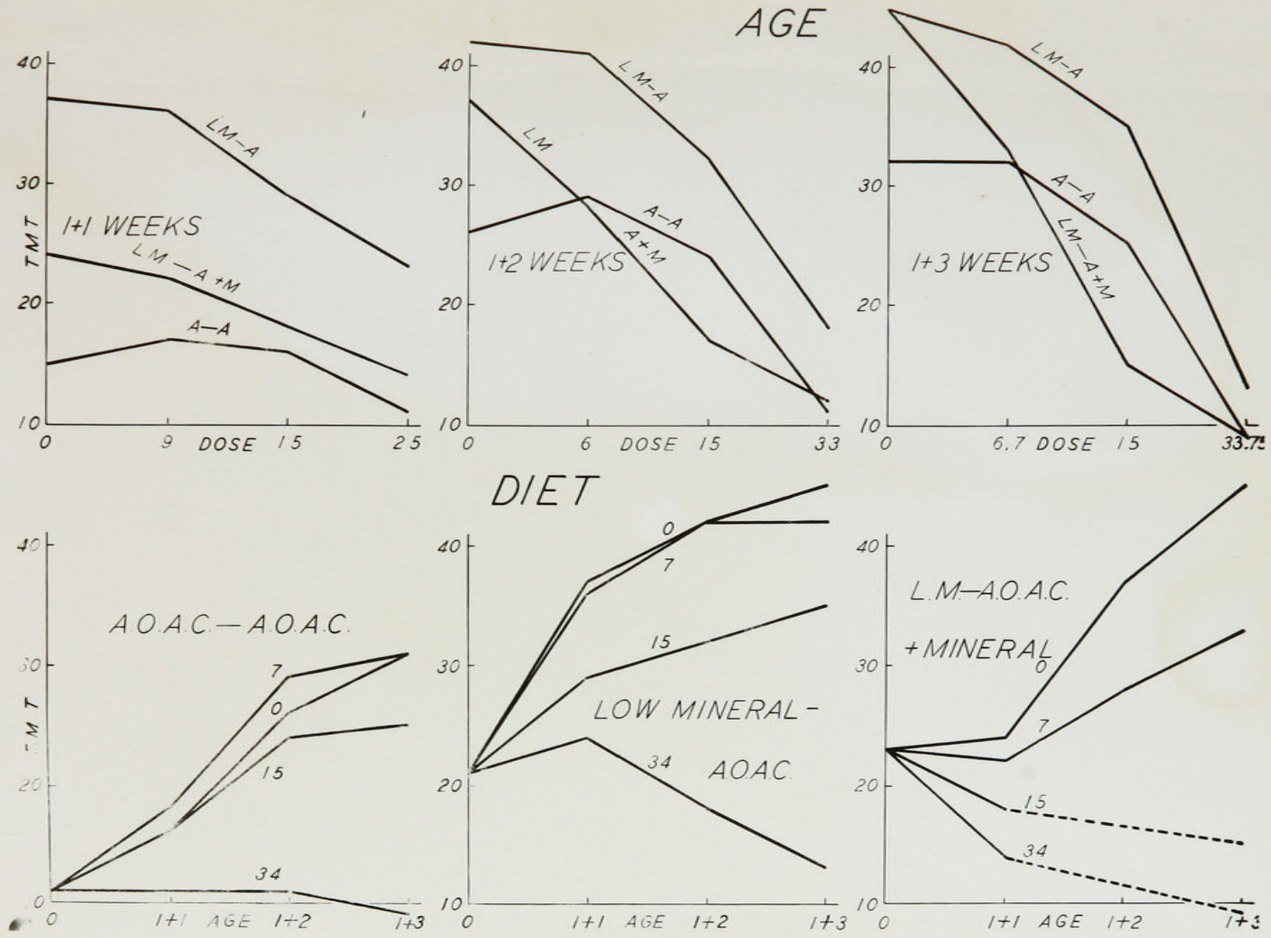


FIGURE 7. Response curves for X-ray measurements on chicks fed 3 diets in a curative feeding period, plotted according to dose and age.

of the low mineral--A.O.A.C. + mineral curve is between 6 and 15 units, while for the other two it is between 15 and 33.75 units. In general, however, there are no great differences evident between the methods as regards slope or precision of response lines. These conclusions in regard to the curative assay are in line with work on various diets in the preventive assay by Migicovsky and Emslie of this laboratory, and by Bliss (1946). Evidently the response line representing the response per unit dose tends to be much the same regardless of diet, at least within fairly wide limits, but changes in the calcium and phosphorus content of the diet does alter its position.

It was postulated earlier in this section that within limits the age of chicks may be of greater importance in determining the slope of the dose-response curve than diet or type of feeding period. To test this hypothesis an experiment was set up comparing the curative and preventive periods on three diets of varying calcium and phosphorus content. The chicks were X-rayed at 1, 2, 3, and 4 weeks of age, and the readings were recorded in millimeters of the magnified radiograph as in the last experiment. The diets were based on the A.O.A.C. (1940) diet, the calcium and phosphorus content being adjusted by additions of calcium carbonate and tricalcium phosphate to the mineral-free basal diet. The percentage composition from analysis of the diets were as follows:

	Calcium	Phosphorus
A.O.A.C.	0.91	0.83
High Calcium	2.95	0.53
Low Calcium	0.52	0.83

The data have been plotted in three ways in Figures 8, 9 and 10 to clarify the comparisons. They have also been analyzed statistically to partition the total variance into two factors, between and within doses, the latter including both the individual and replicate variance. These data are given in Table 18. From Figure 8 it is evident that again there is greater tendency with time for cross over in the curative test than there is in the preventive, although these tests are not particularly good examples. It is difficult to determine from the single test whether the poor type of response curve found on the low calcium curative test is a true condition or is caused by some experimental mishap. There is not as much difference in the two and three week data as is usually evident. When the progress of calcification is plotted against time as in Figure 9 the difference in degree of rickets on the three diets becomes more evident. On the low calcium diet chicks seem to reach a certain degree of rickets and remain there while on the other diets the lower levels become progressively more rachitic. In Figure 10 the curves of Figure 8 have been selected on the basis of age of chicks to make the curves more comparable at the same age. Again no marked differences are evident.

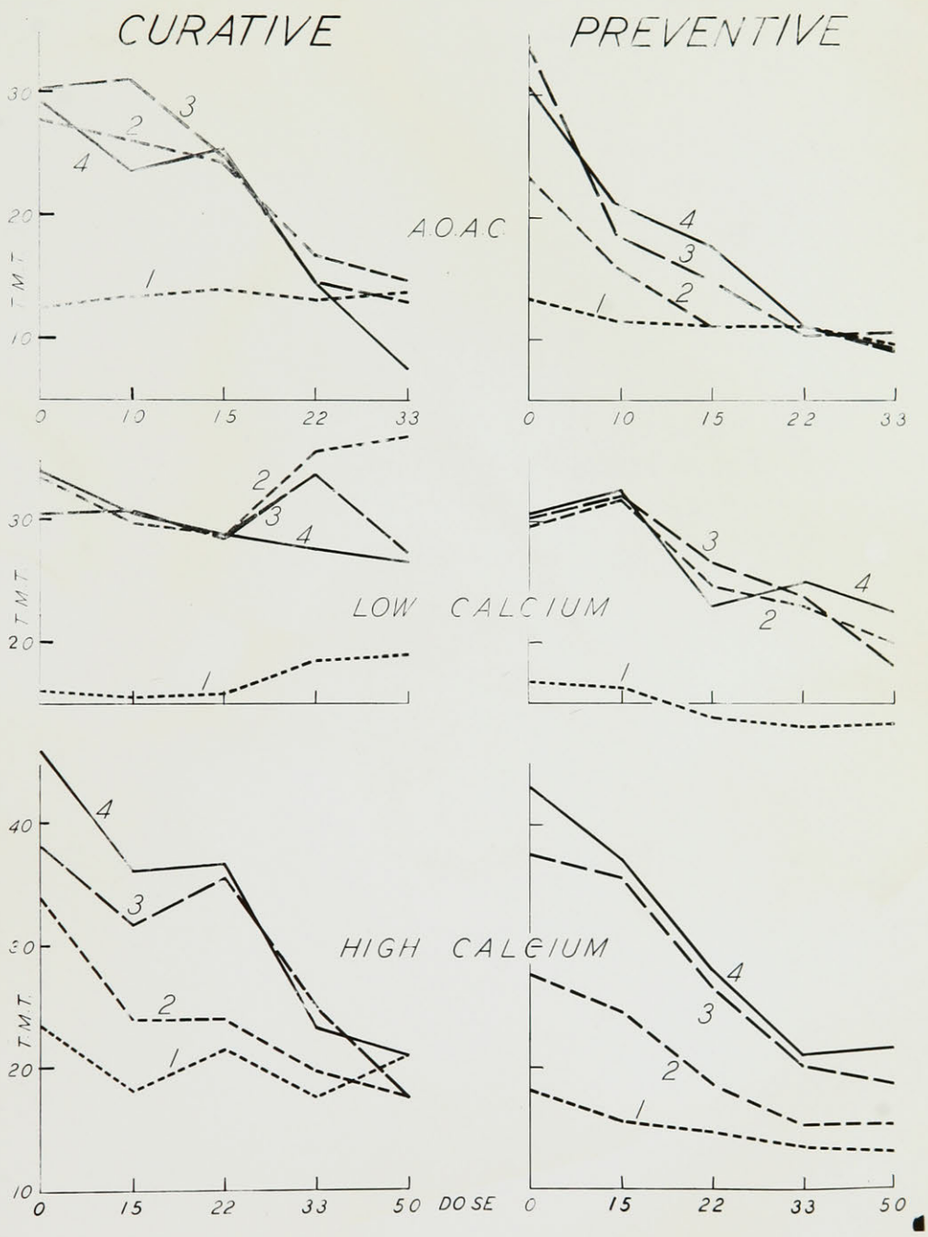


FIGURE 8. Response curves in preventive and curative assays for three diets, plotted against dose. Numbers indicate age of chicks.

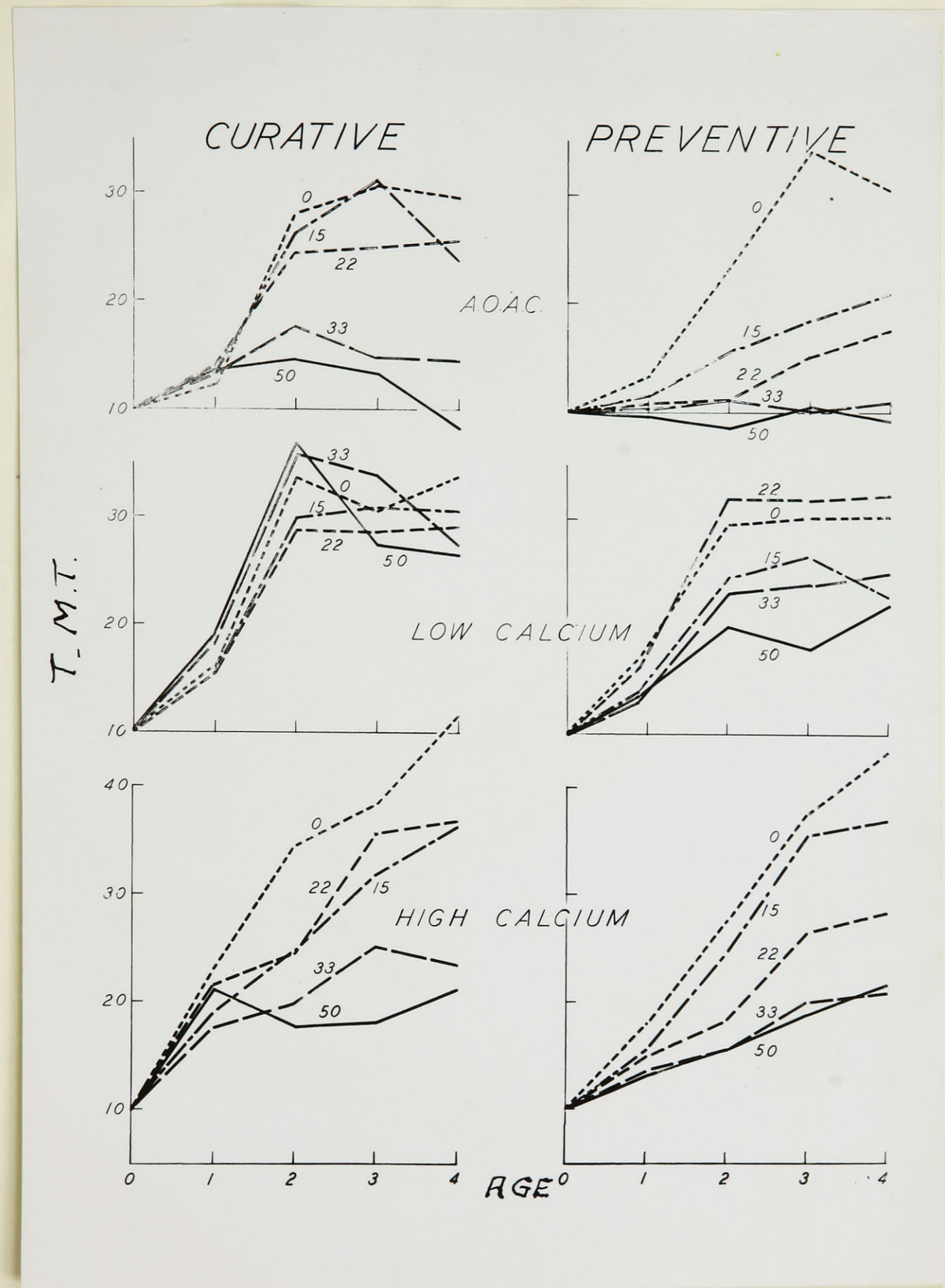


FIGURE 9. Response curves in preventive and curative assays for three diets plotted according to age. Numbers indicate dosage levels of vitamin D.

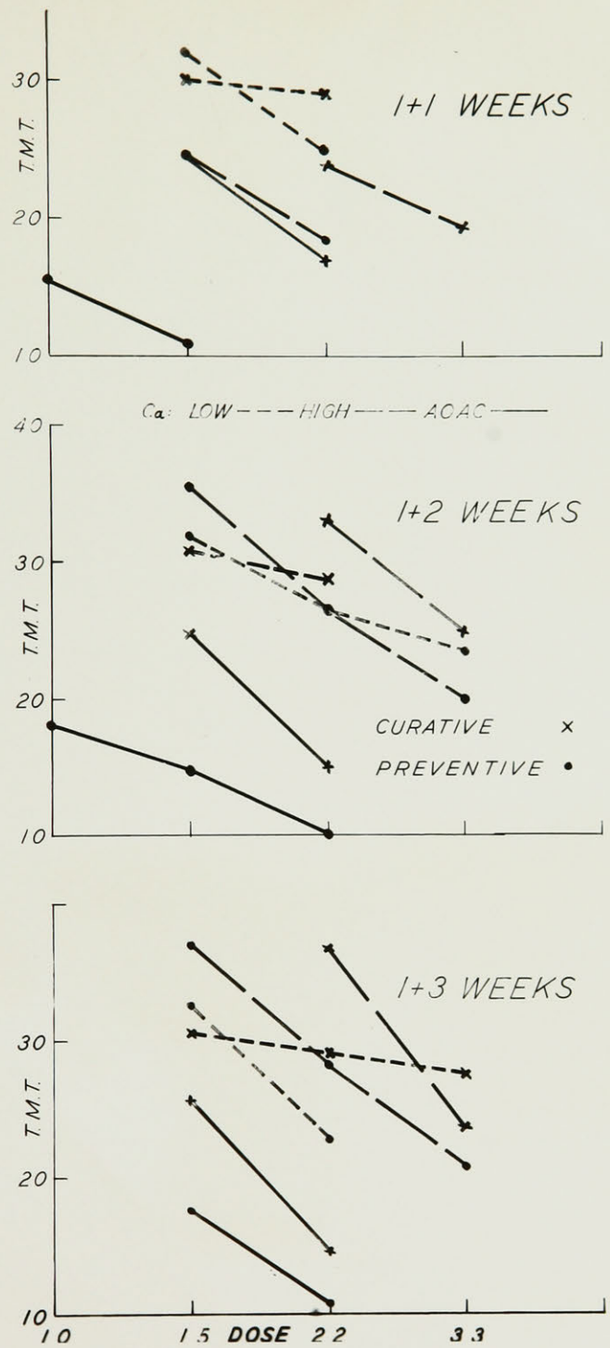


FIGURE 10. Steepest portion of response lines for each age group.

(from Figure 8)

The data in Table 18 show the same trend as the figures. It is also evident that the mean squares for error in the A.O.A.C. diets are much lower than those for the high calcium diets. This indicates that differences between doses need not be so great for the A.O.A.C. diet as for the others to obtain the same degree of precision. The data also show that the A.O.A.C. diet is at least as good as either of the others, but on the basis of only one test no definite conclusions will be drawn.

In general, age seems of greater importance in the chick assay than diet. The curative period makes it possible to obtain an equally precise assay one week sooner. The exact status of the low calcium diet is not clear from these data but the A.O.A.C. diet is as useful as any diet tested.

6. Breed of Chicks

White Leghorn chicks have been used almost exclusively for vitamin D assays in the U.S. as evidenced by the methods proposed by Carver et al. (1939), Massengale and Bills (1936) and A.O.A.C. (1940). Olsson (1941) has also recommended their use. On the other hand, the B.S.I. (1940) method recommends crossbred chicks of one sex. In general chick feeding, it is recognized that crossbred chicks are more vigorous and exhibit more uniform and rapid growth than pure bred chicks, but comparatively little information is available on their behaviour in vitamin D assays.

Heiman and Tighe (1942) reported that the vitamin D requirements of Leghorn chicks were the same as those of crossbred

TABLE 18

Variance ratios for 3 Diets Using Curative and Preventive Feeding Periods

PERIOD	DIET	VARIANCE* DUE TO	AGE OF CHICKS IN WEEKS					
			2		3		4	
			Variance	F	Variance	F	Variance	F
Curative	Low Ca	Dose	--	--	--	--	51	0.3
		Error	--	--	--	--	170	--
	A.O.A.C.	Dose	1052	11.1	2004	25.6	2583	31.0
		Error	95	--	78	--	83	--
	High Ca	Dose	485	4.0	1978	9.6	3031	13.8
		Error	121	--	205	--	230	--
Preventive	Low Ca	Dose	1007	14.6	829	8.7	1659	14.7
		Error	69	--	95	--	113	--
	A.O.A.C.	Dose	408	8.9	408	10.2	924	16.5
		Error	46	--	40	--	56	--
	High Ca	Dose	799	8.9	1176	9.8	1344	9.3
		Error	89	--	120	--	145	--

* In each case, d.f. = 1 for dose
= 75 (approx.) for error.

chicks. The Leghorn had higher bone ash at all levels of vitamin D and reached a higher maximum calcification. The slope of the response lines were quite similar but no estimate of variation was possible. Motzok et al. (1943) carried our preliminary experiments on this subject and suggested that "crossbreeding of two inbred lines may reduce individual variability." A close examination of their data, however, reveals some discrepancies. Consider, for example, their Table 3, page 267. While two of their groups show very low coefficients of variation the third group shows greater variability than the three pure bred lots at the same per cent bone ash. Similar criticism may be levelled at their Table 4. Furthermore, the slope of the response curves of the crossbreds has not been considered at all.

It is possible that crosses of certain breeds may produce more uniform chicks than other breeds. In the same paper, Motzok et al., compared certain breeds as regards suitability for vitamin D assay. The authors conclude that the New Hampshires compared favourably with the White Leghorns but that the Barred Rocks gave a smaller spread. In Table 19 the data have been recalculated and combined and they show that, expressed by the ratio of coefficient of variability/spread, New Hampshires are somewhat more precise in response than the other two breeds. The significance of this result is doubtful, but both experiments show the same trend. The results do suggest that there may be possibilities of getting a better chick than the pure bred White Leghorn.

TABLE 19

Breed of Chicks and Variability of Response

(re-analysis of data by Motzok et al. 1943)

BREED	MOTZOK'S TABLE 3			MOTZOK'S TABLE 4		
	Spread in ash percent	Coefficient of Variation	Coefficient / Spread	Spread in ash percent	Coefficient of Variation	Coefficient / Spread
White Leghorn	13.6	9.15	0.67	6.7	9.07	1.35
Barred Rock	11.6	7.81	0.67	5.0	7.87	1.57
New Hampshire	13.3	7.46	0.56	7.6	7.13	0.94

In view of the importance of this subject two experiments were set up to test the relative merits of pure versus crossbreds. In the first test an assay was set up in duplicate using the A.O.A.C. diet fed to pure breds and to crossbreds. Each part consisted of one negative control group and three levels of three groups each for both the unknown oil and reference oil. The pure breds were White Leghorns, and the crossbreds were obtained by mating Barred Rock males and Leghorn females. In the second test Leghorns were compared with both crosses of Leghorn and Barred Rocks. Eight replicate groups for each breed were fed at each of three levels of vitamin D as the reference oil. The numbers per group varied from 3 to 8 depending on the number available but was the same in all cages for any one breed. By feeding all three breeds in one cage possible pen differences were eliminated and the value obtained for replicates should give a more critical test. This system may, however, be criticised on the basis that if there was any tendency for the crossbreds to be larger and more active, they might take advantage of smaller chicks and any difference would consequently be exaggerated.

