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# Studies on the Analysis of Vitamins D

## 1. THE REACTION OF VITAMINS D AND RELATED SUBSTANCES WITH IODINE TRICHLORIDE

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The problem of the chemical analysis of vitamins D in natural oils and synthetic irradiation products of ergosterol and 7-dehydrocholesterol has received considerable attention over the past 15 years. Relatively little success, however, has been achieved in attempts to find a substitute for bioassays for determining vitamin D potencies, although some progress has been made with the analysis of high potency synthetic concentrates. Ewing, Kingsley, Brown & Emmett (1943) have given a very full summary of the literature dealing with the chemical analysis of vitamin D. So far, the most useful reagent has undoubtedly been the improved antimony trichloride reagent of Nield, Russell & Zimmerli (1940). Since the paper by Ewing *et al.* (1943), the main advances have been due to Sobel, Mayer & Kramer (1945) with their discovery of the glycerol dichlorohydrin reaction, and De Witt & Sullivan (1946), who used a chromatographic separation on magnesia and diatomaceous earth and a modified antimony trichloride-acetyl chloride reagent in ethylene dichloride for determining vitamin D in several products. Ewing, Powell, Brown & Emmett (1948) developed two methods for determining vitamin D in irradiated ergosterol products, one by direct estimation with the improved antimony tri-

chloride reagent of Nield *et al.* (1940) and the other, after a chromatographic separation on 'Superfiltrol', by direct measurement of extinction at 265 mμ.

The four chief problems in the accurate analysis of vitamin D in natural products are (*a*) the elimination of large quantities of interfering sterols, particularly cholesterol, (*b*) the elimination of vitamin A, (*c*) the need to measure minute amounts of vitamin D, because of its great biological potency, and (*d*) to devise a sufficiently reproducible and specific reagent. No reagent so far described is very satisfactory. Spectrophotometric measurements at 265 mμ. are of doubtful value, unless irrelevant absorption is low; the various antimony trichloride reagents must be meticulously prepared to give even moderately reliable results, and no hitherto described procedure is based on a stoicheiometric reaction.

The present work describes a new reagent which undergoes a stoicheiometric reaction with the vitamins D. Part 1 shows how the reaction may be used for quantitative titrations and is the basis (with other work to be described later) of methods of chemical analysis for vitamin D in natural and synthetic products. These will be described in succeeding parts.

In the course of investigations into the action of inorganic halogen compounds on vitamin D, it was found that a dilute solution of iodine trichloride in chloroform, when added to a solution of calciferol in the same solvent, gave an immediate reddish violet coloration, which disappeared on addition of excess of the reagent. The reaction occurred in the cold, and appeared to take place instantaneously. When the chloroform was replaced by a more polar solvent such as acetone, no colour appeared. It seemed, from these observations, that the reaction was a simple addition of chlorine to the unsaturated linkages of the calciferol molecule, with the liberation of free iodine.

The difficulty of estimating free iodine in the presence of iodine trichloride by chemical methods prevented an unequivocal test of this hypothesis, but a study of the reaction in the visual spectrophotometer showed that the red-violet solution gave the typical absorption spectrum of iodine, with a maximum at 518 mμ.

The reaction was obviously different from all previously described reactions of iodine monochloride or trichloride with unsaturated molecules, in all of which there is iodine addition rather than liberation.