The data from the first experiment are presented in two ways. In Table 20 the s/b ratios for the X-ray data favour the crossbreds but those for toe ash data do not. The ratio of variances in Table 21 show a similar trend. Data from the second experiment are given in Tables 22 and 23 and in general show no conclusive differences between the breeds. Neither does there seem to be clear cut evidence of differences in the vitamin D requirements,

TABLE 20

Summary of s/b Ratios for Pure and Cross Bred Chicks

(on basis of individual chick)

BREED	S CALCULATED FROM VARIANCE FOR	X-RAY DATA s	X-RAY DATA b	X-RAY DATA s/b	TOE ASH DATA s	TOE ASH DATA b	TOE ASH DATA s/b
Pure	Replicates	0.4560	-0.4245	1.074	2.9136	5.2695	0.552
	Error	0.2114	-0.4245	0.498	--	--	--
Cross	Replicates	0.3061	-0.4948	0.619	2.5860	4.5547	0.564
	Error	0.1823	-0.4948	0.368	--	--	--

TABLE 21

Analysis of Variance for Pure and Cross Bred Chicks

Breed	Variation Due To	X-RAY DATA			TOE ASH DATA		
		D/F	Mean Squares	F	D/F	Mean Squares	F
Leghorn	Doses	3	0.5790	13.0	3	4.2873	8.08
	Replicates	10	0.2079	4.7	10	0.5306	--
	Error	249	0.0447	--	--	--	--
B.R. x L.H.	Doses	3	1.1450	34.5	3	2.1031	5.03
	Replicates	10	0.0937	2.8	10	0.4180	--
	Error	266	0.0332	--	--	--	--

TABLE 22

s/b Ratios for Pure and Cross Bred Chicks

BREED	TIBIA ASH			TOE ASH		
	s	b	s/b	s	b	s/b
Leghorn	0.9482	10.0041	0.095	0.5282	3.0695	0.172
L.H. x B.R.	1.4894	13.1094	0.114	0.5344	4.8053	0.111
B.R. x L.H.	1.8226	10.7774	0.169	0.4671	3.6413	0.128

TABLE 23

Analysis of Variance for Data on Pure and Cross Bred Chicks

BREED	VARIATION DUE TO	D/F	TIBIA ASH		TOE ASH	
			Mean Squares	F	Mean Squares	F
Leghorn	Between Doses	2	23.5170	26.2	2.3778	8.5
	Within Doses	21	0.8991	--	0.2790	--
L.H. x B.R.	Between Doses	2	43.0151	19.4	5.7777	20.2
	Within Doses	21	2.2185	--	0.2856	--
B.R. x L.H.	Between Doses	2	28.9428	8.7	3.3163	15.2
	Within Doses	21	3.3219	--	0.2182	--

i.e., there is no difference between breeds in the amount of vitamin required to get initial response or optimum response. Both experiments confirm the increased growth of crossbreds. If growth were the criterion of vitamin D action, undoubtedly there would be an advantage in using crossbreds.

As mentioned above, strain differences must not be overlooked. It is possible that this factor was operative in the above experiments. Recent discussions with other workers in this field indicate that much work is being undertaken to study this problem.

7. The Estimation of Error from Replicate Groups.

It has been the general practice in this laboratory, when comparing different criteria of calcification, to feed three or four groups of chicks at each dosage level. It is thus possible for each vitamin D supplement to partition the total variance into (1) variance between individuals of each group, i.e., error variance, (2) variance between replicate groups at each dose, and (3) variance between doses. Fiducial limits are ordinarily calculated from the error variance. However, if the variability between replicate groups exceeds that between individuals of one group, then the error variance does not adequately express the variability of the assay. Under these circumstances, Bliss (1943) recommends that individual data be disregarded and group replication practiced. The error might then be estimated from the variance between the replicates, considering them as individuals.

This procedure would involve considerably less labour by eliminating the need for individual data. It would also eliminate the confounding of treatment effects with possible pen differences as different dosage groups must be kept in different pens. As to whether or not such a procedure should be adopted would depend upon the average relationship between the variance for replicates and variance for error for each individual laboratory.

This study was based on assays and methods which have been described above. Analyses of variance have been carried out on each oil, both reference and unknown, which has been fed to replicated groups of chicks. For both the tibia ash and 3 week radiographic methods more than 30 oils, involving over 4,000 chicks, were analyzed statistically. Lesser numbers were available for the other methods.

To determine the effect of the two variances on the estimation of error, the fiducial limits were calculated by using group means as individual values. The variance between these replicated group means was taken as the error variance and the value of "t" for the 5 per cent points properly adjusted for the degrees of freedom for replicates rather than for individuals. The means were not weighted for the number of chicks. Where the number of chicks in each group is approximately equal, as is the case in the assays reported herein, the error of this procedure is negligible. For purposes of comparison, the limits, calculated in the usual way from error variance as reported previously,

were also tabulated. The B.S.I. (1940) system of calculation was used for both types of data and the approximate formula for fiducial limits was employed. As the potency is the same for both methods fiducial limits are just as valid as the λ ratio for comparison of methods in this case.

The pooled analysis is presented in Table 24. It is evident that for all four methods the variance between replicates, on the average, exceeds the variance for error. The ratio of these two variances is significant in each case at the 5 per cent point on the basis of the pooled degrees of freedom, but is not significant (e.g., tibia ash) on the basis of the average number of degrees of freedom available for the individual oils. The same is true for all criteria of response except that of toe ash. This would indicate that the variance ratios are on the borderline of significance and exhibit values which require more than one assay before their significance can be established. The important fact seems to be that while the ratios are not excessively large (toe ash to the contrary), nevertheless, the variance for error does underestimate the whole variability of the assays.

In order to determine what effect the ratios of the replicate variance to the error variance had on the fiducial limits, part of the series of assays was recalculated using the group means as single individual values. To clarify further the relation between the variance ratios and the limits, the F values for replicates were computed for the combined unknown and standard oils and

TABLE 24

Pooled Data for Comparison of Variances
Between Replicates and for Error

Criterion of Calcification	No. of Oils	Variance Due to	Degrees of Freedom	Mean Squares	F Values	Necessary F (P=0.05)
Tibia ash	31	Replicates	168	17.834	1.71	1.19
		Error	4251	10.449	--	--
Toe ash (extracted)	9	Replicates	53	6.131	2.36	1.35
		Error	1384	2.600	--	--
Radiographs (3 weeks)	35	Replicates	183	0.0527	1.24	1.18
		Error	4140	0.0425	--	--
Radiographs (2 weeks)	13	Replicates	76	0.0361	1.50	1.29
		Error	1571	0.0241	--	--

entered along with the limits. These data are given in Table 25 with means of groups of assays for comparing the different criteria of calcification more critically.

Table 25 shows that the relation of the limits calculated from replicates to those calculated from error varies in proportion to the F value. There is an appreciable difference in the magnitude of the F values of different assays. Averages for all assays at the foot of this table show that for all methods the limits calculated from replicates are on the average wider than those calculated from error. In the comparison of methods the three week X-ray method and the tibia ash method on the average yield quite similar results (assays 87-95). In the next comparison (assays 99-114) limits for replicates for the 3 week methods give the same interpretation of the variability, with tibia ash being a probable exception. This discrepancy seems to be caused chiefly by Assays 105-109 which were carried out at the same time and which may not be entirely representative.

Examples of the methods of calculation are given in the Appendix for both the B.S.I. (1940) procedure, using individual X-ray data, and the Bliss (1940) method, using replicated group (fresh toe) data, to permit comparison of the two methods. Bliss's method is particularly suited to the use of group means as there should seldom be difficulty with missing values. The differences in the calculations are obvious. When these are considered, together with the labour saved in composite ashing, some idea of the advantages

TABLE 25

Comparison of fiducial Limits Calculated from Variance Between Individuals (error) and from Variance Between Replicate Groups

ASSAY NO.	NO. OF CHICKS	LIMITS CALCULATED FROM	APPROXIMATE FIDUCIAL LIMITS[1]					COMBINED F VALUES			
			Tibia Ash	Toe Ash (Extracted)	Radiographic (3 weeks)	Radiographic (2 weeks)	Toe Ash (Fresh)	Tibia	Toe (Extracted)	X-ray (3 wks.)	X-ray (2 wks.)
62	259	Replicates	85-117	78-128	--	--	--	0.84	1.70*	--	--
		Error	86-116	85-118	--	--	--	--	--	--	--
77	400	Replicates	81-124	91-110	--	--	--	4.15*	0.93	--	--
		Error	91-110	91-110	--	--	--	--	--	--	--
87	340	Replicates	84-119	82-123	80-125	--	--	0.94	0.67	1.13	--
		Error	85-118	80-125	83-121	--	--	--	--	--	--
88	332	Replicates	85-118	65-154	80-125	--	--	0.62	3.09*	1.09	--
		Error	85-118	79-126	82-123	--	--	--	--	--	--
89	358	Replicates	89-113	65-153	78-128	--	--	0.40	2.48*	1.26	--
		Error	84-119	78-128	82-121	--	--	--	--	--	--
90	331	Replicates	80-125	78-129	79-126	--	79-126	1.93*	--	1.51	--
		Error	87-115	--	85-118	--	--	--	--	--	--
91	310	Replicates	84-119	84-120	88-114	--	69-145	1.38	--	0.75	--
		Error	85-118	--	84-119	--	--	--	--	--	--
92	307	Replicates	80-125	83-121	85-118	--	79-126	2.49*	--	1.09	--
		Error	86-117	--	85-118	--	--	--	--	--	--
93	341	Replicates	80-125	89-112	80-125	--	--	1.78*	--	1.01	--
		Error	86-116	--	82-122	--	--	--	--	--	--
94	340	Replicates	81-124	--	82-122	--	--	2.26*	--	1.01	--
		Error	88-114	--	84-119	--	--	--	--	--	--
95	319	Replicates	83-121	--	82-122	--	--	1.77	--	1.18	--
		Error	87-115	--	85-118	--	--	--	--	--	--
99	322	Replicates	66-151	71-142	68-146	--	78-128	--	--	1.21	--
		Error	--	--	71-141	--	--	--	--	--	--
100	340	Replicates	72-139	85-117	80-125	--	88-114	--	--	1.02	--
		Error	--	--	74-135	--	--	--	--	--	--
101	325	Replicates	71-141	81-124	70-142	--	82-123	--	--	1.27	--
		Error	--	--	76-132	--	--	--	--	--	--
102	324	Replicates	74-135	82-121	79-127	--	86-116	--	--	0.92	--
		Error	--	--	77-129	--	--	--	--	--	--
103	326	Replicates	58-174	70-142	58-174	--	74-135	--	--	2.02*	--
		Error	--	--	70-142	--	--	--	--	--	--
104	270	Replicates	87-115	85-117	80-125	72-138	87-116	--	--	1.06	1.89*
		Error	--	--	83-121	80-125	--	--	--	--	--
105	245	Replicates	51-195	75-134	67-148	64-156	83-121	--	--	1.95*	1.42
		Error	--	--	79-127	72-139	--	--	--	--	--
106	244	Replicates	30-337	75-133	65-154	58-172	78-129	--	--	1.25	1.14
		Error	--	--	73-138	64-157	--	--	--	--	--
107	241	Replicates	37-271	73-137	65-153	50-200	76-132	--	--	1.62	2.23*
		Error	--	--	76-132	66-152	--	--	--	--	--
108	237	Replicates	41-241	75-133	68-148	63-158	77-130	--	--	1.23	1.12
		Error	--	--	74-135	69-146	--	--	--	--	--
111	429	Replicates	84-119	86-116	74-135	58-172	80-125	--	--	1.59	1.43
		Error	--	--	83-121	76-132	--	--	--	--	--
112	198	Replicates	74-135	82-122	73-138	47-211	58-173	--	--	1.46	1.07
		Error	--	--	80-126	59-171	--	--	--	--	--
114	266	Replicates	80-125	61-164	63-158	72-140	64-156	--	--	1.36	0.44
		Error	--	--	70-144	63-158	--	--	--	--	--
Means of assays for comparing methods											
62-89		Replicates	85-118	76-134	--	--	--	1.39	1.77	--	--
		Error	86-116	83-121	--	--	--	--	--	--	--
87-95		Replicates	83-121	--	82-123	--	--	1.51	--	1.11	--
		Error	86-117	--	84-120	--	--	--	--	--	--
99-114[2]		Replicates	63-175	77-131	70-144	61-168	78-131	--	--	1.38	1.34
		Error	--	--	76-133	69-148	--	--	--	--	--
All assays[3]		Replicates	83-121	76-134	75-135	61-168	--	1.69	1.77	1.27	1.34
		Error	86-116	83-121	79-128	69-148	--	--	--	--	--

* F values significant at 5% point. Significance of mean F values not assessed here.

1 Approximate limits calculated for "t" at P = 0.05.

2 Only 8 assays used in average for 2 week radiographic method.

3 All assays having values for both replicates and error.

of this system may be gained. Furthermore, as mentioned before, because different dosage groups cannot be fed in the same pens the variance within pens may not be a valid error for testing differences between treatment, e.g., in this laboratory the testing error would be underestimated.

8. The Estimation of Error from the Variation in Response of Replicate groups with Time.

It has been observed that the refinements introduced in the preceding section may not explain all the discrepancies found in the chick assay. The variation in repeated assays of the same oil are often greater than would be expected. Discussions with other workers indicate that this difficulty is not confined to this laboratory. Discrepancies in the data of some laboratories mentioned by Bliss (1946) suggest the presence of some factor or factors which cause differences between assays greater than the internal errors of the assays. The impression is that an estimate obtained from a series of assays may be more reproducible than one which is determined at any one time, because of the possible bias of a single test. Bliss (1945) has suggested the use of a combined slope from several assays. Waddell and Kennedy (1947a) showed that this procedure has advantages. Whether it would be applicable to all laboratories would depend on the relative magnitude of the factors involved. In view of the importance of this problem, it seemed desirable to study the variability of response when assays are repeated at different times.

Series of assays for each of the three criteria of calcification, tibia ash, toe ash and X-ray, were selected from data extending over two years work. Details of the assay procedures were given in earlier sections. Only those assays were selected in which four groups of chicks had been fed the Canadian Standard Reference Oil at each of three dosage levels. The dosage levels (9, 15 and 25 units per 100 gm. diet) and the distribution of groups in the various pens were the same in all assays. Under these restrictions there were 12 monthly tests available for tibia ash, 19 tests for toe ash and 6 tests for the radiographic method. Analysis of variance was carried out in each case on the group means. Assuming that the variance within groups determined in the previous section was a reliable estimate, this variance was converted to the group basis by dividing by the mean of the number per group (Snedecor and Cox, 1935). As the numbers per group were very uniform the error of this method was considered relatively unimportant. Data from an analysis of an assay which had been replicated three times are also included. In this assay 3 groups were fed at each of three doses during each of three months. The dosage levels were 10, 15 and 22.5 units per 100 gm. diet.

In the study of the reference oil alone, four groups at different cage levels were fed each of three doses of the standard oil. Accordingly, the sources of variation in each month may be partitioned as follows:

Factor	D.F.	
Cage level (replicate)	3	
Slope (b)	1	)
		) Dose
Curvature	1	)
Cage X Slope	3	)
		) Cage X Dose
Cage X Curvature	3	)

It was found that although cage X slope interaction tended to be somewhat greater than the cage X curvature interaction, they were similar enough to be pooled for an error term. This cage X dose variance was then determined for each assay and the series of values so obtained tested for homogeneity by Bartlett's χ^2 test. The χ^2 values for tibia and X-ray methods were 17.4 (11 d.f.) and 4.16 (5 d.f.), respectively. These were not significant at the 5 per cent level but the χ^2 for toe ash, 44.17 (18 d.f.), was significant. Nevertheless, these cage X dose variances were pooled over the whole length of the experiment to furnish an over all error term with 72 degrees of freedom for tibia ash 114 for toe ash and 36 for the X-ray data. The complete analysis of variance is given in Table 26. For comparative purposes, the variances within subclasses and within groups of chicks discussed in the previous section are also listed.

The significance of cage level and months may be judged by the magnitude of the interaction of the two factors. Only in the case of tibia ash is the cage level significant and then just on the borderline. The month factor is highly significant in all

TABLE 26

Analysis of Variance of Response to Canadian Standard Reference Oil in Several Assays for Three Criteria of Calcification

SOURCE OF VARIATION	TIBIA ASH		TOE ASH		X-RAY	
	D.F.	Mean Squares	D.F.	Mean Squares	D.F.	Mean Squares
Months	11	24.93**	18	6.759**	5	0.2483**
Cage level (Reps)	3	1.78	3	0.125	3	0.0002
Cage x Month	33	0.61	54	0.199	15	0.0023
Slope b_c	1	664.97**	1	156.350**	1	0.6211**
Curvature	1	0.21	1	0.377	1	0.0182*
Months x Slope	11	2.86**	18	1.180**	5	0.0102**
Months x Curvature	11	2.80**	18	0.488**	5	0.0053
Error	72	1.09	114	0.236	36	0.0030
Within dose month subclasses	108	0.96	171	0.222	54	0.0026
Within groups	2300	0.580	3600	0.145	1200	0.0024

* Significant at P = 0.05
** " " P < 0.01

cases indicating that the response is subject to periodic variation. This should not affect the precision of the assay as both standard and unknown oils should be affected equally.

The slope is highly significant. The fact that the curvature is negligible except for X-ray data indicates that, on the whole, the assays were valid as far as linearity was concerned. The higher curvature for the X-ray data suggests a difference in the sensitivity of this criterion to the levels of vitamin fed. Examination of the curves show that somewhat lower levels would furnish a more sensitive assay when the X-ray criterion is used. The month X slope interaction is significant in all cases, indicating that the slope of the dose response curve varies significantly from month to month. The month X curvature interaction is also significant for toe and tibia ash. It is suggested that this combination of conditions may be caused by changes in the sensitivity of the chick to vitamin D, i.e., the amount of vitamin required to give measurable response. That this is probably the most important single factor is demonstrated by the fact that when the slope X month interaction is recalculated using only the steepest part of each response curve, its significance disappears in the case of tibia ash and X-ray data and is greatly reduced in the toe ash data although it still holds a probability of approximately 0.01 per cent. The slope X month interactions become 0.87, 0.556 and 0.0030, resp. for tibia, toe and X-ray data. The reason for the higher variability of the toe ash response is not clear but as the same assays

were not available for all criteria their comparison is not as critical as would be desired. The stability of slope in the tibia and X-ray data are in line with the findings of Bliss (1945).

Table 27 presents an analysis of the response to two oils. In each assay an error term was calculated with 10 degrees of freedom consisting of the pooled interactions involving the replicate or cage level factor. These terms were essentially homogeneous from one assay to another and were, therefore, pooled to obtain an error term for the whole experiment with 30 degrees of freedom. The difference between the assumed potencies of the oils is larger than desirable and possibly contributes to the significant lack of parallelism. This condition, however, should not invalidate tests of other factors. Variation of slope from month to month is not significant, although for tibia ash the potency changes appreciably. The interaction of months with other effects (6 degrees of freedom) is significant for the X-ray data and fairly high for tibia ash.

Because of the high month X curvature effects in Table 26 and the relatively high interaction of months with other factors in Table 27, it is evident that under the conditions of this laboratory the most sensitive part of the curve is not always in the same part of the range of 9, 15, and 25 units as used here. Possibly there should be four doses in this range for this laboratory. The difficulty of determining the exact position is obvious when the factors controlling these changes are not understood. Experimental conditions may vary in other laboratories to produce different

TABLE 27

Analysis of Variance of Response of Two Oils
in Three Assays by Three Criteria

SOURCE OF VARIATION	D.F.	MEAN SQUARES		
		Tibia Ash	Toe Ash	X-ray
Months	2	17.659**	2.688*	0.0514**
Cage Level (Reps)	2	0.164	0.221	0.0005
Cage x Month	4	0.752	0.336	0.0010
Oils	1	417.667**	52.412**	0.9600**
Combined Slope	1	68.476**	18.233**	0.2147**
Parallelism	1	11.776**	1.751	0.0289**
Combined Curvature	1	1.078	0.013	0.0018
Opposed Curvature	1	0.163	0.475	0.0000
Months x Oils	2	6.798*	0.542	0.0034
Months x Slope	2	0.864	1.294	0.0000
Months x Other Effects	6	3.028	0.394	0.0105**
Other Interactions	30	1.393	0.519	0.0018

* Significant at P = 0.05

** " " P < 0.01

effects from those observed here. Studies on the factors causing this change in the position of the response level should aid a great deal in improving the assay technique. Undoubtedly they include such influences as season, temperature, diet of parent stock, supply of vitamin and mineral in the egg and possibly others.

As the slopes are actually homogeneous for tibia and X-ray data the use of a combined slope as suggested by Bliss (1945) is permissible. It would mean, however, that more dosage levels should be fed but only the sensitive portion of the curve used in calculations. This may mean discarding some groups of chicks. If 3 doses are used for calculations as is customary, there remains the question as to what should be done until dosage responses can be predicted accurately, or can be controlled at constant levels. Alternative suggestions include the use of only the steepest part of the response curves, or the replication of assay groups over a period of time. The latter method may be particularly effective in aiding in the study of the factors involved, particularly the variation from month to month. This method would also seem to be indicated for assays such as the determination of the potency of D_3, where the most accurate and reproducible work is required. When the variance is increased by the use of month factors in this way the fiducial limits of the assay will be found somewhat wider. However, there should be greater probability of duplicating an assay within predicted limits, i.e., greater reproducibility.

That the month factor is of considerable importance in this laboratory is further substantiated by studies on the growth of chicks on vitamin D tests. Data on the increase in weight in 3 weeks of chicks fed the A.O.A.C. diet with various levels of the Canadian Standard Reference Oil were selected from 21 assays involving almost 5,000 chicks. The standard oil had been fed to 3 or 4 groups of chicks at each of three dosage levels. These levels were the same in each assay and were considered as different diets for the purpose of this study. An analysis of variance presented in Table 28 was made on the group means. The variance for individuals, based on 11 assays, was calculated in the usual manner and divided by the harmonic mean (18.8) of the number per group to bring it to the group basis.

It is evident that, using the within group variance as testing error, there is a significant variance between replicates or within subclasses. Using the latter variance as testing error, there is a significant variability for the diet X month interaction. This indicates that the difference between diets in different months varies significantly more than would be expected from random sampling.

It may, therefore, be concluded that until conditions can be controlled and understood, the error of an assay may be found to be greater than predicted from the variance within or between replicate groups of chicks. The replication of groups over a period of time may furnish a more reproducible type of assay and be particularly useful in experimental studies of the method.

TABLE 28

Analysis of Variance of Repeated Tests on Growth of Chicks on Three Diets[1]

Variance Due to	D.F.	Mean Squares
Diet	2	2007**
Month	20	1373**
Diet x Month	40	81*
In subclasses	165	55*
Within groups	2090	27.6

1 Analysis consists of 2 series of assays, 3 groups per diet for 8 months and 4 groups per diet for 13 months. Interaction and main effects compared with subclasses and subclasses with groups.

* Significant at $P = 0.05$
** " " $P < 0.01$

DISCUSSION

The review of literature has indicated that a great deal of research has been put into the chick assay for vitamin D yet the methods are still cumbersome and far from satisfactory. In many experiments there has been little if any adequate statistical control. In this thesis an attempt has been made to critically assess various modifications of the assay method with the aid of recently developed bioassay techniques.

Four criteria of calcification have been compared. All methods show considerable and approximately equal variability in their estimate of the potency of the oils and in their reproducibility. For an estimate of the precision of an assay involving individual data, the radiographic technique has unquestioned advantages in manipulation and practically equal precision to other methods. If X-ray equipment is not available, the use of toe ash on the extracted basis offers considerable simplification and equal precision to the official tibia ash procedure. If a purely routine procedure is required, the use of toes on the fresh basis undoubtedly furnishes the most rapid procedure. The 2 week feeding period seems of particular value for use with fresh toes although it also is applicable to the other procedures for estimates of potency involving composite data. Its wide application, however, may be limited as the precision is relatively low.

A comparison of the preventive and curative types of feeding periods indicate that the age of chicks is more important

than the type of period. The spread between dosage levels increases with age. It is possible to obtain a 35 to 40 per cent increase in precision by the use of a 3 week curative period as compared to the 3 week preventive, for the chicks are a week older at the start of the curative period. Provided diets produce a reasonable degree of rickets there is little difference for diets of widely differing calcium and phosphorus contents in the slope per unit dose. The position of the response line, however, may may vary markedly. The A.O.A.C. diet is as good as any which have been tested. The advantage of crossbred chicks does not seem to be as important as has sometimes been postulated. It is suggested that the strain of chicks may be of equal or greater importance.

The calculation of fiducial limits in the usual manner from the error variance may very definitely underestimate the whole variance of an assay. For this laboratory, the adoption of routine replication of groups to be used for the estimation of error seems to be indicated. Besides expressing the error of an assay more adequately, the use of replicate groups will cut the labour of the toe ash methods to less than 1/15 of that required for individual data. The latter may then be disregarded. As there is fair agreement in most cases between limits calculated from composite fresh toe data and from other criteria of response, the use of fresh toes should merit wider use as a rapid routine procedure.

The applicability of this system of analysis to data obtained in other laboratories will, of course, depend on the relation between the two variances in those laboratories. However, in view of the differences found between replicate groups in collaborative assays, e.g., Baird and Barthen (1941), it may be expected that the use of replicate groups for the estimate of error should have a wide applicability.

The importance of the variation in response of replicate groups with time has been demonstrated. The results indicate that the chick assay for vitamin D is not as precise as it has sometimes been considered. Until these sources of variation are known and understood, it is suggested that repetition of assays may be necessary for reliable results. One group of chicks at each of three levels of each oil repeated in 3 or 4 months should offer a reproducible assay. This would make assays somewhat slower to complete but the increase in reliability of the result should warrant the time if a particularly reliable estimate is required. In view of the greater variability of slope and curvature with time, it will be evident that fiducial limits calculated from a variance involving months will be somewhat wider than if calculated from replicates in one assay or from individuals. The wider limits indicate that the chick assay for vitamin D is not as precise as it has sometimes been considered. It suggests the need for repetition of experiments before general conclusions are drawn. It also suggests that some of the lack of agreement found in

collaborative assays and chick experiments may be attributed to variation within the laboratories themselves causing a bias in individual tests.

Since this system of assay was proposed, Waddell and Kennedy (1947b) with the collaboration of Bliss, published a method of analysis involving the estimation of error from the deviations of group means from the log. (dose)-response curve. There was no essential difference between this method and the calculation of error from replicates when applied to the same assays, i.e., both criteria gave the same measure of experimental error. If several replicates or months are used for each dose then the method proposed in this thesis would be the logical one to use. However, if replication is not practiced then the method of Waddell and Kennedy must be used. The question then follows, is replication necessary? It is the author's opinion that it is necessary until such time as more is known about the chick assay method. The variability of the variance for error in the work of Waddell and Kennedy (1947a) indicates that their method does not estimate it as reliably as would be desired. The use of the combined slope markedly improves the estimate of error and eliminates some of the rather impossible values. However, as pointed out in the last section, to use a combined slope it must be relatively uniform from assay to assay. To obtain this uniformity, certain doses may need to be discarded. This again reduces the available data.

It is pointed out that our knowledge of vitamin D assay is still far from complete. The most logical approach to the problem would seem to be a comprehensive study by several laboratories of all factors which cannot be held under control but which may cause variability in the assay results. For this purpose repeated assays of oils should be carried out using replicated groups at each dosage level. It is with this idea in view that the method outlined in the Appendix has been proposed.

In view of the advantages of using a crystalline material rather than a cod liver oil for reference standard, the following recommendations are made: (1) That crystalline D_3 be used as a standard for vitamin D tests with chicks, (2) that in place of the International Standard for D_2, vitamin D_3 be used as the International Standard for all vitamin D tests, (3) that the standard solution be made up in the same way as the B.S.I. (1940) provisional standard, i.e., each mg. solution to contain 0.000025 mg. crystalline D_3, (4) that the weight system of expressing potency be adopted with the descriptive term "rat test" or "chick test" appended to indicate the type of test applied, (5) that if the unit system is to be adhered to, the units be designated "International chick unit" and "International rat unit" of vitamin D.

The response range of 10 to 20 A.O.A.C. units would be equivalent in the weight system to approximately 0.2 to 0.4 micrograms as one unit would be equivalent to approximately 0.02 micrograms. Of course, as mentioned above, if the weight system were used it would eliminate the use of units entirely.

APPENDIX

The following method is proposed on the basis of a consideration of the literature and of the results reported in this thesis. This method is not to be considered as a final or complete procedure but rather as a tentative one which may serve as a guide in future studies of the method. That there is great need for such detailed studies is evident from the foregoing discussion. The method should be especially useful where a particularly reliable estimate of potency is required, e.g., in the estimation of potency of pure vitamin D_3.

The system of statistical analysis chosen to handle the data is that of Bliss and Marks (1939a, b) and Bliss (1940). Other methods give approximately the same results and the B.S.I. method, which has been used to a considerable extent in this thesis, is also outlined here (Tables IV to VII) for reference purposes. In view of recent changes in the concept of error in chick assays, which have been outlined in this thesis, and of the need for statistical studies of the procedure the method of Bliss was thought to offer certain advantages.

TENTATIVE PROCEDURE FOR CHICK ASSAY OF VITAMIN D_3

The assay is conducted on groups of chicks kept in screen bottom cages away from sunlight or other source of actinic light. There should be constant temperature control throughout the room during the test period. Artificial light is supplied

for 12 hours *per diem*.

Chicks

Chicks, from stock of uniform breeding which has received an optimum level of vitamin D, are placed in batteries at one or two days of age and fed the rachitogenic diet *ad libitum* for one week. At this time the chicks are distributed at random into groups of ten to twenty and fed the rachitogenic diet with various levels of vitamin D supplements.

Diet

The A.O.A.C. diet is recommended. The composition is as follows:

	Per cent
Ground corn	57
Middlings	25
Casein	12
Non-irrad. yeast	2
$Ca_3(PO_4)_2$	2
Iodized salt	1
Corn oil	1
$MnSO_4$	0.2 gm./kilo.

The ingredients must be thoroughly mixed. The calcium: phosphorus ratio should approximate 1.2:1 with a phosphorus content of 0.7-0.9 per cent. Only one batch of diet should be used for all groups of any one test. To facilitate mixing with the diet, the oils containing vitamin D are diluted to one per cent of the

diet with corn oil. Negative control diet is mixed with one per cent corn oil alone. Fresh water is supplied ad libitum to all groups.

System of Assay

All data will be on the basis of group means. All responses must be on the linear part of the log. (dose)-response curve, and dosage levels should be spaced at equal logarithmic intervals to permit coding in the calculations.

For routine tests, three or four groups of chicks should be fed at each of three dosage levels of the standard reference oil. It is desirable to have the same distribution of groups for the unknown oil.

For assays where greater accuracy and reproducibility are desired the groups should be replicated over a period of weeks or months, using different lots of chicks, diet, etc. This will tend to eliminate the possible bias of an assay carried out completely at one time.

The length of the feeding period on the curative diet may be varied from 2 to 4 weeks according to the precision required. At the end of the period the chicks are killed by chloroforming in groups.

Criteria of Calcification - Toe Ash

Centre toes are removed at the third joint from the claw, taking care not to cut the bone on either side of the joint. The determination of ash may be made on the fresh basis when the toes

are put into tared weighing bottles. After weighing, they are ashed at 800° C. to constant weight, the temperature being raised slowly at first. If ashing cannot be carried out immediately the toes may be put into crucibles and dried to constant weight. When convenient the toes are ashed in these crucibles. The moisture-free fat-free basis may also be employed to express toe ash, in which case the toes are wrapped in hardened filter paper by groups and preserved in 95% ethyl alcohol until extraction can be carried out. Extraction with alcohol and ether may be carried out in a large Soxhlett or Bailey-Walker type extractor. The use of both centre toes serves to increase the accuracy of the test with little extra labour.

Estimation of Potency and Error of Assays

Bliss's (1940) factorial analysis has been applied to the chick assay for vitamin D. The calculations of an assay in terms of fresh toe ash data are given in Tables I and II. It is necessary that equal numbers of responses be available for each dose of both standard and unknown. Dosages should be equally spaced on the logarithmic scale to facilitate coding. The responses, i.e., group means, are put down under their appropriate headings. In this case there are three replicate groups for each of three doses of standard (S) and unknown (U). Analysis of variance is then carried out on these group means. The total sum of squares is partitioned into that between doses and within doses or error. This partition is used where replicate groups are fed

in one complete assay. If these replicates constitute different months or positions then the variance for the three months can also be segregated from the error variance. As pointed out previously, this may result in considerably greater reproducibility of the estimate of potency. The error would then have ten instead of twelve degrees of freedom and be made up as follows:

	D.F.
Month X dose	4
Month X oil	2
Month X dose X oil	4

In Table II, Bliss's factorial coefficients for the sources of variation are given and factors are obtained for calculating potency and limits. The totals of the 3 responses are put down under the appropriate headings. The column headed "Divisor" is obtained by multiplying the number of responses for each dose by the square of the coefficient (x) for that dose. The next column is the sum of the products of each total multiplied by its appropriate coefficient. The values may be positive or negative. The variance column is the square of the sum of products divided by the divisor for each item, and the last column is the square root of the variance. Factors D and B take the sign of the corresponding sum of products, e.g., for X-ray data, B is negative. The significance of the various factors is tested by comparing their variances with that for error determined from the analysis of variance. The first value in the variance column

indicates the difference between unknown and standard. The second shows the significance of the slope of the curves and should be large for a good assay. The last three are tests for non-parallelism, curvature, and opposed curvature. In an ideal assay, variances for these factors should be less than the testing error. In this case there is significant curvature of the response lines (1 degree of freedom) when compared to error (12 degrees of freedom).

Using the values obtained, the potency and limits are then calculated by substituting in the formulae. K for a 3 point assay is 1.6330 and I is the log. interval between doses. Data from this assay indicate that the potency may be expected to lie, with a probability of 95 per cent, between the limits of 98 and 136 units. The most probable potency is 115 units per gram.

The calculation of precision of response lines is illustrated in Table III. Standard deviation (s) is taken from the analysis of variance. The formula for slope (b) for an uneven number of doses is given and the factors are taken from the appropriate columns of Table II. I is the log. interval. The value of 0.069 is found for the s/b ratio on the group basis. This may be converted to the basis of the individual chick by multiplying by the square root of the number per group. The standard error is obtained by substituting in the formula for symbols derived earlier. The number of responses for each oil is given by n. The s/b ratio is the most valid measure for comparing the precision of response lines for different treatments or methods.

TABLE I

Group Response and Analysis of Variance for Toe Ash Data

(fresh basis)

REPLI-CATES	RESPONSE						
	S_1	S_2	S_3	U_1	U_2	U_3	Totals
1	3.72	3.83	4.62	3.85	4.00	4.52	
2	3.44	3.75	4.40	3.67	4.19	4.52	
3	3.69	4.10	4.47	3.73	3.92	4.68	
Totals	10.85	11.68	13.49	11.25	12.11	13.72	73.10

$$\text{Correction} = T^2/N = \frac{(73.10)^2}{18} = 296.8672$$

Total sum of squares = 299.4048 - C = 2.5376

Doses sum of squares = 299.1926 - C = 2.3254

Variation due to	D.F.	Sums of Squares	Mean Squares	S.D.
Total	17	2.5376	--	--
Between doses	5	2.3254	0.4650	--
Within doses (error)	12	0.2122	0.0177	0.1330

TABLE II

Factorial Analysis of Response and Calculation of Potency and Error

Effect	Factorial Coefficients (x) for Dose S_1	S_2	S_3	U_1	U_2	U_3	Divisor $NS(x^2)$	Sum of Products $S(x\ Y_p)$	Variance $\frac{S^2(x\ Y_p)}{NS(x^2)}$	$\sqrt{\text{Variance}}$
Samples	-1	-1	-1	+1	+1	+1	18	1.06	$0.062 = D^2$	$0.249 = D$
Slope	-1	0	+1	-1	0	+1	12	5.11	$2.176 = B^2$	$1.475 = B$
Parallels	+1	0	-1	-1	0	+1	12	-0.17	0.002	
Curvature	+1	-2	+1	+1	-2	+1	36	1.73	0.083	
Opposed Curvature	-1	+2	-1	+1	-2	+1	36	-0.23	0.001	
Total Y_p	10.85	11.68	13.49	11.25	12.11	13.72		Error	$0.0177 = s^2$	$0.133 = s$

$$M = \frac{K\ I\ D}{B} = (1.6330)\ (0.2219)\ \frac{0.249}{1.475} = 0.0612$$

Potency = Antilog. M x Expected Potency = 1.152 x 100 = 115 units/gm.

$$s_M = \frac{s_K\ I \sqrt{D^2 + B^2}}{B^2} = (0.133)\ \frac{(1.6330)\ (0.2219)\sqrt{0.062 + 2.176}}{2.176}$$

$$= \frac{(0.04820)\ (1.4959)}{2.176} = 0.03314$$

$t = 2.179$ (for $n = 12$)

$ts_M = 0.0722$ and 0.9278

Limits = antilog. ts_M x 100 = 118% and 85% or 136 and 98 units.

TABLE III

Calculation of Precision of Response Lines According to Bliss and Marks (1939)

s = Standard deviation = 0.133

For 3 doses, slope = $b = \frac{S\ (x\ Y_p)}{I\ NS\ (x^2)} = \frac{5.11}{(0.2219)\ (12)} = 1.919$

On group basis $s/b = \frac{0.133}{1.919} = 0.069$

On individual basis $s/b = 0.069 \times \sqrt{N}$ per group = 0.308 (for 20 chicks/group)

Variance of ratio $= V_{(s/b)} = \frac{s^2}{b^2} \left(\frac{V_{(s)}}{s^2} + \frac{V_{(b)}}{b^2} \right)$

where $V_{(s)} = \frac{s^2}{2n}$

and $V_{(b)} = \frac{s^2}{I^2 NS\ (x^2)}$

$$V_{(s/b)} = \frac{s^2}{b^2} \left(\frac{1}{2n} + \frac{s^2}{b^2} \cdot \frac{1}{I^2 NS\ (x^2)} \right)$$

$$= \frac{0.0177}{(1.919)^2} \left(\frac{1}{(2)\ (9)} + \frac{0.0177}{(1.919)^2} \cdot \frac{1}{(0.2219)^2\ (12)} \right)$$

$$= 0.004806 \left(0.05556 + \frac{0.004806}{0.59088} \right)$$

$$= 0.004806\ (0.06369) = 0.0003061$$

S.E. $= \sqrt{V_{(s/b)}} = 0.0175$

$s/b = 0.069 \pm 0.018$ (group basis)

or 0.308 ± 0.081 (individual basis)

TABLE IV

Calculations on Responses to Reference Oil from Individual Data from Radiographic Method

(B.S.I., 1940, procedure)

Group No.	Dose Units D/100 gm. Feed	x_1	y_1	n_1	n_1x_1	n_1y_1	$n_1x_1^2$	$n_1x_1y_1$	$n_1y_1^2$	$S_1(t.m.t.)^2$	$f_1s_1^2$	f_1
1	9	-1	1.165	12	-12	13.985	12	-13.985	16.2984	16.9515	0.6531	11
2	9	-1	1.257	14	-14	17.601	14	-17.601	22.1282	22.3852	0.2570	13
3	9	-1	1.233	15	-15	18.489	15	-18.489	22.7895	23.2958	0.5063	14
				41		50.075			61.1587			
4	15	0	1.214	15	0	18.207	0	0.000	22.0997	22.5611	0.4614	14
5	15	0	1.065	14	0	14.914	0	0.000	15.8877	16.5178	0.6301	13
6	15	0	1.171	14	0	16.397	0	0.000	19.2044	19.5961	0.3917	13
				43		49.518			57.0240			
7	25	+1	0.962	13	+13	12.505	13	+12.505	12.0288	12.3524	0.3236	12
8	25	+1	0.901	15	+15	13.519	15	+13.519	12.1842	12.7332	0.5490	14
9	25	+1	0.989	15	+15	14.839	15	+14.839	14.6797	15.0558	0.3761	14
				43		40.863			38.8322			
	Sums			127	+2	140.456	84	-9.212	157.0149		4.1483	118
	Correction						0.032	2.212	155.3577			
	Difference						p_1	q_1	r_1			
							83.968	-11.424	1.6772	s_1^2 = 0.03516		

$\bar{x_1}$ = +0.0157

$\bar{y_1}$ = 1.106

$\frac{q_1^2}{p_1}$ = 1.5543

Difference = A_1 = 0.1229

TABLE V

Calculations on Responses to Unknown Oil from Individual Data from Radiographic Method

(B.S.I. 1940 Procedure)

Group No.	Dose Units D/100 gm. Feed	x_2	y_2	n_2	n_2x_2	n_2y_2	$n_2x_2^2$	$n_2x_2y_2$	$n_2y_2^2$	$S_2(t.m.t.)^2$	f_2s_2	f_2
11	9	-1	1.139	15	-15	17.088	15	-17.088	19.4666	19.8796	0.4130	14
12	9	-1	1.190	15	-15	17.850	15	-17.850	21.2415	21.7522	0.5107	14
13	9	-1	1.188	15	-15	17.826	15	-17.826	21.1844	21.6749	0.4905	14
				45		52.764			61.8675			
14	15	0	1.125	14	0	15.756	0	0.000	17.7323	18.6082	0.8759	13
15	15	0	1.053	13	0	13.683	0	0.000	14.4019	14.7854	0.3835	12
16	15	0	1.179	14	0	16.508	0	0.000	19.4653	19.9546	0.4893	13
				41		45.947			51.4909			
17	25	+1	0.950	15	+15	14.250	15	+14.250	13.5375	13.9675	0.4300	14
18	25	+1	1.005	14	+14	14.065	14	+14.065	14.1303	14.7589	0.6286	13
19	25	+1	0.911	15	+15	13.665	15	+13.665	12.4488	12.7105	0.2617	14
				44		41.980			40.0527			
Sums		130	-1	140.691	89	-10.784			153.4111		4.4832	121
Corrections					0.008	-1.082			152.2612			
Differences					P_2	q_2			r_2		$s_2^2 = 0.03705$	
					88.992	-9.702			1.1499			

$\bar{x}_2 = -0.0077$

$\frac{q_2^2}{P_2} = 1.0577$

$\bar{y}_2 = 1.082$

Difference = $A_2 = 0.0922$

Pooled s^2 (239 d.f.) $= \frac{4.4823 + 4.1483}{121 + 118} = 0.0361112$

Pooled $s = 0.1900$

Pooled $b = Q/P = \frac{-9.702 - 11.424}{88.992 + 83.968} = \frac{-21.126}{172.960} = -0.1221$

Pooled $s/b = -1.5561$

TABLE VI

Calculation of Potency and Fiducial Limits (approximate) from Radiographic Data (B.S.I. 1940 Procedure)

Potency

$$M = I\left(\bar{x}_1 - \bar{x}_2 - \frac{\bar{y}_1 - \bar{y}_2}{b}\right)$$

$\bar{x}_1 - \bar{x}_2 = +0.0234$, $\bar{y}_1 - \bar{y}_2 = +0.024$

$b = -0.1221$, I = Log. Interval = 0.2219

$$M = 0.2219\ (0.0234 - \frac{0.024}{-0.1221})$$

$$= 0.2219\ (0.0234 + 0.1966)$$

$$= 0.2219\ (0.2200) = 0.0488$$

Potency = Antilog. M x Expected Potency

$= 1.119 \times 100$

$= 112$ units/gm.