## EXPERIMENTAL

### *Materials and apparatus*

(1) *Calciferol*, m.p. 115–116°. Supplied by Glaxo Laboratories Ltd., 'Puriss. in Nitrogen' grade. (2) *Vitamin $D_3$*. Supplied by Glaxo Laboratories Ltd., and also by E. I. Dupont de Nemours, U.S.A. (3) *Lumisterol*, m.p. 117–118°. Obtained, by alkaline hydrolysis, from a sample of lumisteryl dinitrobenzoate, supplied by the National Institute for Medical Research, London. Recrystallized from acetone. (4) *Tachysteryl dinitrotoluate*, m.p. 151°. Supplied by G. Merck, Darmstadt. (5) *Suprasterol II*, m.p. 110–111°. Obtained, by alkaline hydrolysis, from a sample of the dinitrotoluate supplied by G. Merck, Darmstadt. (6) *Suprasterol I*. Isolated as the allophanate from the irradiation of calciferol in ether by a mercury arc lamp. (7) *Sitosteryl acetate*. (8) *Pyrocalciferyl acetate*, m.p. 79–80°. (9) *iso*Pyrocalciferyl acetate, m.p. 110°. (10) *Ergosterol*, m.p. 158–160°. Supplied by Glaxo Laboratories Ltd. Recrystallized from methanol. (11) *7-Dehydrocholesterol*, m.p. 147°. Obtained pure, by fractional crystallization from ether of a 60% concentrate, supplied by Glaxo Laboratories Ltd. (12) *β-Carotene*. By fractional crystallization of a pure commercial grade, supplied by British Crop Driers Ltd. (13) *Vitamin A concentrate*. Molecular distillate 1,015,000 i.u./g. supplied by British Drug Houses Ltd. (14) *Iodine trichloride*. Rechlorinated until pure. (15) *Carbon tetrachloride*. B.P. grade, washed with $H_2SO_4$, six times with water, dried over $MgSO_4$, and redistilled in glass apparatus.

Instruments used for colorimetric measurement were the Spekker absorptiometer, the Hilger-Nutting visual spectrophotometer, and the Hilger medium quartz ultraviolet spectrophotometer.

### *Preliminary qualitative studies*

Several classes of substance, containing different unsaturated systems, were tested to see if they gave the $ICl_3$ reaction under the same conditions as calciferol (i.e. simple mixing in $CHCl_3$ solution).

*Sterols.* The reaction was given by calciferol, vitamin $D_3$, ergosterol, 7-dehydrocholesterol, lumisteryl and tachysteryl dinitrotoluates, suprasterols I and II, pyrocalciferyl acetate, and *iso*pyrocalciferyl acetate.

The reaction was not given by cholesterol or sitosteryl acetate, although the former very slowly liberated iodine on shaking with a solution of $ICl_3$.

*Vitamin A* gave a positive reaction.

*β-Carotene.* The reaction of $ICl_3$ with a dilute solution of β-carotene was found to follow an unusual course. When the reagent solution is added slowly to the carotene solution, a transient greenish-blue colour occurs at the point of mixing and disappears in less than a tenth of a second. As the reagent is added, no pink coloration is observed, but the golden solution of the β-carotene becomes progressively paler. Finally, with the addition of one drop of reagent, a threshold is reached and the whole solution very suddenly turns a deep pink, as the free iodine is liberated.

The transient greenish-blue colour is, in all probability, related to an initial reaction similar to the chromogenic reactions with carotenoids given by other inorganic halides, such as $SbCl_3$; this reaction is then followed by a second reaction involving addition of the whole $ICl_3$ molecule to the conjugated

system, and this in turn is followed by a third reaction involving liberation of iodine. The second-stage reaction product, formed by the addition of the whole $ICl_3$ molecule, appears to be highly unstable near the iodine-liberation threshold, since even sudden shaking of the solution at this point serves to liberate the iodine.

*Vegetable and animal oils.* Almost all natural oils and fats tested reacted to some degree to liberate iodine from $ICl_3$. In addition, most oils absorbed very large amounts of the reagent. The only exception found was castor oil, which absorbed large amounts of the reagent, but liberated no iodine.

*Aromatic hydrocarbons, and heterocyclic bases.* Very pure benzene, in dilute $CCl_4$ solution, liberated iodine only very slowly from $ICl_3$. Ordinary reagent benzene reacted positively, as did toluene and xylene. When solid $ICl_3$ was thrown into benzene or toluene, iodine was liberated with almost explosive violence.

Pyridine and quinoline, in solution, immediately absorbed the reagent, with the separation of yellow precipitates.

*Benzene derivatives.* In compounds such as aromatic phenols, ketones, etc., no reaction takes place.