Limits

$$S^2 = \frac{s^2}{b^2}\left(\frac{1}{N_1} + \frac{1}{N_2} + \frac{1}{P}\left(\frac{\bar{y}_1 - \bar{y}_2}{b}\right)^2\right)$$

$$= (1.5561)^2\left(\frac{1}{130} + \frac{1}{127} + \frac{1}{172.960}(0.1966)^2\right)$$

$$= 2.4214\ (0.00769 + 0.00787 + 0.00022)$$

$$= 2.4214\ (0.01578)$$

$$= 0.038210$$

$S = 0.1954$ t(at $P = 0.05$) $= 1.97$

$tSI = 0.197 \times 0.1954 \times 0.2219$

$= 0.085$ and 0.9150

Limits = Antilog. (tSI)

$= 121.6\%$ and 82.2%

$= 136$ and 92 units.

In 95% of the cases the potency will lie between 92 and 136 probable value being 112 units.

TABLE VII

Tests for Linearity and Parallelism of Dose-Response Lines from Radiographic Data

(B.S.I., 1940, procedure)

Linearity of log. dose-response line for reference oil

$$\frac{A_1}{s_1^2} = \frac{0.1229}{0.03516} = 3.50* \qquad (118 \text{ d.f.})$$

Linearity of log. dose-response line for unknown oil

$$\frac{A_2}{s_2^2} = \frac{0.0922}{0.03705} = 2.49 \qquad (121 \text{ d.f.})$$

Parallelism of lines

$$\frac{A_3}{s^2 \text{ (pooled)}} = \frac{q_1^2}{p_1} + \frac{q_2^2}{p_2} - \frac{Q^2}{P} = \frac{1.5543 + 1.0577 - 2.5804}{0.03611} = 0.88 \qquad (239 \text{ d.f.})$$

Combined linearity and parallelism

$$A = \frac{R - \frac{Q^2}{P} \times \frac{1}{\text{doses} - 3}}{s^2 \text{ (pooled)}} = \frac{\frac{1.1499 + 1.6772 - 2.5804}{3}}{0.03611} = \frac{0.08223}{0.03611} = 2.28** \qquad (239 \text{ d.f.})$$

* necessary F (at P = 0.05) = 3.92

** necessary F (at P = 0.05) = 2.64

PART II

THE CHEMICAL ESTIMATION OF VITAMIN D

REVIEW OF LITERATURE

The vitamin D potency of a preparation may be determined by measuring the amount of light absorbed at the wave length of maximum absorption or by the intensity of colour produced in a chemical reaction. Both methods have many advantages over biological procedures but the results may be affected by interfering substances present in the vitamin D preparation. These substances must be removed or corrections made for their presence. With high potency products the error is relatively slight, but low potency products, such as are encountered in control work in connection with the administration of the Feeding Stuffs Act, contain a relatively large amount of these interfering substances and they provide a very real problem in the satisfactory testing of the product. These vitamin D products are sold to promote the mineral metabolism of livestock and poultry. So it is important that the chemical method of estimation be checked against biological assays.

1. Spectrographic Estimation

In 1932, Reerink and Van Wijk determined the concentration of vitamin D_2 by measuring the absorption maximum at 265 mu. The following year Fuchs and Beck (1933) and Fuchs

(1933) reported that the spectrographic method had an accuracy of 20-30 per cent and agreed with the biological method within these limits. Töpelmann and Schuhknecht (1935) also used a spectrographic procedure. Bowden and Snow (1934) studied the characteristics of the absorption bands but did not use the procedure for analytical purposes.

Haman and Steenbock (1935) showed that many substances were formed on irradiation of ergosterol and that vitamin D_2 could not be accurately determined by the spectrographic method. Similar conclusions were arrived at by Brockmann and Chen (1936). Brockmann and Busse (1938) showed that the molecular extinction coefficients of vitamin D_2 and D_3 were the same. Marcussen (1939) estimated both vitamins by the spectrographic procedure after removing interfering substances. He used calciferol as the standard and reported values for several oils. Crews and Smith (1939) pointed out that calciferol is highly unstable under ultraviolet light, and to avoid loss, minimum exposure times must be used. They suggested a continuous flow method of exposing the solution during photometry. Nakamiya and Takizawa (1939) confirmed the work of Haman and Steenbock and found that there was no relationship between the extinction coefficient of an oil, or its unsaponifiable fraction, and its biological potency. However, Nakamiya and Koizumi (1940) suggested that the interference by vitamin A in the vitamin D determination could be reduced by making the light absorption measurements after the sample had been irrad-

iated for some time. Ol Khin (1941) found that the vitamin D_2 content of irradiation products as determined by the spectrographic method agreed with the biological potency.

2. Colorimetric Reactions

One of the first attempts to determine the antirachitic activity of irradiated materials by means of a chemical reaction was made by Yoder (1926). He attempted to measure the peroxide content of various oils by means of the starch iodide reaction. It was found that peroxide content and antirachitic potency were not correlated but it was postulated that the capacity to form peroxides was a function of the antirachitic potency. Peroxide formation was produced by heating as well as by irradiation. It occurred with mineral oil as well as with animal and plant oils. As knowledge of vitamin D progressed it became evident that this method was not a reliable measure of antirachitic potency.

Shear (1926) suggested a colorimetric method based on the red colour given by vitamin D in liver oils with a mixture of aniline and hydrochloric acid (15:1). This colour seemed to be correlated with degree of irradiation but no biological tests were carried out. Later Levine and Shaugnessy (1933) reported that the reaction was not specific and that it was given by many substances including all the fat soluble vitamins. Stoeltzner (1928) used phosphorus pentachloride to produce a reddish-brown colour reaction but Christiansen (1928) showed that other components of oils gave the same reaction. According to Meesemaecker (1930)

the addition of zinc chloride or phosphoric anhydride to a chloroform solution of ergosterol results in a rose colouration. If the solution was exposed to ultraviolet light a green colour appeared, the intensity of which was proportional to the length of time of irradiation. Cruz-Coke (1930) obtained a green colour when hydrochloric acid was added to an alcoholic solution of irradiated ergosterol. With a trace or an excess of hydrochloric acid a white precipitate formed. The green colour did not appear with ergosterol. Rosenheim and Callow (1931) found that when acetic acid or mercuric acetate in nitric acid was added to irradiated ergosterol a transient red colour was followed by a permanent olive green. Oxidized products give the same reaction. Valentin (1931) tested for the presence of "vitamin" by adding chloroform and sulphuric acid to an extract after removal of ether. The next year Brandrup (1932) showed that the blue colour was a function of the oil and not its vitamin content. It was subject to many interferences. Bruckner (1934) showed that vitamin D_3 in benzene or chloroform with addition of acetic anhydride, acetone, cupric acetate (or silver acetate) and zinc chloride gave a yellowish colour which rapidly changed to red brown with an absorption band extending from green to blue. This test was also given by calciferol. Ergosterol developed a permanent blue violet colour with reddish fluorescence. Halden (1936), Halden and Tzoni (1936), and Tzoni (1936) reported a colour reaction which they claimed was specific for vitamin D. The solution containing vitamin D was treated with

5-10 drops of 0.1 per cent solution of pyrogallol in absolute alcohol and concentrated to 0.1 ml., 2-4 drops of fresh aluminum trichloride in absolute alcohol were added and the solution heated. If conditions were kept anhydrous a deep violet colour appeared in the absolute alcohol solution. The reaction was not affected by the presence of cholesterol, ergosterol or lumisterol. Suprasterol II gave only a slight reaction, and vitamin A must not be present. The colour was compared with a dye standard and the reaction was sensitive to 2 micrograms of vitamin D. Recently Sobel et al. (1945) have reported that the reagent was difficult to prepare and the reaction generally unsatisfactory.

Robinson (1937) reported a colorimetric method based on the yellow colour formed when an alcohol solution of vitamin D was boiled with sodium nitrite and acetic acid and then made alkaline. The reaction was not given by cholesterol, ergosterol, dehydroergosterol or lumisterol but was given by vitamin A (orange) and the non-saponifiable fractions of olive and arachis oil. The reaction was about one-tenth as sensitive as the antimony trichloride reaction. Solyanikova (1939) used the Tortelli and Jaffee (1914) reaction for vitamin D but Rutkovskii (1940) showed that this reaction was also given by provitamins D. An acetic acid solution of the vitamin when mixed with a 2% solution of bromine in chloroform gave a green colour with both forms of vitamin D.

The use of the antimony trichloride reaction for the estimation of vitamin D was first suggested by Brockmann and Chen

(1936). A saturated solution of antimony trichloride in chloroform yielded a pink colour (maximum 500 mu) with both vitamins D. Tachysterol behaved similarly but other sterols gave weaker bands. It was claimed that vitamin A did not interfere in the reaction unless present in more than six-times the concentration of vitamin D. The orange yellow colour reached its maximum in about 10 to 15 minutes. Emmerie and Van Eckelen (1936), however, found that the reaction could be used with only relatively concentrated preparations of vitamin D. They also showed that vitamin A must be removed. They suggested the use of acetic anhydride (1 drop) to intensify the colour. Wolff (1938) used the Brockmann and Chen reaction for the determination of vitamin D_2 in samples of irradiated ergosterol. The results of the chemical method varied from 8 to 40% from the bioassay values. Vitamin A, carotenoids and sterols were found to interfere. Ritsert (1938) claimed that the method was not applicable to low potency fish oils or to products obtained by the irradiation of the provitamins. The antimony trichloride reagent was found to be unstable and the limit of sensitivity was 15 micrograms.

Raoul and Meunier (1939) further modified the antimony trichloride reagent, adding sulphuric acid and using the following proportions:

Saturated solution of $SbCl_3$ in $CHCl_3$	- 30 ml.
Acetic anhydride	- 3 ml.
Concentrated H_2SO_4	- 5 drops

This reagent must be freshly prepared. These authors studied the rates of reaction of various sterols and irradiation products using a blue filter (400-450 mu). For example, sterols containing one double bond reacted slowly while those containing two double bonds reacted rapidly and the colour intensity reached a constant value. Vitamins D_2 and D_3 yielded a colour of high initial intensity which faded rapidly. The authors did not consider the effect of vitamin A but suggested the removal of non-irradiated sterols of trans configuration with digitonin. (Provitamins are more usually referred to as having cis configuration). Nield, Russell and Zimmerli (1940) also had difficulty with the $SbCl_3$ reagent and found that acetyl chloride stabilized and sensitized the reagent. They added 2 ml. of acetyl chloride to 100 ml. of a 15 to 20% solution of antimony trichloride in chloroform. With vitamins D_2 and D_3 this reagent gave identical absorption curves with maxima at 500 mu. The colour reached a maximum in 30 seconds and was stable for 4-5 minutes. This reaction was about three times as sensitive as the Brockmann-Chen reaction and could determine 0.2 micrograms of vitamin D when a special cell was employed. To make this reagent applicable in the presence of sterols, Zimmerli et al. (1943), added a reducing agent such as metallic tin, zinc, etc., to reduce the pentavalent antimony which was causing the transient colour with sterols. In this work the reaction was not applied to fish oils but to relatively pure solutions of the vitamins. Gudlet (1941) and Aranov and Geshelina (1944) also used the antimony tri-

chloride reaction and pointed out that the chief sources of error in the determination of vitamin D in low potency oils were the saponifiable oil, sterols and vitamin A.

Milas, Heggie, and Raynolds (1941) have used two methods for the estimation of vitamin D, both employing the antimony trichloride reaction. In one method vitamin A and sterols are estimated at 620 mu and 480 mu, respectively, and their presence corrected for in the estimation of vitamin D. This procedure has been criticised by Morton (1942) as being empirical. In the other method the interfering substances are removed and vitamin D estimated in the purified solution. The latter method seemed more successful and yielded results more in line with the biological potency. Vacher and Lortie (1943) used a 14.40% solution of antimony trichloride in chloroform as a reagent. They plotted the whole absorption curve of the coloured solution and used two methods to estimate the calciferol. In the first method, "Heterochromatic Photometry", the difference between intensities at two wave lengths on either side of the maxima was plotted against wave length. In the second, increments of calciferol were added to the unknown. The variation in intensity during the course of the reaction was studied. The authors claimed that these methods minimized the influence of interfering substance and only slightly reduced the precision. No data was given to show the reliability of the method in practice.

A very detailed procedure has been proposed by Ewing

et al. (1943) using the reagent of Nield et al. (1940) and specially purified reagents. Vitamin A and sterols were removed by chromotographic adsorption. Using this method they analyzed 51 samples of oil and compared the results with the biological potency. They obtained fair agreement with oils containing at least 5,000 units of vitamin D per gram. However, the method is lengthy and seems rather empirical, judging from the comments of the authors on the importance of details, and furthermore, the purification of the reagents is cumbersome.

Shantz (1944) drew attention to some of the difficulties inherent in the antimony trichloride reaction which have not always been considered. Using the original antimony trichloride reagent in chloroform he pointed out that the calibration curve is a straight line only with concentrations of 1 and 5 micrograms of vitamin D, that the maximum colour was reached in 4 minutes after addition of chloroform and that light or temperatures above or below 42° C., tended to depress the colour. The application of these conditions to the modified reagents is not known although Nield et al. (1940) pointed out that their reagent also gave maximum colour intensity in 4-5 minutes. More recently, DeWitt and Sullivan (1946) have further modified the antimony trichloride reagent by using ethylene chloride as solvent. Anhydrous calcium chloride was added to absorb any moisture. The reagent was stable and with vitamin D gave a pink colour with an absorption maximum at 500 mu. A chromatographic procedure was used for removing

interfering substances. The results were in good agreement with biological tests but only four oils were reported containing less than 1,000 units per gram. The authors admit that the limitations of the method have not been fully determined. The reaction of antimony trichloride with other sterols has been studied by Lamb et al. (1946).

Other reactions for vitamin D have also been suggested recently. In 1943, Beall and Grant, reported a method based on Lifschütz' (1921) ferric chloride reagent. This had been used by Bergström and Wintersteiner (1942) for the estimation of 7-hydroxy cholesterol. Beall and Grant found that the transient pink colour (maxima 540 mu) appeared to be a function of the vitamin D_2 or D_3 content of the solution. Halpern (1944) made preliminary studies on the reaction. Recently, the same authors (Beall and Grant, 1946) presented a modification of the method in which the vitamin solution was first purified by running through specially activated alumina using ethylene dichloride as solvent. The results obtained when the modified method was applied to high potency preparations agreed well with biological assays. The reagent, although readily prepared, is not suited for low potency oils because of its low sensitivity, (100 micrograms per ml. of test solution). Guillot and Saias (1943) observed a similar type of reaction with the Liebermann reagent and suggested the use of the transient rose colour as a measure of vitamin D activity. No attempt was made to develope the method. Sobel et al. (1945) have suggested the use of glycerol dichlorohydrin in the presence of

acetyl chloride as the basis of a colour reaction for vitamin D. A transient yellow colour was formed which turned to a stable green in one minute. The latter was used for the estimation of the vitamin. Ergosterol and 7-dehydrocholesterol gave comparatively little colour in this reaction but factors such as solvent-reagent ratio, time, and the amount of acetyl chloride affected the intensity of colour.

Schaltegger (1946) has proposed a reaction for sterols with an aromatic aldehyde in the presence of sulphuric acid. It was interpreted as a condensation of the aldehyde with the carbenium ion of the sterol. The colour reactions of many aldehydes and sterols were studied. The reaction seemed rather cumbersome for a routine determination and it was necessary that the reagents be highly purified. It was not as sensitive as the antimony trichloride reaction (10 to 20 micrograms per ml.) and was subject to interference by sterols and vitamin A.

3. Separation of Interfering Materials

Interfering compounds are of three chief types, saponifiable matter, sterols and vitamin A. Vitamin A and sterols give interfering absorption curves. All three substances react with many of the reagents to give similar or interfering colour reactions. It is, therefore, important that these substances be removed regardless of the method used to determine the vitamin. Because of their similarity in constitution the provitamins D constitute a particular problem.

Fuchs (1933), Tzoni (1936), and Raoul and Meunier (1939) and Morton (1942) used digitonin in alcoholic solution to precipitate the non-irradiated sterols from the unsaponifiable fraction of oils. The irradiation products of sterols do not react with digitonin. Natelson and Sobel (1935) suggested the conversion of the sterols and vitamin D to the potassium salts of their sulphuric acid esters by means of pyridine chloro-sulfonate and potassium hydroxide. The vitamin salts were soluble in ether while the sterol salts were insoluble. This method has not been applied to the estimation of vitamin D.

The removal of vitamin A by the addition of maleic acid anhydride or citric acid anhydride has been suggested by Brockmann (1936) although Brockmann and Chen (1936) claimed that unless present in great excess the removal of vitamin A is not necessary. Wolff (1938) found that it was necessary to remove vitamin A and carotene in benzene solution with Montana earth. In low potency oils the sterols were precipitated with digitonin. Ritsert (1938) removed vitamin A by chromatographic adsorption on aluminum oxide. Marcussen (1939) removed interfering substances by passing a heptane solution of the non-saponifiable fraction through a Tswett column filled with "hydraffin K_4" as adsorbent. To minimize the effect of vitamin A, Nakamiya and Kaizumi (1940), have suggested that vitamin D be determined after irradiating the sample. They do not mention the possible formation or destruction of vitamin D on prolonged irradiation.

Milas, Heggie and Raynolds (1941) used two methods for eliminating vitamin A and sterol interference. In the first method, vitamin A and sterols were estimated in the antimony trichloride reaction at 620 and 480 mμ, respectively, and correction made for their presence. In the second method sterols were removed by freezing in methyl alcohol at -10° C. and vitamin A was eliminated by coupling with maleic anhydride in dioxane. Gudlet (1941) suggested that oils be saponified, the sterols frozen out and vitamin A removed with maleic anhydride; quantitative removal of vitamin A was claimed. Aronov and Geshelina (1941) suggested that there should be vigorous stirring during saponification, sufficient absolute methyl alcohol to dissolve the non-saponifiable fraction and excess maleic anhydride to remove vitamin A. Morton (1942) considered maleic anhydride particularly useful for removing vitamin A and suggested the formation of a lithium chloride insoluble addition compound for the removal of sterols. Vacher and Lortie (1943) minimized the effect of impurities at the expense of sensitivity by measuring the absorption of the antimony trichloride reaction colour at two wave lengths one on each side of the maxima.

An extremely detailed procedure has been given by Ewing et al. (1943) for the removal of vitamin A and sterols. The non-saponifiable fraction of the vitamin oil was taken up in a skelly-solve-ether-absolute alcohol mixture (50:10:1) and passed through a standardized column of Superfiltrol using Sudan III as a dye for the location of vitamin D. Vitamin A and sterols were removed from the top of the column and the vitamin D and other sterols eluted

and determined together using the $SbCl_3$ reaction. An aliquot was transferred to a mixture of skellysolve-benzene (1:2) and passed through Superfiltrol again. Vitamin D was retained on the adsorbent and the potency obtained from the difference between the combined reaction and the reaction of sterols alone. Thibaudet (1945) used an acid clay to separate calciferol and tachysterol. The calciferol was adsorbed more strongly than the latter which was eluted with cyclohexane and alcohol (79:10). DeWitt and Sullivan (1946) reported a comparatively simple procedure using ethyl ether as solvent and a mixture (1:1) of magnesia and diatomaceous earth (Celite) as adsorbent. Five bands were obtained and the presence of vitamin D (4th. band) was detected by using ultra-violet radiations. Vitamin D gave a colour which was stable for 5 minutes, after this time the interference by sterols was apparent.

Since the completion of part of the work presented in this thesis, Beall and Grant (1946) reported the use of a specially activated alumina for the removal of substances which interfere in their modified Lifschütz reaction. With high potency materials good agreement with biological data was obtained.

The foregoing review of literature shows that many reactions have been suggested for the estimation of vitamin D. Most of them are not specific and much of the work is contradictory. Only the antimony trichloride reaction has been tested with any degree of thoroughness. Fair agreement with biological data can be obtained using this reagent with high potency oils, but many

improvements are needed before it can be applied to low potency fish oils. There is no comparative data by which the relative merits of these methods may be assessed. One of the chief problems is the removal of the sterols and vitamin A which interfere with all colour tests and with the direct determination of the absorption maxima of vitamin D. It is also evident that difficulty has been experienced by workers in duplicating the results of others. In almost all the investigations there is a significant lack of detail as to what influence the various steps in the methods have on the absorption characteristics of vitamin D and interfering substances in the oil. Without this detail, methods become empirical.

Accordingly, in this investigation it was planned to study the factors affecting the estimation of vitamin D by the application of three colour reactions and the ultraviolet absorption method. The colour reactions chosen for study were those employing the Lifschütz reagent, antimony trichloride and glycerol dichlorohydrin.

EXPERIMENTAL

Reagents

Lifschütz reagent - 1 gm. $FeCl_3$ was dissolved in 900 ml. glacial acetic acid and 100 ml. concentrated sulphuric acid added (Beall and Grant, 1943). The reagent is stable for 2 to 3 months.

Antimony trichloride reagent - This was prepared according to DeWitt and Sullivan (1946) using ethylene dichloride as the solvent.

Glycerol dichlorohydrin reagent (G.D.H.) - One per cent acetyl chloride was added to the activated G.D.H. prepared by Shohan Labs., N.J. according to the recommendations of Sobel et al. (1945).

Apparatus

Evelyn Photoelectric Colorimeter.- Filter 540 mu was used for measuring the pink colour in the Lifschütz reaction and 440 mu for the green colour. Filters 420 and 620 mu were used for the G.D.H. reaction.

Coleman Universal Spectrophotometer.- This was used for the estimation of peroxides by the Chapman and McFarlane reaction (1943).

Beckmann Quartz Spectrophotometer, Model DU, with Corex and silica cells of 1.000 ± 0.004 cm. thickness. Minimum band width was used where possible but below 260 mu it was necessary to use a 2 mm. setting. This was decreased to approximately 1.0 mm. at 300 mu and much less at higher wave lengths. Sensitivity

control was used at 1 to 3 turns from clockwise limit where possible. Readings are indicated in the figures and were generally taken at intervals of 5 mu. Between 258 and 270 mu the intervals were 2 mu.

(a) LIFSCHÜTZ REAGENT

1. Vitamin D_2

Preliminary studies were made with crystalline vitamin D_2 as it was more readily available at the time. The Lifschütz reagent was used and chloroform was the solvent for the vitamin. On addition of the Lifschütz reagent to vitamins D a transient pink colour appears which reaches a maximum intensity and fades in less than half a minute, i.e., the galvanometer swings downward, stops momentarily and then swings back. After approximately one minute the galvanometer again swings downward in response to a green colour of increasing intensity. According to Beall and Grant (1943) this double reaction is characteristic of vitamins D while provitamins show only the green colour. They further specify that if the galvanometer does not return after indicating the maximum intensity of the red colour, the reaction is atypical and the result should be discarded. Reaction curves were determined for three samples of vitamin D_2 and are shown in Figure 11. There is considerable difference between two presumably pure samples (1 and 2). Another sample (3) which had been exposed to the air for about 6 months but kept under refrigeration showed very low potency.

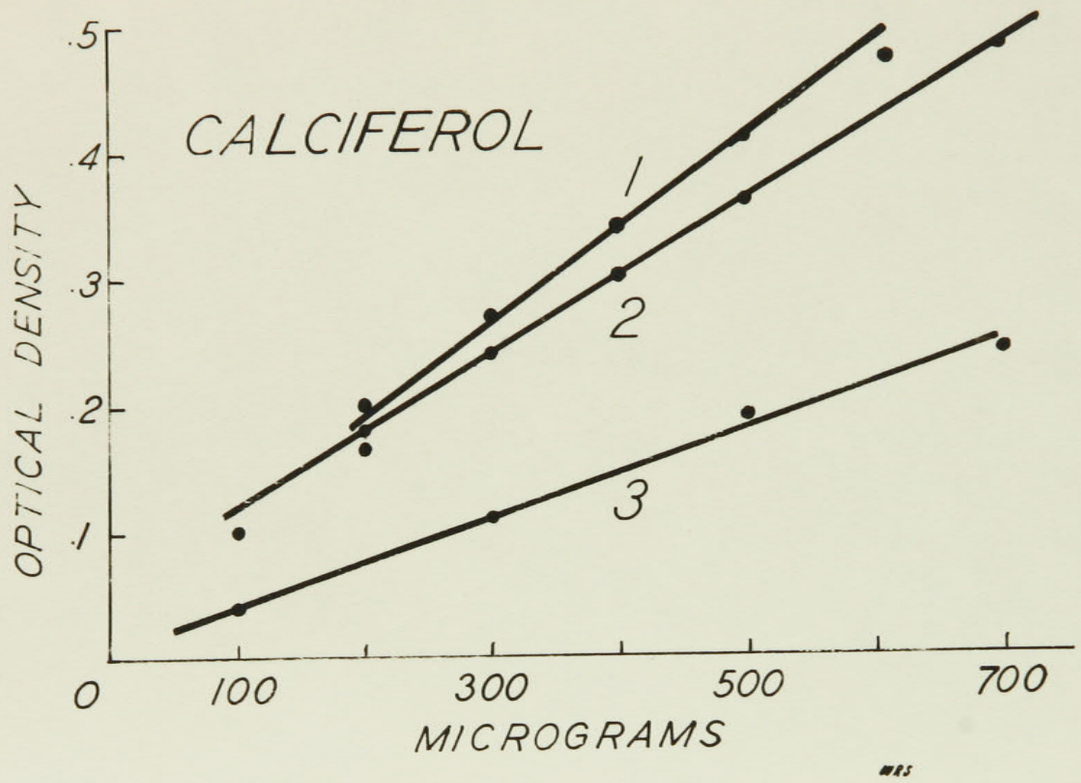


FIGURE 11. Density-concentration curves for Lifschütz reaction with calciferol from three sources: 1. Ayerst, 2. Frosst, 3. A sample exposed to air, under refrigeration, for 6 months.

The Chapman and McFarlane (1943) thiocyanate reaction for the detection of peroxides was used to determine the oxygen uptake by this exposed sample of vitamin D_2. A peroxide value of approximately 1080 mg. equivalents per kilo. of vitamin was found for this sample while the fresh samples gave a scarcely detectable reaction. For one double bond in the vitamin to be completely oxidized, 2520 mg. eq. of oxygen would be required. This means that about 2/5th of the vitamin was oxidized in the exposed sample leaving 3/5th in the original form. If it can be assumed that the oxygen taken on at any one of the four double bonds will cause loss of potency, then the potency of the material should be about 3/5th of its original value. The potency of the product as determined by the Lifschütz reaction showed that only 2/5th remained unchanged. The difference may indicate that the loss of potency is not entirely accounted for by oxidation. It is possible, however, that in this preliminary work the difference could be accounted for by variations in the two procedures.

Further studies on the oxidation of vitamin D_2 under various conditions indicated that it was slowly oxidized on standing for more than four hours after the solvent was removed. The oxidation was more rapid at higher temperatures or when aerated and was slower in vacuum. The changes are indicated by the fact that the galvanometer return became less marked, finally disappearing altogether, i.e., the reaction became atypical of vitamins D.

2. Vitamin D_3 in Oil Solution

Vitamin D_3 produces a more intense colour with the Lifschütz reagent than vitamin D_2 and, therefore, a separate calibration curve must be used. This is shown in Figure 12 and was used for estimating the potency of all preparations reported here. It can be seen that the reaction is variable as was pointed out by Beall and Grant (1943), i.e., the points do not all fall on the line. As these authors suggested, however, a fairly reliable value may be obtained if the average 3 readings at different concentrations are used. When the optical density is plotted against concentration in micrograms of vitamin D, a straight line is obtained only within certain limits, i.e., between 100 and 300 micrograms or 200 and 400 micrograms. From the values of K ($\frac{2-\log. G}{\text{concentration}}$) also plotted in Figure 12, it is evident there is considerable variability and that the best straight line portion to use is probably 100 to 300 micrograms.

One of the chief objectives of this investigation was to apply the method to low potency oils. Solutions of crystalline D_3 in corn oil were prepared and attempts made to recover vitamin D quantitatively. The saponification procedure consisted in adding 3 ml. 50% KOH and 30 ml. 95% ethyl alcohol to varying quantities of the oil up to 5 gm. and boiling on a water bath until saponification was complete, which sometimes required as long as two hours.

Much difficulty was experienced in reproducing results at

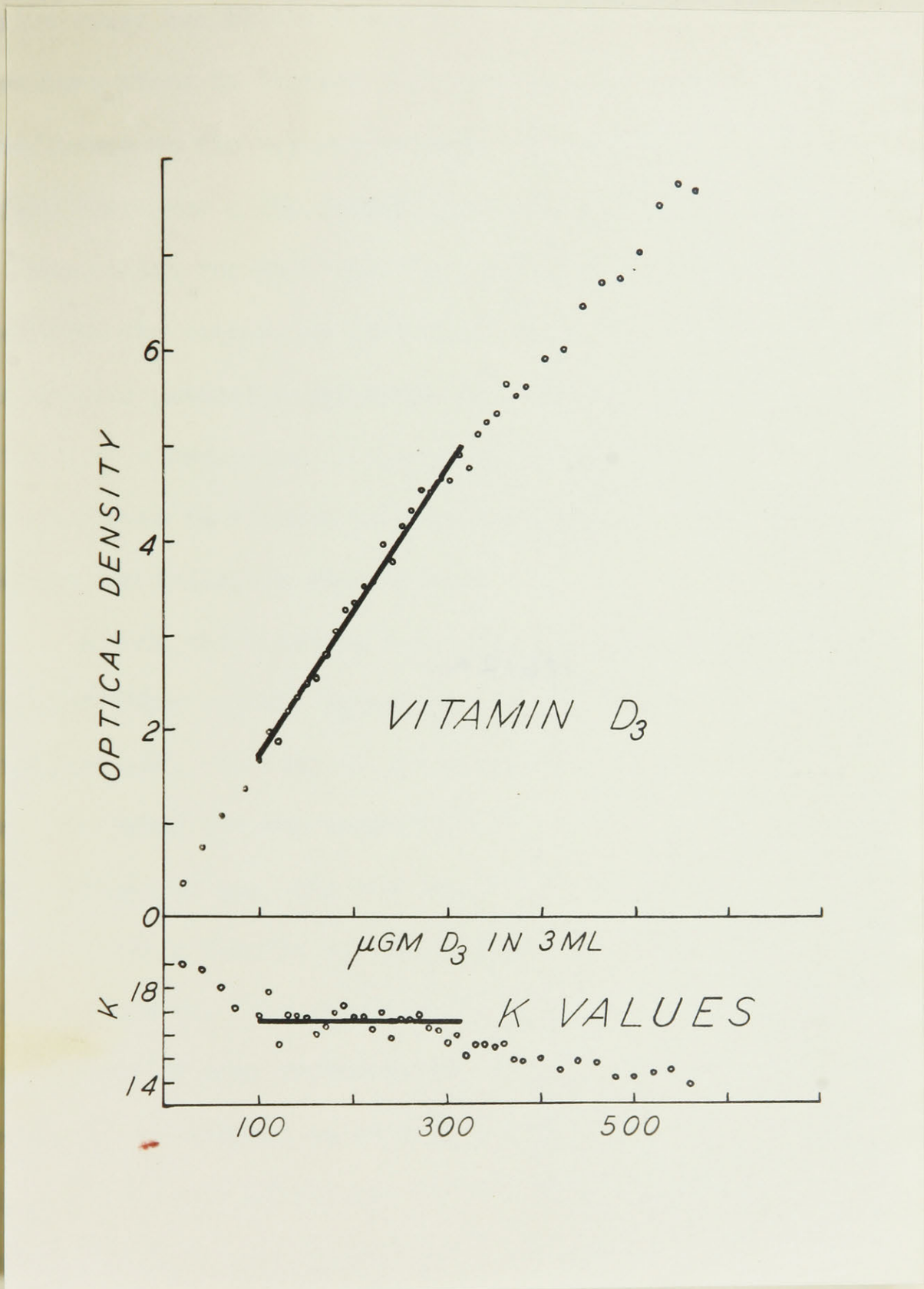


FIGURE 12. Density-concentration curve and K values for Lifschütz reaction with vitamin D_3.

different times but it was found that the length of time the saponified mixture stood in contact with the alcoholic KOH had a significant influence on the value obtained. For example, on standing overnight there was a 20% reduction in the apparent vitamin D content. The reason for this was that sterols and related substances reacted with the reagent to give high values. As vitamin D_3 is stable in cold potassium hydroxide and the extraneous material is not, there is a reduction of the material determined as vitamin D when the mixture is allowed to stand overnight. This was demonstrated in the following experiment:-

A corn oil solution was prepared containing 1,000 micrograms crystalline vitamin D_3 per gm. Eight samples of 0.5 gm. each were weighed out. To four of these samples, 5 gm. of oil was added to each, to bring the concentration of vitamin D_3 to approximately 100 micrograms per gm. All the samples were saponified and let stand for varying lengths of time before the non-saponifiable fraction was extracted. The results of this experiment shown in Table 29 are typical of many experiments. The data also indicate that for solutions of vitamin D_3 in oil the Lifschütz reagent gives a fairly good estimate of vitamin D content provided two conditions are met, i.e., that the saponified oil stand overnight before extraction and that the concentration be at least in the neighborhood of 1,000 micrograms (50,000 A.O.A.C. units) per gram.

That it was some compound other than vitamin D, which was decomposing on standing, was shown by taking crystalline D_3

TABLE 29

The Effect of Cold Alcoholic Potassium Hydroxide after Saponification on the Apparent D_3 Content of oil Solutions

Time (hrs.)	Recovery at 2 Concentrations of D_3. (%)	
	1000 ugm. D_3 in 1 gm. oil	100 ugm. D_3 (approx.) in 1 gm. oil
0	117	217
6	120	150
18	107	162
42	101	125

solution in chloroform, removing the chloroform and carrying out the saponification procedure in the usual way. After two hours on the boiling water bath there was no loss of vitamin D_3. The loss on standing is considered to be within the error of the method. The results were as follows:

	micrograms of vitamin D_3
In original aliquot	1600
After 2 hours saponification	1650
After 2 hours saponification and 24 hours standing	1550

To prove that the reagents, i.e., alcohol and potassium hydroxide, were not causative factors in the decomposition, determinations were carried out using freshly mixed reagents and others that had been prepared for some time. No difference was found. Further studies on the effect of alkali on oils are reported in the section on ultra-violet absorption measurements.

The Lifschütz reagent reacts with ergosterol to give a bright red colour. This colour is extremely transient and cannot be accurately measured in the Evelyn colorimeter. It appears to be of similar intensity to that obtained with equal amounts of vitamins D. The green colour also increases in intensity very rapidly. It is suggested that this type of reaction may be responsible for the disappearance of the return of the galvanometer, mentioned earlier, in low potency samples with a corn oil base.

The increase in colour with the Lifschütz reaction in corn oil solutions may also be due to ergosterol or closely related sterols.

Cholesterol had no effect on the reaction in concentrations up to ten times that of the vitamin D_3 present. This substantiates the work of Grant and Beall. However, in greater amounts, when tested alone, cholesterol did give the red colour as well as the green colour but showed no typical return of the galvanometer as with pure vitamin D. It is evident that cholesterol or similar animal sterols in fish oils react in a manner similar to that of ergosterol or other plant sterols in corn oil.

Attempts were also made to make a blank reading with the corn oil alone, so as to obtain a correction factor which could be applied in the estimation of the vitamin. Duplicate samples of 10 and 20 gm. of corn oil were weighed out. To one of each, 500 micrograms of vitamin D_3 was added in oil solution and then all were subjected to the saponification procedure and the Lifschütz reaction. The galvanometer readings were recorded at intervals up to 6 minutes and the optical density plotted against time as shown in Figure 13. The curves show the effect of the oil in obscuring the reading of the initial pink colour with 10 grams corn oil. The optical density measured at 540 mu reaches a plateau and remains constant for a few seconds, then gradually increases as the density of the green colour increases. With pure vitamin D the intensity of the red colour decreases in the manner indicated by the dotted line. When the quantity of oil is doubled

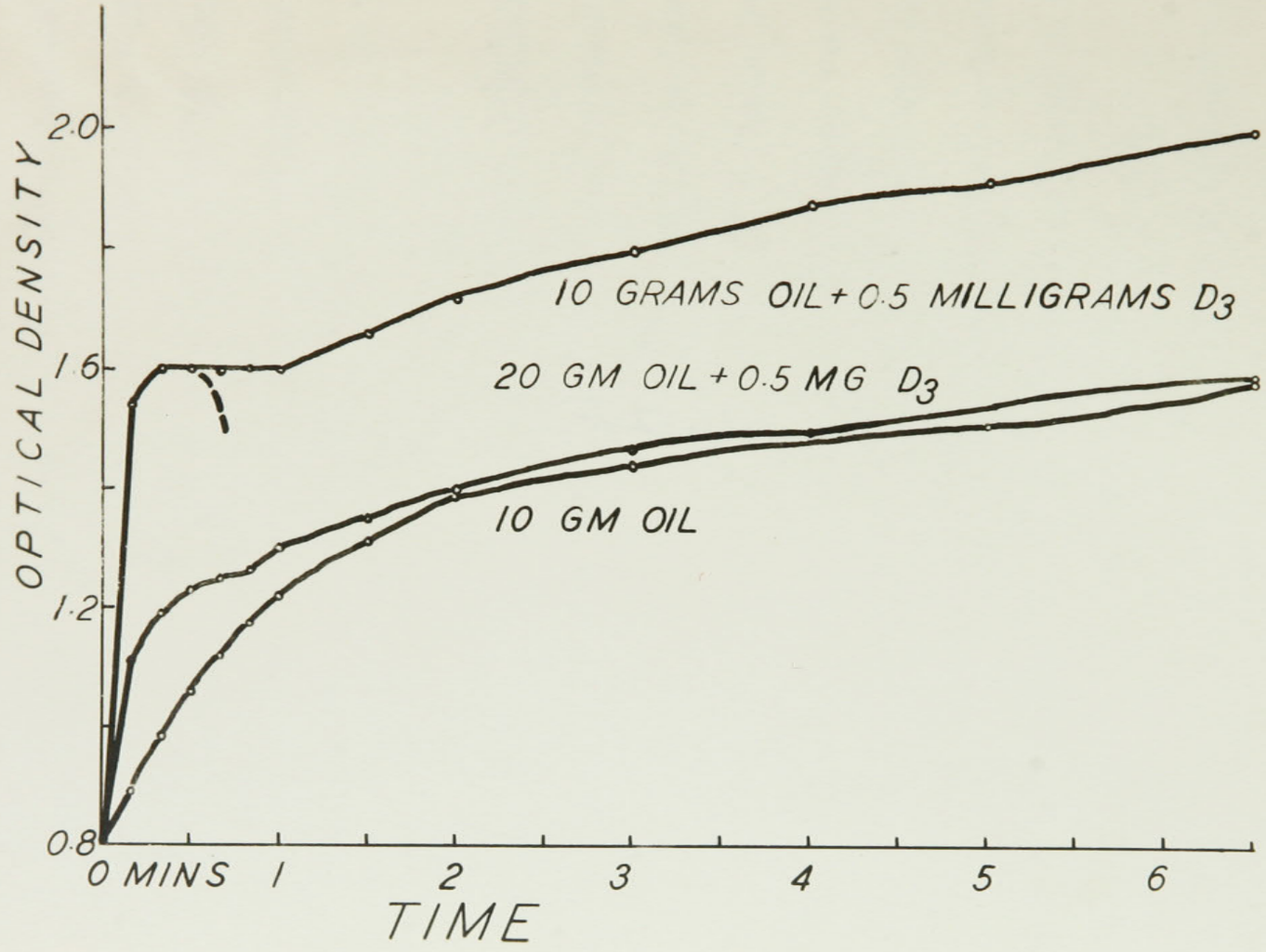


FIGURE 13. Density-time curves for corn oil solutions and corn oil + vitamin D_3.

the curve shows no levelling off and in general cannot be distinguished from the one containing no vitamin D. There seemed no possibility of obtaining a correction factor from these data. It is possible that, if the wave bands isolated by the 540 filter were narrower, the interference offered by the green colour would not be present and the reaction would be more specific.

Further studies on the green colour reaction of cholesterol and fish oils likewise gave negative results. The idea had been to determine the apparent cholesterol content of the oil by comparison with a concentration curve of the green colour reaction. The value obtained was then subtracted from the apparent vitamin D content to give a corrected vitamin D value. However, no constant relation was found which could be applied and in most cases the correction was much too large.

3. Removal of Interfering Compounds

Various procedures have been suggested for the separation of vitamins A and D but the adsorption technique seemed the most promising. Several attempts were made to use the procedure of Ewing et al. (1943) but it was not found satisfactory. It is possible that this was due in part to differences in the adsorptive properties of the Superfiltrol. After many preliminary experiments, it was found that a chloroform solution of vitamins A and D, when put through a column of Superfiltrol, gave the best separation. The columns were prepared as outlined by Ewing et al.

The non-saponifiable fractions of two oils were used to

study the separation of vitamins A and D. One oil was a vitamin A and D concentrate and was used to study the conditions for removal of vitamin A. The other oil was a solution of vitamin D_3 (crude) in corn oil and was used to study the recovery of vitamin D in the eluate. Using the vitamin A and D concentrate it was found that vitamin A was removed completely (as indicated by the antimony trichloride reaction) when an aliquot of a chloroform solution of the non-saponifiable fraction was passed through a tightly packed two centimeter column of Superfiltrol. The recovery of vitamin D from the same type of column was then tested using the second oil. It was found that the more Superfiltrol used the greater was the adsorption of vitamin D. Typical results are given in Table 30. By carefully washing the column and using a 5-10 ml. aliquot of solution it was possible to reduce the loss to 5% or less using the 2 cm. column.