*Mono-ethenoid compounds.* Hydrocarbons such as *cyclo*hexene absorb the reagent without iodine liberation. Styrene, which contains a side-chain double bond in conjugation with the benzene ring, reacts similarly. Non-hydrocarbon molecules, such as maleic anhydride, do not react.

*Di-ethenoid compounds.* Isoprene (two conjugated double bonds) absorbed $ICl_3$ without liberation of iodine. Methyl linoleate (two conjugated double bonds) reacted similarly.

*Tri-ethenoid compounds.* Methyl linolenate (three unconjugated double bonds) absorbed the reagent with no iodine liberation. Methyl elaeostearate (three conjugated double bonds) liberated iodine immediately from $ICl_3$, without absorbing an excess of reagent.

### *Preliminary quantitative studies*

For quantitative work $CCl_4$, purified as described, was found to be the best solvent. $ICl_3$ in this solvent is far more stable than in $CHCl_3$. $CCl_4$ is also the safest solvent for preparing stable dilute solutions of provitamins and vitamins D, as Paterson & Harvey (1944) have already demonstrated for ergosterol.

First studies of the reactions were made by estimating liberated iodine on the Spekker absorptiometer, using O.B. 2 filters and cells of 10 ml. capacity and 1 cm. optical depth.

(*a*) *Stability of colour.* Solutions of calciferol in $CCl_4$ (15·5 mg./50 ml.) and of $ICl_3$ in $CCl_4$ (46·8 mg./50 ml.) were prepared. Equal volumes (4 ml.) of each solution were mixed in the test cell and the reading taken on the absorptiometer, matching against pure $CCl_4$. The cells were covered with ground-glass slides to prevent evaporation, and readings were taken at intervals. Table 1 shows that the reading is reasonably stable for at least 1 min., after which a drift occurs.

Table 1. *Stability of iodine colour, obtained by interaction of calciferol and* $ICl_3$, *measured on absorptiometer*

(For details see text.)

| Time (min.) | Reading (*E*) |
|---|---|
| 0 | 0·121 |
| 0·25 | 0·121 |
| 0·50 | 0·122 |
| 0·75 | 0·122 |
| 1·0 | 0·122 |
| 5·0 | 0·130 |
| 10·0 | 0·134 |
| 15·0 | 0·139 |
| 20·0 | 0·144 |
| 25·0 | 0·144 |
| 30·0 | 0·150 |

(*b*) *Shape of titration curve.* $CCl_4$ solution (1 ml.), containing 2 mg. of vitamin $D_3$, was titrated in the test cell with a solution of $ICl_3$ in $CCl_4$ (0·052 g./100 ml.). The procedure was to add to the 1 ml. of vitamin $D_3$ solution a measured volume, from a burette, of the reagent, make up to 10 ml. with pure $CCl_4$, and immediately read on the absorptiometer.

Table 2 shows the typical titration results obtained.

Table 2. *Vitamin* $D_3$ (2 *mg.*) *titrated with* $ICl_3$. *Total volume made up to* 10 *ml. with* $CCl_4$

(For details see text.)

| $ICl_3$ solution added (ml.) | Reading (*E*) |
|---|---|
| 1·0 | 0·055 |
| 2·0 | 0·114 |
| 3·0 | 0·180 |
| 3·5 | 0·207 |
| 4·0 | 0·235 |
| 4·5 | 0·263 |
| 5·0 | 0·289 |
| 5·5 | 0·292 |
| 6·0 | 0·281 |
| 6·5 | 0·293 |
| 7·0 | 0·271 |
| 7·5 | 0·202 |

Stepwise addition of reagent produces a peak reading which is quickly followed by a second peak very nearly identical in maximum reading, and finally there is a rapid fall-off in readings as excess reagent re-absorbs the iodine liberated. Such two-peak curves are nearly always observed when the titration is carried out using small stepwise additions of reagents.

The slope of the upward portion of the curve is independent of the original amount of vitamin D titrated.

(c) *Attempts to remove excess reagent.* Attempts were made, with only moderate success, to remove excess $ICl_3$ quantitatively from the reaction mixture without removing the already liberated iodine. Small quantities of a substance such as castor oil or *cyclo*hexene were added to the titration solution, but although these substances did not absorb free iodine from simple $CCl_4$ solution, in the presence of $ICl_3$ both the latter reagent and iodine were always absorbed together. On addition of sufficient $ICl_3$ to a solution of *cyclo*hexene or castor oil and iodine, complete absorption may be obtained, leaving a colourless solution. If vitamin $D_3$ is titrated, with the addition of a small amount of *cyclo*hexene before $ICl_3$ addition, both substances compete for the reagent and the maximum quantity of iodine liberated is considerably lower than that liberated by vitamin $D_3$ alone. If the stepwise titration is carried out, adding *cyclo*hexene after the $ICl_3$, liberated iodine is only absorbed beyond the end point and, in this case, the maximum quantity determined is only slightly lower than the theoretical amount. Further experiments on these lines might lead to a simpler quantitative titration method, but it was not considered necessary to investigate the matter in detail, since the end point is relatively easy to determine.

It is possible that pure vitamin $D_3$ could be electrometrically titrated against $ICl_3$ in a suitable solvent, since, for example, $ICl_3$ is conducting in nitrobenzene, whereas iodine is not. Electrometric titration in $CCl_4$ should not be impossible, using perhaps high frequency (electrodeless) titration methods.

*Quantitative spectrophotometric studies*

Examination of the mixture of $ICl_3$ and calciferol in $CCl_4$ solutions with the Hilger-Nutting visual spectrophotometer showed, in the presence of a slight excess of calciferol, the normal iodine absorption spectrum, with a maximum at 518 m$\mu$.: there were no other bands to be seen in the visible region. The ultraviolet absorption of the same solution showed a strong band at 265 m$\mu$. and a faint band at 310 m$\mu$. The absorption spectrum of the chlorinated calciferol is unknown and may account for either or both of the latter two bands. No peak was observed at 330 m$\mu$.: this indicates the absence of free chlorine from the reaction mixture. Pure iodine in $CCl_4$ was found to have a molar extinction coefficient of 1116, which figure was used throughout the following calculations. Earlier determinations by Brode (1926) gave considerably lower values: the discrepancy could not be accounted for.

(a) *Vitamin $D_3$.* The vitamin $D_3$ molecule contains a conjugated tri-ethenoid system. If the $ICl_3$ reaction is quantitative and stoicheiometric, the equation followed will be

$$\text{`}D_3\text{'} + 2ICl_3 \rightarrow \text{`}D_3\text{'}\,Cl_6 + I_2.$$

For experimental test of the equation, the $ICl_3$ solution used contained 91·1 mg./250 ml., and the vitamin $D_3$ solution 40·2 mg./50 ml. The vitamin $D_3$ solution (1·5 ml.), mixed with 4 ml. of the reagent, theoretically gives an $ICl_3/D_3$ molar ratio of 2.

Table 3. *Determination of* $ICl_3$:$I_2$:$D_3$ *molar ratios*

| Vitamin $D_3$ (mg.) | $ICl_3$ added (mg.) | Total vol. (ml.) | $E_{518\,m\mu}$. | $I_2$ liberated (mg.) |
|---|---|---|---|---|
| 1·206 | 1·456 | 5·5 | 1·21 | 0·766 |
| 1·206 | 1·602 | 5·9 | 1·25 | 0·849 |

The vitamin $D_3$ solution (1·5 ml.) was placed in a test cell (2 cm. optical depth) and 4 ml. reagent added. Liberated iodine was estimated by measuring the optical density of the solution at 518 m$\mu$. The experiment was repeated using a 10% excess of reagent. Table 3 shows the results obtained. The maximum amount of $I_2$ was formed when 10% excess $ICl_3$ was added: the molar ratio, $I_2$/vitamin $D_3$ was then

$$\frac{0{\cdot}849 \times 384}{1{\cdot}206 \times 254} = 1{\cdot}06.$$

The purity of the $ICl_3$ was checked by measuring its extinction at 455 m$\mu$. at an optical depth of 4 cm. The solution was found to contain only 88% of pure $ICl_3$. Using this correction, at the maximum point the molar ratio $ICl_3$/vitamin $D_3$ is therefore,

$$\frac{1{\cdot}602 \times 384 \times 88}{1{\cdot}206 \times 254 \times 100} = 1{\cdot}8.$$

Although this figure is slightly low, it is in good agreement with the value expected from the equation above. The slight difference between the figure found in this experiment and the theoretical value of the molar ratio is due to the presence of some monochloride in the reagent.