Various other adsorbents and solvents were tried in an effort to obtain a more complete separation of vitamins A and D, but none were satisfactory. It was found that vitamin A in chloroform solution was not adsorbed by Brockmann's alumina and only slightly adsorbed on a silica-alumina adsorbent. In petrol ether both vitamin A and D were adsorbed by Superfiltrol but could not be eluted with ethyl ether or chloroform.

Direct adsorption of vitamin A from the oil solution was tried in an effort to simplify the procedure by eliminating the saponification of high potency oils containing vitamin A. It

TABLE 30

Recovery of Vitamin D after Passing Through Superfiltrol Column

Amount Present (micrograms)	Amount Through Column (micrograms)	Depth of Superfiltrol (cm.)
154	104	7
154	140	4
154	145	2
173	164	2
155	145	2

was found that, under these conditions, vitamin A diffused rapidly down the column and separation was not possible. However, vitamin D came through with little loss.

Carotenoids are removed by Superfiltrol but do not interfere greatly in the reaction for vitamin D. It is the sterols, in lower potency oils which cause most of the difficulty as discussed earlier and as shown in Figure 13. Various procedures have been suggested for their removal, including digitonin precipitation, freezing out, and selective adsorption. Ewing et al. (1943) outlined a procedure involving selective adsorption using Superfiltrol but in our hands it did not prove successful. Preliminary tests were made on the freezing out method using both methyl alcohol and petrol ether. Better separation was obtained using the latter, as filtration was very difficult with methyl alcohol.

In general, the Lifschütz reaction is not sufficiently sensitive for low potency oils and the results are variable. The sterols of corn oil have been shown to react with the Lifschütz reagent and to be decomposed on standing in cold alkali after saponification. Ergosterol reacts with the Lifschütz reagent to give a very transient red colour. It is this type of reaction which may obscure the typical colour reaction of vitamins D alone. Sterols have not been satisfactorily separated from vitamin D but vitamin A may be removed from a chloroform solution by Superfiltrol while vitamin D may be washed through the column.

(b) ANTIMONY TRICHLORIDE REACTION

1. Factors Affecting Reaction

The antimony trichloride reagent was prepared according to the method of DeWitt and Sullivan (1947). These authors point out that the limitations of their method have not been fully determined. It was found that the colour intensity reached a maximum in 1 to 2 minutes (Figure 14) and in about 5 minutes began to decrease gradually. The decrease was not great up to about 1 hour. On standing 24 hours all concentrations of vitamin D gave the same intensity of colour.

The calibration curve was found to be linear over a range of concentrations from 2 to 20 micrograms but the K values were uniform only from 8 to 20 micrograms. This differs somewhat from the findings of DeWitt and Sullivan using ethylene dichloride as solvent and of Shantz (1944) using chloroform. The variability of individual determinations was not great but the reagent lost sensitivity rapidly after standing for more than two weeks. It was of approximately the same sensitivity as that used by Nield et al. (1940). It was found that in comparing blanks using different solvents petrol ether gave an increase in colour on standing over 10 minutes but this would not affect the reaction as ordinarily used. In ethylene dichloride and chloroform there was no change in colour up to 90 minutes. The effect of solvent on the reaction of antimony trichloride with the vitamin was not great as ethylene dichloride gave approximately the same reading

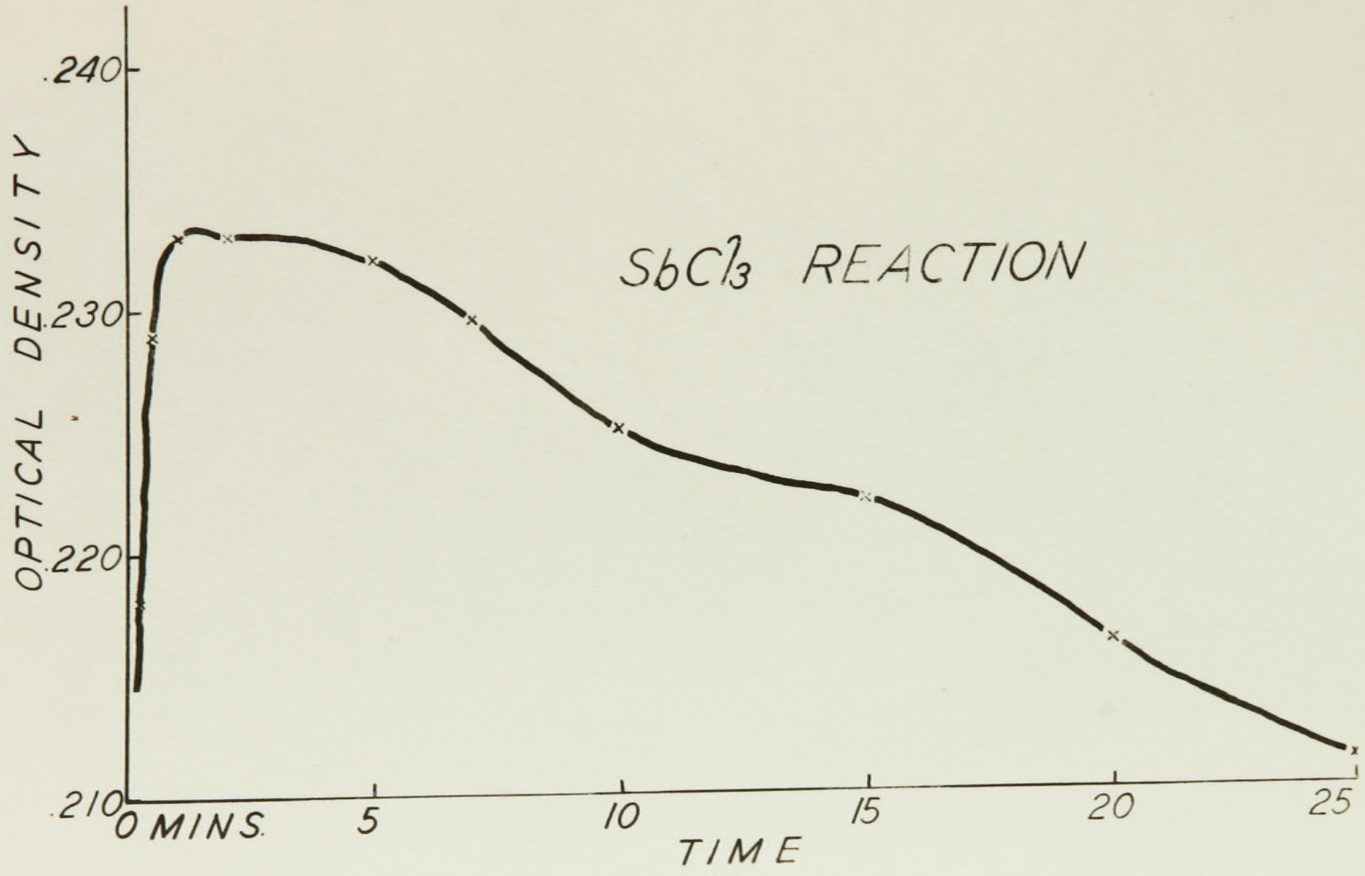


FIGURE 14. Density-time relation for the antimony trichloride reaction with vitamin D_3.

as the same concentration in petrol ether.

DeWitt and Sullivan recommended dissolving the sample in 0.5 ml. petrol ether for the reaction with 9.5 ml. antimony trichloride solution. It is often more convenient where lower dilutions are used to employ a greater volume of solution. It was found that within certain limits the ratio could be changed without affecting the reaction as shown in Table 31. A narrower ratio than 2:8 of solvent to reagent seemed to affect the sensitivity of the reagent. The effect of temperature on the reaction was also investigated and the density of the colours formed at approximately 20° C. and 40° C. were compared. Both the solvent and reagent were held at those temperatures until mixed. The results were somewhat variable but the indication is that raising the temperature to 40° C. increases the density of colour. This is in agreement with the work of Shantz (1944) using as the reagent antimony trichloride in chloroform. The differences indicate that the slight variations which obtain under usual laboratory conditions may not be of great importance.

TABLE 31

Effect of Reagent:Solvent Ratio in
Antimony Trichloride Reaction

(Galvanometer set at 100 without colorimeter tube)

Sample ml.	Solvent ml.	Reagent ml.	Galvonmeter Reading Per cent Transmission
0.1	0.4	9.5	62.0
0.1	0.9	9.0	62.5
0.1	1.4	8.5	62.0
0.1	1.9	8.0	62.5
0.1	2.4	7.5	66.0
0.1	2.9	7.0	66.0

(c) GLYCEROL DICHLOROHYDRIN REACTION

1. Sensitivity of Method

Sobel et al. (1945) proposed the glycerol dichlorohydrin (G.D.H.) reaction for vitamin D as having certain advantages. They recommended a solvent to reagent ratio of 3:2 as being best suited for the estimation of vitamin D. In Figure 1 of their paper it may be noted that the density of the colour obtained in the G.D.H.-calciferol reaction increased rapidly with lowered wave length in the vicinity of 400 mu. The maximum is higher than at 625 mu which is the wave length they recommend for making the reading. There seemed a possibility of making the method more sensitive by reading at about 400 mu if the maximum in this region was significantly higher than that at 625 mu.

Vitamins D_2 and D_3 and a high potency Dupont oil were dissolved in chloroform and G.D.H. added. After about 20 minutes, absorption measurements were made in the Beckmann spectrophotometer at wave lengths from 300 to 800 mu. The extinction coefficients are plotted in Figure 15; the values for the oil being adjusted to plot on the same scale. The E value at 410 mu is much greater than that at 625 mu for both vitamins, the latter maxima being low and ill defined. Absorption measurements at 410 mu would thus greatly increase the sensitivity. Furthermore, vitamin D_3 has a slightly higher extinction coefficient at 410 mu while at 625 mu the reverse is true. The same figure also illustrates the similarity in the reaction of a high potency oil. Sobel et al. (1945)

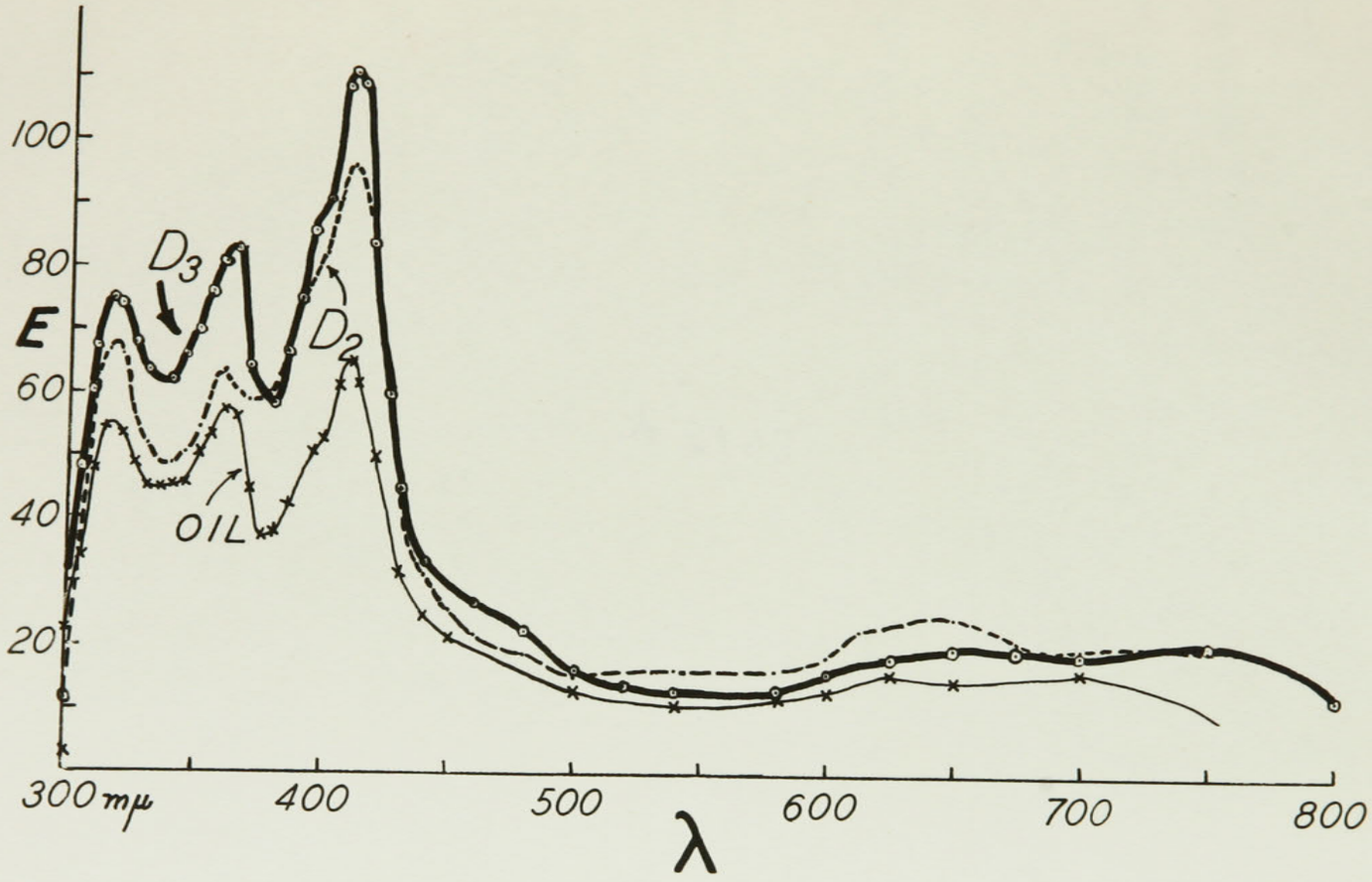


FIGURE 15. Extinction coefficients for the glycerol dichlorohydrin reaction at various wave lengths. Solvent:reagent ratio = 3:2. (Beckman Spectrophotometer).

chose 625 mu on the basis that ergosterol and 7-dehydrocholesterol interference was less here than at 400 mu. In fish oils the presence of ergosterol may be disregarded. The fact that the maxima of the oil at 410 mu is not obscured is conclusive evidence for this type of oil that the measurement of colour at this point would be much more sensitive and satisfactory than at 625 mu. The reaction with ergosterol produces a series of colours that change slowly over a period of several hours. It may also be noted that possible interference offered by vitamin A in this reaction is at a minimum at both 410 and 625 mu (Sobel and Werbin, 1946).

Time studies of the G.D.H. reaction, using a 3:2 ratio of solvent to reagent, measured at four wave lengths 310, 363, 410 and 625 mu suggest it is even more complex than indicated by Sobel et al., (1945) and is subject to the influence of many factors. With time the optical density at 310 mu reaches an initial peak in less than one minute, fades rapidly in the first 20 minutes and more gradually thereafter. This initial peak of absorption is higher than at any other wave length but was not considered suitable for general use. It is difficult to get the solution well mixed and read in the Beckman spectrophotometer before the colour fades and it would have no application to filter colorimeters such as the Evelyn. The reaction at 410 mu is a convenient one as it reaches a peak in about 25 minutes and remains relatively constant for 10 minutes. At this time the colour is seven times as intense as at 625 mu. The density at 363 mu is somewhat greater than at

410 mu but it does not reach a definite plateau and is still gradually increasing at 90 minutes. At 625 mu the colour density is low and relatively constant.

When the ratio of solvent to reagent is changed to 1:4 the situation is somewhat different. The effect of time on the reaction at different wave bands is relatively the same as found with the 3:2 ratio. However, the density at 410 mu is greatly increased, amounting to approximately 20 times that at 625 mu with a solvent to reagent ratio of 3:2. For example, 50 micrograms vitamin D_3 per ml. solution yields a colour with a density of 0.07 at 625 mu and 1.43 at 410 mu. The reaction at 410 mu is influenced much less by the constituents of fish oils and no more by the constituents of corn oil than it is at 625 mu. By increasing the ratio to 1:9 and 0.5:9.5 only slight increase in density is found. By employing these high ratios of solvent to reagent this reaction is made equal in sensitivity to ultra-violet absorption measurements and much more comparable to the antimony trichloride method.

As colorimetric measurements are more conveniently made in the Evelyn colorimeter the relation between the two wave lengths was studied in this instrument. The filters used were 420 mu and 620 mu and a 3:2 ratio of solvent to reagent was used. As was expected, the calibration curves in Figure 16 were much steeper and more sensitive at 420 than at 620 mu. The increase in sensitivity using the Evelyn colorimeter, however, is not equal to that record-

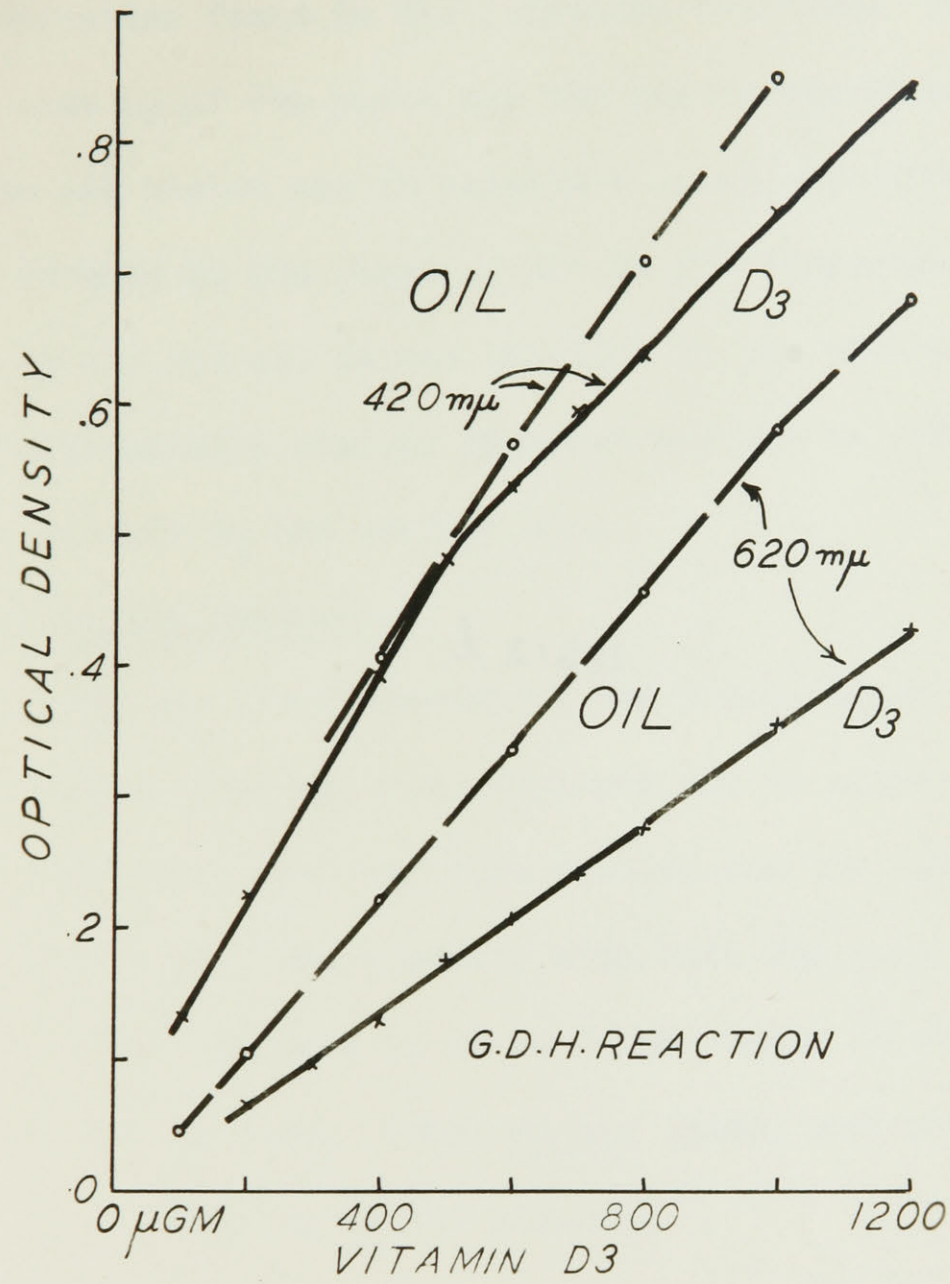


FIGURE 16. Density-concentration relations for the G.D.H. reaction with vitamin D_3 and an oil at 420 and 620 mu.

(Evelyn Colorimeter)

ed on the Beckman spectrophotometer which isolates narrower wave bands. It is also evident that the individual determinations all fall on a straight line suggesting that this reaction is not subject to the variation found in the Lifschütz reaction. The curve at 420 mu is made up of two parts and the points representing the values for the oil tested are in excellent agreement with those for the pure vitamin in the range of 100 to 500 micrograms. However, the curve for the oil is not parallel to the vitamin curve at 620 mu. Substantially similar observations may be made from Figure 17 for vitamin D_2 and another oil.

2. Factors affecting Reaction

Sobel et al. (1945) developed the G.D.H. colour in the dark. It was found, however, that daylight had no effect on the reaction. The reaction time was also studied and the results are presented in Figure 18. The reaction with both the pure vitamin and the oil reaches a maximum in 45 to 60 minutes. In 90 minutes there is a tendency to fade. Sobel et al. (1945) did not observe any fading of the colour up to 120 minutes.

From these studies, it is evident that the G.D.H. reaction is convenient and reliable. The colour formed is sufficiently stable to facilitate reading and is not subject to the variations of the Lifschütz reaction. When the absorption is measured at 410 mu with a solvent to reagent ratio of 1:4 the sensitivity is increased about 20 fold.