It was clear, from these results, that the reaction was stoicheiometric and could be used for titration, using the maximum iodine liberated as a means of measuring the end point. Fig. 1 shows the full titration curve obtained by titrating 3 ml. of the vitamin $D_3$ solution with $ICl_3$. The technique used was to place the vitamin $D_3$ solution in a small conical flask, add a measured amount of reagent and mix by swirling. The absorption at 518 m$\mu$. was then rapidly measured in the test cell. Titration was continued until the peak had been passed. Simple calculation gave the iodine liberated at each point and this was plotted against volume of reagent added. The characteristic double peak is clearly shown. Beyond the end point, the absorption falls off slowly for a considerable time, since $ICl_3$ itself contributes about one-third of its maximum absorption at 518 m$\mu$.

This makes it relatively easy to determine the precise end point, although, of course, immediately after the peak in Fig. 1, the measured 'iodine liberated' is made up from an 'iodine + $ICl_3$' component.

(*b*) *Iodine trichloride-iodine back reaction.* Excess reagent combines with liberated iodine to form ICl: if this reaction were stoicheiometric, equimolecular proportions of $I_2$ and $ICl_3$ would be necessary to form three molecules of ICl. The complete vitamin $D_3$-$ICl_3$ reaction to monochloride formation would then involve 50 % excess reagent over the amount necessary for quantitative iodine liberation.

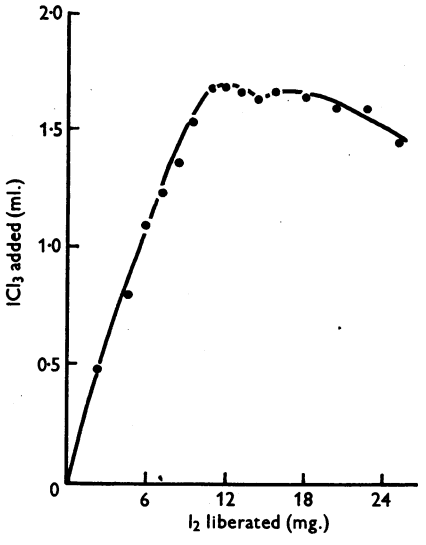


Fig. 1. Full titration curve produced by titrating 2·412 mg. of vitamin $D_3$ with $ICl_3$. Measurements carried out on visual spectrophotometer.

The vitamin $D_3$ solution (1·5 ml.) was mixed rapidly with 6·6 ml. of the $ICl_3$ solution and the mixture examined spectroscopically at 2 mm. optical depth. Only the iodine band at 518 m$\mu$. was observed, and indeed, visually the solution still appeared to contain much free iodine. Experiment showed that several molecular proportions excess reagent were necessary to remove all the iodine, and the solution then showed the typical band at 460 m$\mu$. due to ICl and an additional band at 330 m$\mu$. (due to dissociation of excess $ICl_3$). The $ICl_3$-$I_2$ back reaction is thus not stoicheiometric: iodine is re-absorbed slowly, but eventually completely.

(*c*) *Calciferol.* The calciferol molecule contains a conjugated tri-ethenoid system in the sterol nucleus and one double bond in the sterol side chain. In most samples of calciferol examined, only the conjugated system could be titrated with $ICl_3$, and analytical results obtained were almost identical with those described for vitamin $D_3$. However, anomalous titration of all four double bonds has been found to occur with one sample of calciferol. Studies of the calciferol reaction are referred to in more detail later.