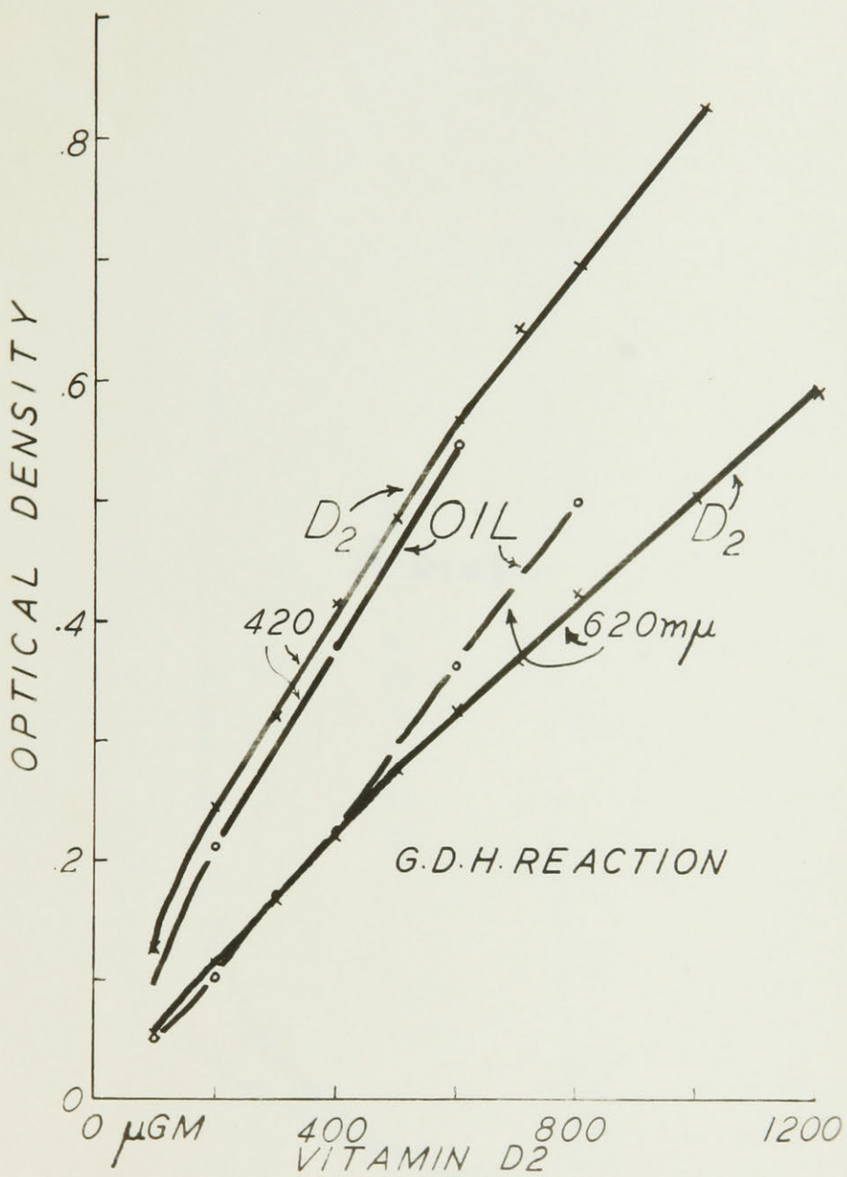


FIGURE 17. Density-concentration relations for the G.D.H. reaction with vitamin D_2 and an oil at 420 and 620 mu.

(Evelyn Colorimeter)

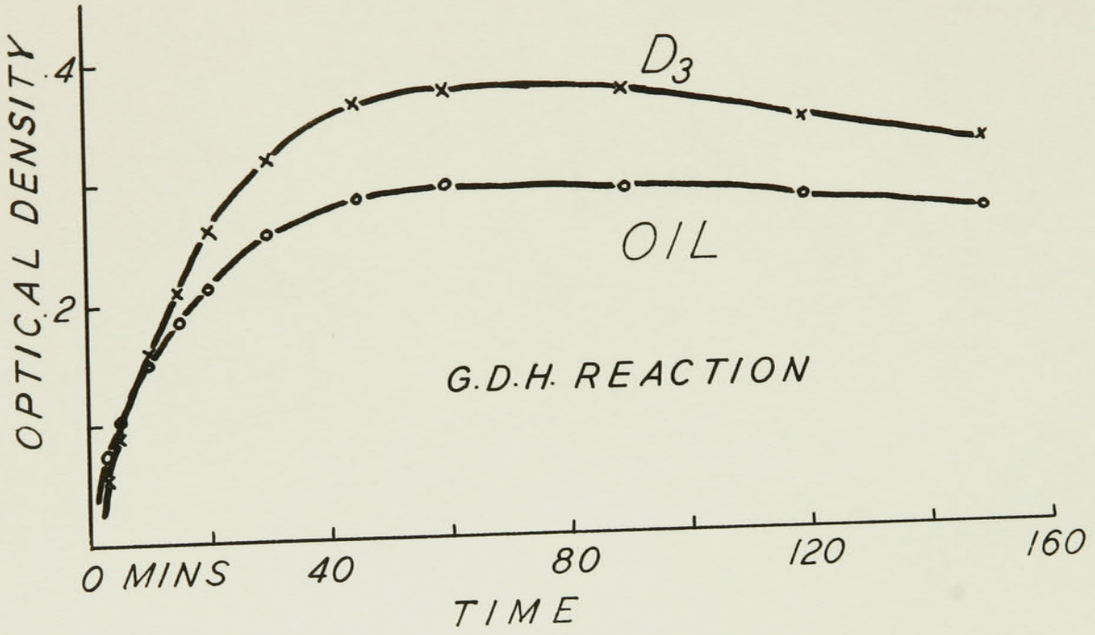


FIGURE 18. Density-time relation for the G.D.H. reaction with vitamin D3 and an oil. Readings were taken in the Evelyn colorimeter using the 420 mu filter.

(d) ULTRA-VIOLET ABSORPTION STUDIES

1. Solvents

In view of the results reported above and the lack of detailed absorption data in the literature it seemed essential to investigate, in some detail, the characteristics of the ultra-violet absorption curve of vitamin D and related compounds.

The Beckman quartz spectrophotometer with silica cells was used. An average E value of 467 was found for vitamin D_3 which checked fairly closely with the value of 481 as reported by Huber, et al. (1945). According to these authors vitamin D_2 and D_3 in alcohol or hexane solution give indistinguishable absorption curves. They further state that purified skellysolve B (i.e., essentially n-hexane) should be optically transparent down to 225 mu. This was found impossible to attain by the procedure given by the authors (i.e., alternate treatment with fuming sulphuric acid and alkaline permanganate) or any other known purification. The transparency of several solvents was investigated and compared with distilled water with the results shown in Figure 19. Hexane (petrol ether) was only slightly improved by purification and none of the solvents except 95% alcohol were very satisfactory for absorption measurements below 260 mu including commercial absolute alcohol. The product used was prepared from 95% ethyl alcohol. Fractions of hexane, with different boiling points, did not differ greatly in light absorption except when the boiling point was above 68° C. This fraction is somewhat less transparent. Because

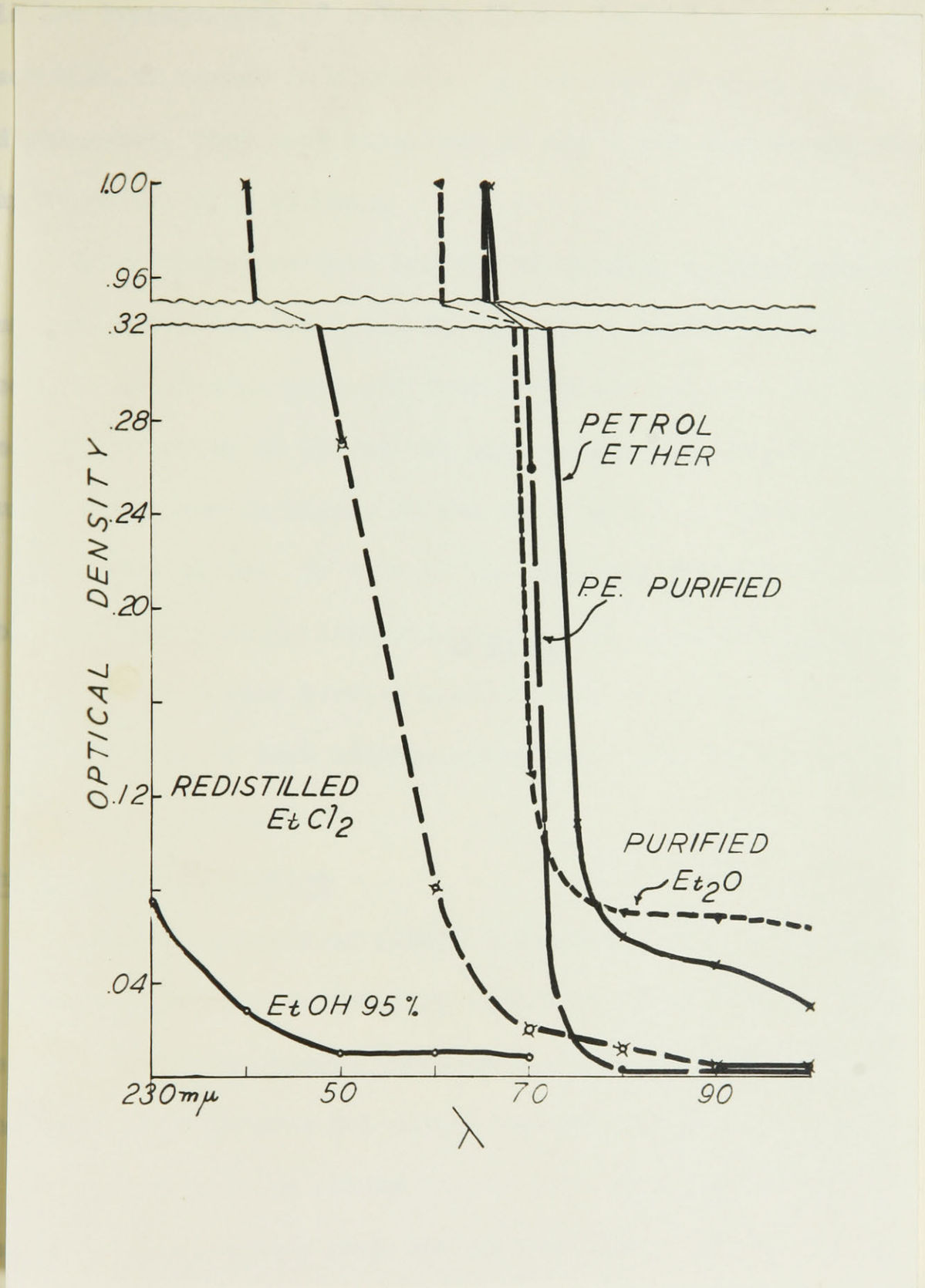


FIGURE 19. Density of solvents compared with distilled water in ultra-violet range.

of this low transparency of solvents it was decided to use a 1 per cent solution of hexane in alcohol. In the case of oils, unless stated otherwise, they were dissolved in the 1 per cent hexane then made up to volume with alcohol.

Using this procedure samples of vitamin D_3 from Ayerst, McKenna, and Harrison, Ltd., and Dupont were compared spectrophotometrically. The maxima were slightly different and also the height of the peak, although at 265 mu the extinctions were identical. The curves are given in Figure 20 and are similar to curves determined in alcohol alone. In view of the observed differences and the possible difficulty in purification of vitamin D_3 (Huber et al., 1945) it would seem that further study on the purification of D_3 would be desirable so that uniform preparations can be obtained for a biological standard.

2. Chromatographic Studies

DeWitt and Sullivan (1947) indicated that vitamin D_3 could be separated from interfering substances by a mixture of MgO and Celite. Beall and Grant (1946) suggested activated alumina (Harshaw) for this purpose but neither method was found satisfactory. It was difficult to remove the vitamin from the adsorbent in both cases. In the latter case the temperature of activation for the alumina may have been rather high. However, this difficulty in duplicating conditions between laboratories is not confined to this laboratory and from discussions with other laboratories (e.g., Dr. K. Morgareidge, National Oil Products, Ltd.) seems to be one

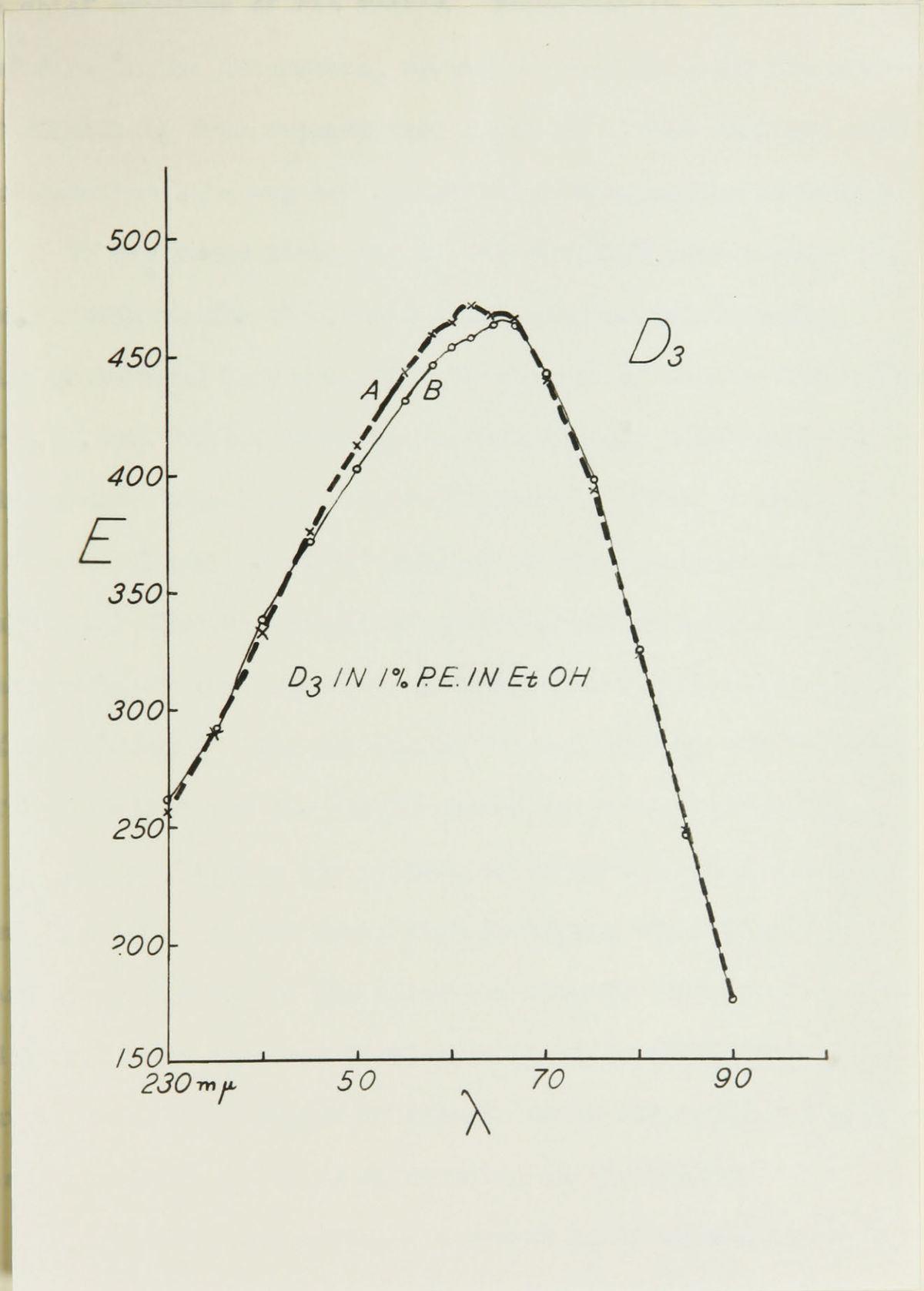


FIGURE 20. Extinction coefficients for two samples of vitamin D3.

A = Ayerst, and B = Dupont.

of the chief problems of the method. Nevertheless, in view of the lack of data in the literature, it was decided to study the recovery of vitamin D_3 from Superfiltrol although it was realized that results obtained here may not be entirely reproducible elsewhere.

It was found necessary to use purified petrol ether for elution as well as for the actual spectrophotometric reading. This is illustrated in Figure 21. Thirty ml. of petrol ether were evaporated, the residue taken up in 95% alcohol, containing 1% purified petrol ether, and compared against a blank of the solvents alone. There was marked density in this solution as compared to a similarly treated aliquot of purified petrol ether. The latter was identical with the check sample which was alcohol containing 1% petrol ether. The two latter curves indicate the reliability of the results and the slight effect of solvent differences.

The adsorption and elution of vitamin D_3 from Superfiltrol was studied. It was found that purifying the petrol ether decreased the affinity of the adsorbent for the vitamin. It required 30 per cent ethylene dichloride in commercial petrol ether to elute a detectable amount of vitamin while with purified petrol ether alone vitamin D_3 could be detected in the eluate.

Adsorption columns were prepared by attaching small bore tubes to the base of 16 mm. test tubes. The Superfiltrol was tightly packed in the test tube to a depth of 1.5 to 2 cm. One ml. of a solution of oil in purified petrol ether was added after the column was wet with petrol ether. To elute the vitamin the

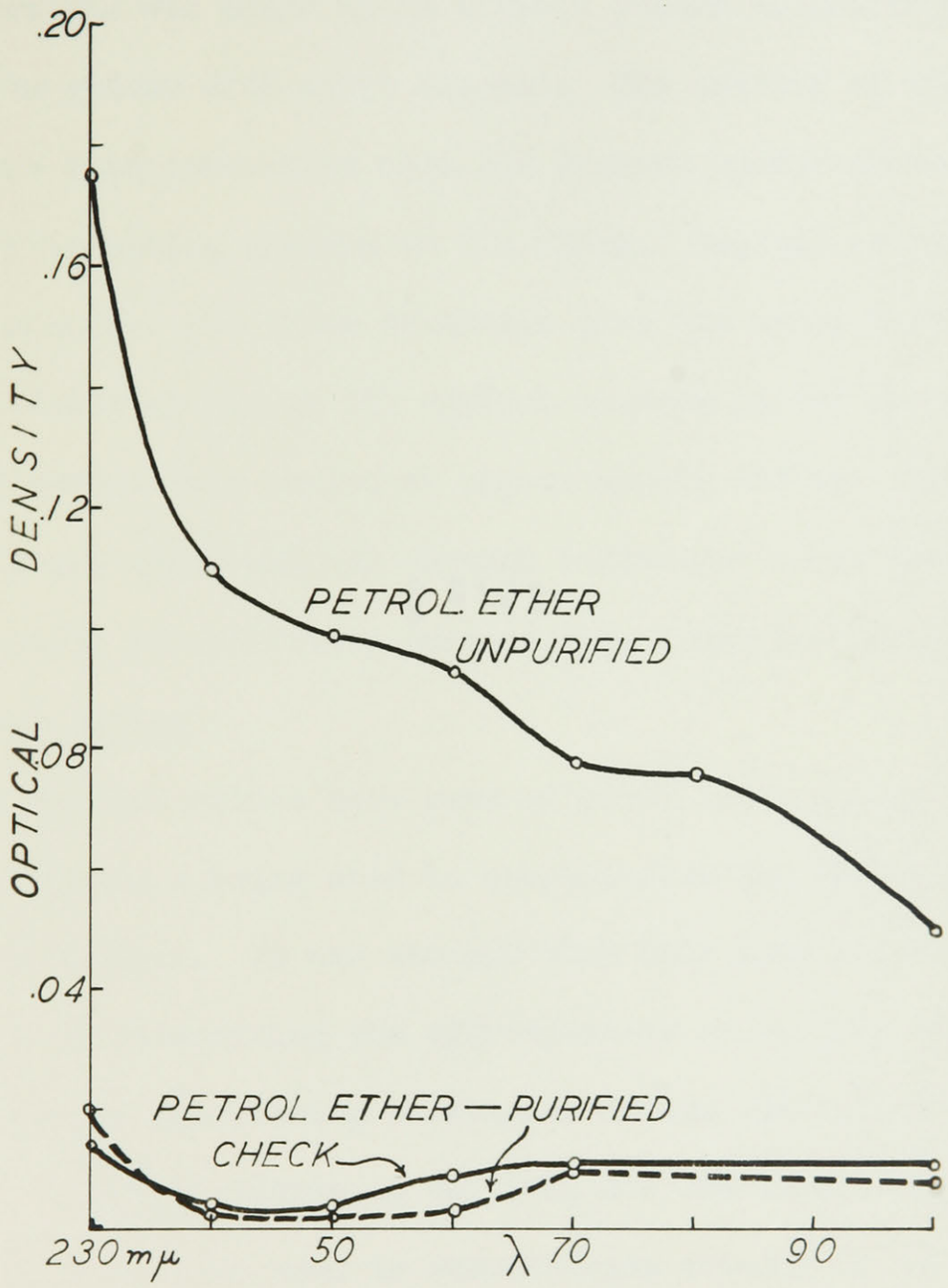


FIGURE 21. The density of the residue of 30 ml. petrol ether, purified and not purified. (See text)

column was washed with forty ml. of petrol ether followed by the same volume of ether containing 1% and 10% ethylene dichloride. These fractions were collected separately and the solvent evaporated. The residue was taken up in a small volume of petrol ether and made up to volume with ethyl alcohol. The density of these solutions were then determined with the Beckman spectrophotometer using the corresponding solvent as the blank. Typical results are shown in Figure 22. All three fractions gave the $SbCl_3$ test. The petrol ether fraction has an ill defined maximum at 265 mu and the other two fractions have maxima at approximately 255 mu. This separation is not clear cut and further refinement seems indicated before a fraction of relatively pure vitamin D can be obtained.

3. Extinction Ratios

Extinction ratios have been of great value in the determination of vitamin A being used to characterize the absorption curves of the vitamin. It was thought that this approach would offer a means of determining the effectiveness of various methods for the removal of interfering substances in the estimation of vitamin D. The $E^{1\%}_{1\ cm.}$ ratios at 255/265, 275/265 mu and 250/265 mu, 280/265 mu have been used to characterize vitamin D_3 before and after saponification. The data are given in Table 32. It is evident, even in these high potency oils, that the absorption curve of the vitamin is not well defined. Saponification does not greatly increase the similarity to the pure vitamin. The separation of various fractions with Superfiltrol is not effective as

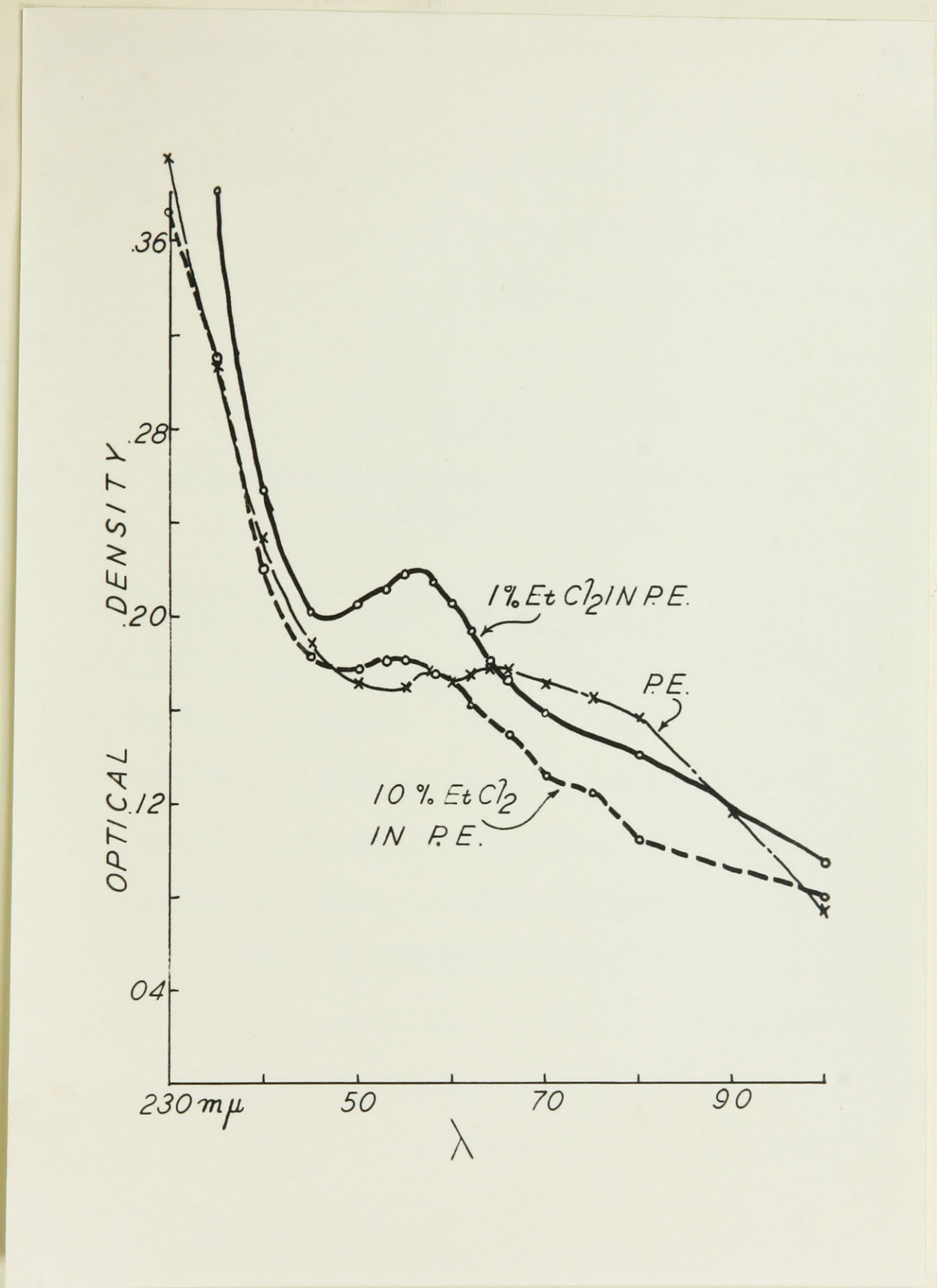


FIGURE 22. Absorption curves of different fractions of a high potency oil eluted with the solvents indicated from superfilterol.

TABLE 32

Extinction Ratios for Samples of Vitamin D_3 and High Potency Oils

Ratio (mu)	Vitamin D_3	Oil 1	Oil 3	Oil 4 Saponified
255/265	0.96	0.85	0.97	0.89
275/265	0.85	0.90	0.91	0.92
250/265	0.89	0.86	1.00	0.90
280/265	0.70	0.80	0.80	0.81

far as vitamin D is concerned.

Alkali Treatment

In earlier studies with the Lifschütz reagent it was found that certain compounds in corn oil reacting with the reagent were unstable in cold alkali when the samples were allowed to stand after saponification. The same samples of corn oil were not available but other samples of corn oil and fish oils were saponified and let stand for periods up to 96 hours before extracting the non-saponifiable fraction with ethyl ether. The ether was removed and the residue redissolved in 2.5 ml. purified petrol ether and made up to 50 ml. with ethyl alcohol. Absorption measurements were made at wave-lengths from 230 to 350 mu. Several types of fish oils, containing 200 to 400 A.O.A.C. units of vitamin D and 1,000 to 3,000 I.U. of vitamin A, and corn oil were also tested in this manner. The curves varied considerably but typical examples are given in Figures 23 and 24. The treatment with alkali seems to produce absorption maxima in the range of 250 to 270 mu. The sample of corn oil showed high absorption only below 260 mu and the curves were not greatly different whether the oil had been allowed to stand in contact with alkali or not.

In general, the absorption studies have indicated some of the difficulties in the applying of these procedures to the estimation of vitamin D. The use of extinction ratios has been shown to be advantageous. Unless the proposed methods include such data

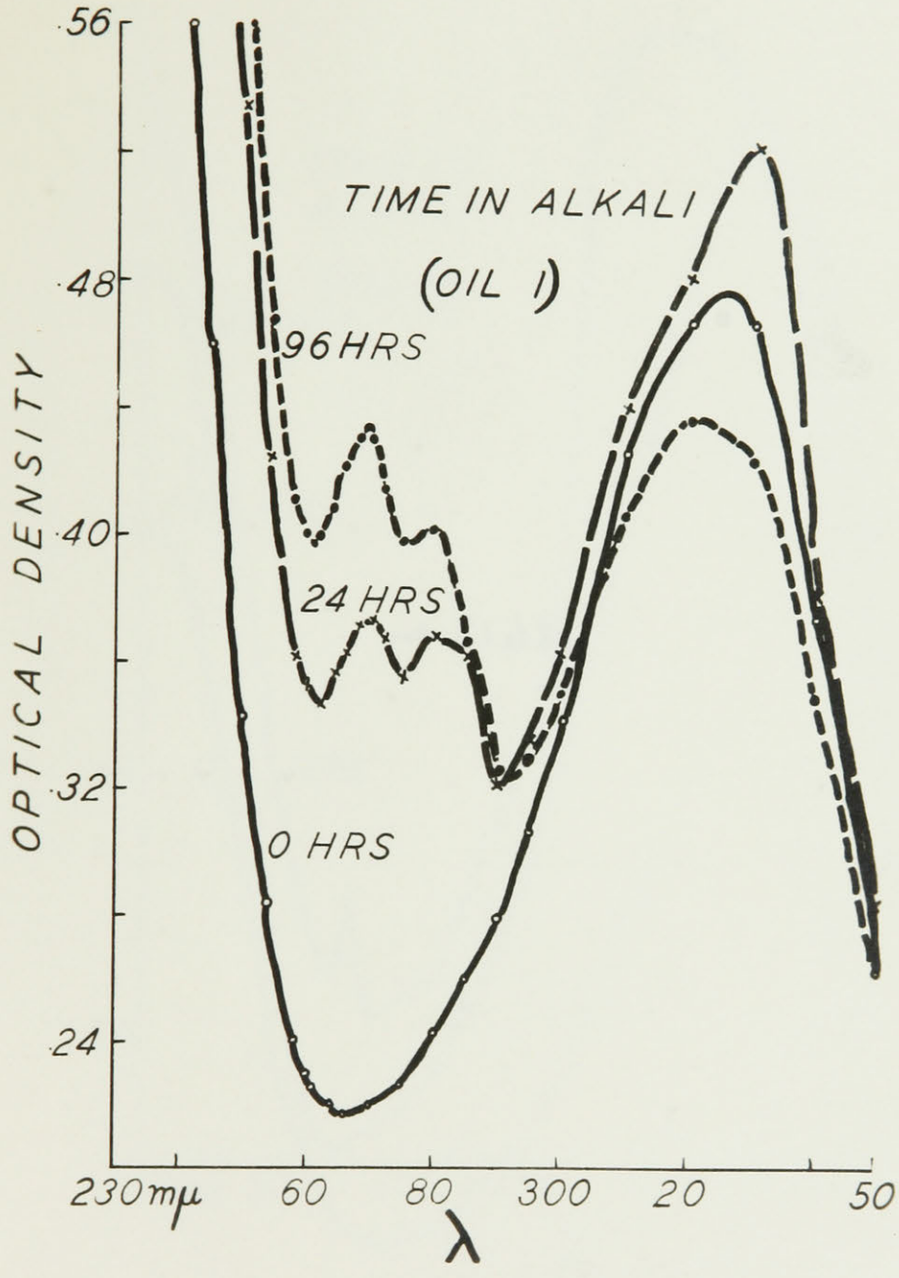


FIGURE 23. Absorption characteristics showing effect of time in alkali after saponification of sample of fish oil (No. 1).

they become empirical. Cold alkali treatment after saponification, slowly changes the absorption characteristics of fish oils, with varying effect.

(e) COMPARISONS WITH CHICK ASSAYS

The estimation of vitamin D by the Lifschütz reaction was compared with the chick assays using a series of oils. Chemical tests were carried out on duplicate samples of oil. Saponification was unnecessary for the high potency oils used in this work with the exception of the oil containing vitamin A. The oil was diluted to necessary volume with chloroform and the test applied to three different aliquots each of which were made up to 3 ml. Nine ml. of the Lifschütz reagent was added to each tube. The lowest point of swing of the galvanometer was noted after setting to 100 with the blank and an average of three values was taken. Biological assays were carried out at Ottawa completely in duplicate. Over 2,000 chicks were used to assay the seven oils. The ash contents of dry and extracted toes (see Part I) were used as criteria of calcification.

Collaborative studies were arranged with Dr. G.A. Grant of Ayerst, McKenna and Harrison, Ltd., Montreal, who supplied both chemical and biological data. The collaborative data presented in Table 33 thus furnish a fairly reliable comparison of the estimates of potency by the two procedures. The ratios of biological to chemical results have also been calculated and give an average value of 0.83, i.e., on the average the biological potency is only about 80 per cent of that of the chemical estimate. For the Ottawa laboratory the value is 0.70. If the ratios had been fairly uniform, then it might have been possible to use a different factor than 40

TABLE 33

Comparison of Lifschütz Reaction and Chick Assays of Oils
Potency in Million A.O.A.C. Units per gm.

(40 million units = 1 gm. D_3)

	Ayerst Laboratory			Ottawa Laboratory		
Sample	Chemical	Bioassay*	Bioassay / Chemical	Chemical	Bioassay**	Bioassay / Chemical
A[1]	-	-	-	1.32	1.21	0.92
D	-	-	-	1.71 (direct)	1.25	0.73
				1.47 (sap.)	-	-
1	2.24	1.16	0.51	2.23	0.99	0.44
2	2.36	1.6	0.68	2.25	1.26	0.56
3	1.84	1.5	0.82	2.09	1.43	0.68
4	1.4	1.7	1.21	1.84	1.41	0.77
5	1.7	1.7***	1.00	1.82	1.44	0.79
6	1.0	0.9	0.90	-	-	-
7	3.1	2.6	0.84	-	-	-
8	1.9	2.3	1.21	-	-	-
9	8.0	8.0	1.00	-	-	-
10	2.08	1.84	0.88	-	-	-
11	1.84	1.66***	0.90	-	-	-
12	2.0	1.6***	0.80	-	-	-

* A.O.A.C. Assay.

** Chick Toe Assay - Average of Two Assays.

*** Average of Two Assays.

1 Sample contains vitamin A - all others contain vitamin D only.

million units per gram D_3. In this case 33.2 million (i.e., 0.83 x 40) might have been suitable. However, some of the chemical results are so variable that this would only exaggerate the difference. It is obvious then that further refinement is needed before this reaction can be applied to even high potency oils. The biological values as reported by the two laboratories check within 15 to 20 per cent which is what would be expected, although Ayerst's values are consistently higher.

The variability in results with individual aliquots of oil as analyzed by the chemical method was also evident. There was a tendency for the more concentrated aliquots to give a higher value. Undoubtedly, these variations would affect the comparison with the biological data.

At the time these tests were conducted, 1944-45, the factor of 40 million A.O.A.C. units per gram was in general use although it was realized that the true value was higher. If the higher factor were used the difference between the methods would be greater. In subsequent tests the factor of 55 million was used.

Four samples of high potency oils (Dupont) were used to compare the other chemical criteria with the results by chick assay. The assigned potencies were based on a mean of assays in three laboratories and, therefore, may be considered reliable estimates. The results of comparisons with the antimony trichloride method are given in Table 34. The latter values agree well with the assigned potencies in spite of the fact that when tested at two separate

TABLE 34

The Potencies of Dupont Oils as Determined by Biological and Antimony Trichloride Procedures

(results in million A.O.A.C. units per gram)

Oil	Bioassay[2]	Antimony Trichloride Test[1]
3381	1.45	1.41
3387	1.30	1.31
3389	1.30	1.38
3395	1.35	1.44

1 Factor = 55 million units per gram vitamin D_3.

2 Means of assays in 3 laboratories.

concentrations the value at one concentration may be 5 to 10 per cent greater than that at another. The values were generally greater for the lower concentration, although both were in the central portion of the transmission scale. This is the opposite to what was found with the Lifschütz reagent. Saponification did not influence the position of the curve.

The glycerol dichlorohydrin reaction was also used to determine the potencies of the Dupont oils. The procedure as already outlined was used and the colour read at 620 and 420 mu. The results are presented in Table 35 under G.D.H. reaction. The values are all high when the factor of 55 million units is used. On the average the bioassay values are 81.8 and 87.7 per cent of the potencies determined at 620 mu and 420 mu, respectively. Using these factors adjusted G.D.H. values have been obtained which are also given in Table 35. The potencies, as measured at 620 mu, now very closely approximate the biological values. At 420 mu the agreement is not quite so good. It is doubtful, however, if the differences in results at the two wave lengths can be considered significant in view of the small number of samples involved.

Ultra-violet absorption measurements were made on the four oils dissolved in absolute alcohol containing 1% purified petrol ether. The E values were calculated and multiplied by a factor of 55/467, i.e., the ratio of potency to E value of crystalline vitamin D_3. The results are recorded in Table 36 under direct

TABLE 35

Potencies of Dupont Oils as Determined by Biological and Glycerol Dichlorohydrin Reaction

(results in million A.O.A.C. units per gram)

Oil	Bioassay	G.D.H. Reaction1 620 mu	G.D.H. Reaction1 420 mu	G.D.H. (adjusted) 620 mu	G.D.H. (adjusted) 420 mu
3381	1.45	1.76	1.69	1.44	1.48
3387	1.30	1.63	1.36	1.33	1.19
3389	1.30	1.58	1.70	1.29	1.49
3395	1.35	1.61	1.41	1.32	1.24

1 Factor = 55 million A.O.A.C. units per gram vitamin D_3.

TABLE 36

The Potencies of Dupont Oils as Determined by Biological and Ultraviolet Spectrophotometric Procedures

(results in million A.O.A.C. units per gram)

Oil	Bioassay	Ultraviolet Absorption at 265 mu	
		Direct[1]	Adjusted
3381	1.45	1.88	1.51
3387	1.30	1.46	1.17
3389	1.30	1.85	1.48
3395	1.35	1.54	1.24

1 Factor = 55 million A.O.A.C. units per gram.

method. They show a very similar trend to the G.D.H. reaction at 420 mu. In the last column the direct values have been adjusted as was done with the G.D.H. data to bring them in line with biological potency. The results indicate that this method may be classed as equivalent to the G.D.H. reaction. It may be mentioned, however, that the absorption maxima of these oils were not well defined and in some cases were little more than a straight line from 258 to 266 mu.

In general, the antimony trichloride reaction yields results as close to the biologically determined potencies as any method. It is evident that the Lifschütz method may not always indicate the same differences between oils as does the chick assay. Although the chick assay data reported here may be considered as reliable as is normally possible to obtain, nevertheless, they are not entirely satisfactory as a standard of comparison for chemical and physical methods which by their nature are much more precise.

DISCUSSION

Three chemical methods and the direct ultra-violet absorption method have been applied to the estimation of vitamin D in fish oils. With high potency oils, the antimony trichloride reaction probably gives the closest agreement with chick assays. It is sensitive to approximately two micrograms of vitamin D. Nevertheless, it is more difficult to handle than the other reagents. High potency oils do not exhibit typical absorption curves for vitamin D. The glycerol dichlorohydrin method as defined by us is more sensitive than the Lifschütz reaction and is not subject to the same variation. It is the most convenient to use. The determination of the extinction coefficient of vitamin D at 265 mu is about one-sixth as sensitive as the antimony trichloride reaction. Extinction coefficients for the reaction products of vitamin D_3 with the several reagents and that by direct ultra-violet absorption measurements may be summarized approximately as follows:

Method	Extinction Coefficient
Antimony trichloride	2570
Direct absorption	467
G.D.H. at 410 mu (ratio 1:4)	350
Lifschütz reaction	198
G.D.H. at 625 mu (ratio 3:2)	16

It is evident that the G.D.H. reaction is now almost equal in sensitivity to the ultra-violet absorption procedure.

The problem of low potency oils remains unsolved. Antimony trichloride reacts with many constituents of the oils including vitamin A, carotene and sterols, to give a variety of colours. Most of the available reagents react somewhat similarly. It has been shown that vitamin A may be removed fairly effectively with Superfiltrol but the removal of the sterols constitutes a major problem. Several methods have been suggested but none seem entirely satisfactory. A more serious problem lies in the fact that a method perfected in one laboratory cannot readily be duplicated in another. This difficulty is evident in this thesis and has been confirmed in discussions with other workers. This thesis is unique in that it constitutes the first major attempt to compare existing chemical methods.

In view of the experience gained here, it is suggested that chromatographic and other procedures be accompanied by studies of the ultra-violet absorption of the materials. The use of extinction ratios may also be helpful in making the procedures less empirical.

GENERAL SUMMARY

Factors influencing the results of various assay techniques for vitamin D_3 have been discussed. The need for greater statistical control in chick tests has been pointed out.

A series of assays and experiments involving over 15,000 chicks has been carried out on various types of vitamin D feeding oils to study statistically various factors in the chick assay and to compare four criteria of calcification, namely, ash per cent of tibiae, fresh toes and extracted toes and the Olsson radiographic technique.

It has been shown that on the average the four criteria of response give approximately the same estimate of potency, and are equally reproducible and precise as judged by the error slope (s/b) ratio. The two week assay period is comparable to the three week period for an estimate of potency but the precision is lower. The curative feeding period yields equally precise results a week sooner than the preventive assay. The A.O.A.C. diet is as satisfactory as any that have been proposed. The breed of chick does not seem to be an important factor.

There is greater variation between replicate groups than between individual chicks within the groups and still greater variability is found when the replicate groups represented different months. New concepts for the expression of error of assays have been proposed to take into account these sources of variation.

It is recommended that crystalline vitamin D_3 be used as reference standard for all vitamin D tests and that potency be expressed in weight rather than units.

It is concluded that, while both the toe ash and X-ray criteria offer advantages over the tibia ash method, the simplest method for vitamin D available at the present time is the toe ash procedure. By using the variance between replicate groups as error the precision of assays can be estimated readily and more precisely than heretofor. For the most reproducible type of assay the replicate groups should be handled at separate times. A method, based on the investigations reported herein, is presented in detail.

Factors influencing the results of chemical procedures for the determination of vitamin D have also been discussed. The need for a less empirical approach has been stressed.

The antimony trichloride, Lifschütz and glycerol dichlorohydrin colour reactions and the ultra-violet absorption procedure have been studied using various materials containing vitamins D. The antimony trichloride procedure is probably the most useful for low potency materials. The glycerol dichlorohydrin method has been made 20 times as sensitive for vitamin D_3. The importance of extinction ratios and ultra-violet absorption data have been illustrated.

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