(*d*) *Vitamin A.* The reaction of vitamin A was studied, using the molecularly distilled concentrate having $1{\cdot}015 \times 10^6$ i.u./g. A solution (0·0400 g./25 ml. $CCl_4$) was titrated with the same $ICl_3$ solution, in 1 ml. quantities. Since the theoretical potency of pure vitamin A is $3{\cdot}3 \times 10^6$ i.u./g., the solution contains 0·488 mg. of vitamin A/ml. Table 4 presents the titration data.

Table 4. *Titration of vitamin A with* $ICl_3$ *on the visual spectrophotometer*

(1 ml. vitamin A solution taken.)

| $ICl_3$ added (ml.) | Total vol. (ml.) | $E_{518\,m\mu.}$ | $I_2$ liberated (mg.) |
|---|---|---|---|
| 3·0 | 4·0 | 1·2 | — |
| 4·0 | 5·0 | 1·2 | — |
| 4·5 | 5·5 | 1·26 | — |
| 5·0 | 6·0 | 1·15 | 0·789 |
| 5·5 | 6·5 | 1·06 | 0·787 |
| 6·0 | 7·0 | 1·06 | 0·848 |
| 6·5 | 7·5 | 0·94 | 0·806 |
| 7·0 | 8·0 | 0·84 | 0·768 |

Calculation gives the $I_2$/vitamin A molar ratio at the end point as 1·71. The reaction,

$$3\,\text{'A'} + 10\,ICl_3 \rightarrow 5I_2,$$

requires a ratio of 1·66. In view of the impurity of the vitamin A source, the experimental result suggests that the stoicheiometric reaction is probably obeyed in the case of vitamin A.

Table 5. *Titration of β-carotene with* $ICl_3$

(4·5 ml. carotene solution taken.)

| $ICl_3$ added (ml.) | Total vol. (ml.) | $E_{518\,m\mu.}$ | $I_2$ liberated (mg.) | Remarks |
|---|---|---|---|---|
| 0·5 | 5·0 | 3·3 | Nil | Colour, golden. General absorption below 580 m$\mu$. |
| 1·0 | 5·5 | 1·65 | Nil | General absorption below 495 m$\mu$. |
| 1·5 | 6·0 | 0·85 | Nil | — |
| 2·0 | 6·5 | 0·80 | 0·594 | Colour, pink. No general absorption |
| 2·5 | 7·0 | 0·80 | 0·640 | — |
| 3·0 | 7·5 | 0·76 | 0·653 | — |
| 3·5 | 8·0 | 0·70 | 0·640 | — |
| 4·0 | 8·5 | 0·68 | 0·661 | — |
| 5·0 | 9·5 | 0·59 | 0·652 | — |

(*e*) *β-Carotene.* The reaction of β-carotene was studied by titrating 4·5 ml. of a solution containing 0·0880 g./100 ml. $CCl_4$. The extraordinary reaction of β-carotene with $ICl_3$ has already been discussed, and is shown clearly by the spectrophotometric study, as presented in Table 5.

A study of the table shows that almost all the theoretical quantity of iodine is liberated at one point, shortly before the end point is reached: further addition of reagent then liberates normal quantities of iodine up to the end point, which is prolonged.

Calculation gives the $I_2$/carotene molar ratio in this experiment as 3·48. Within the limits of experimental error, therefore, the reaction is followed according to the relationship

$$3\text{ 'carotene'} + 22\ ICl_3 \rightarrow 11\ I_2.$$

*Quantitative determinations, using the Spekker micro-absorptiometer*

In the reaction between vitamins D and $ICl_3$, the maximum absorption reading at 518 mμ. is constant over a fairly prolonged end point. The estimation of vitamins D by titration depends on an accurate determination of the maximum, which can, under suitable conditions, be readily carried out with a Spekker absorptiometer. Determinations of absorption must, because of inherent difficulties caused by reagent instability, slight drift of readings and volatilization, be made within about a minute.

The importance of determining the smallest possible amounts of vitamin D has necessitated the use of the micro-absorptiometer for titration purposes, with the use of 0·5 ml. optical cells. However, for larger quantities of material, the Sensitive Model absorptiometer, using 5 ml. cells, may be used.

It has been found convenient to take all readings on the instrument within the range 0·075–0·250 on the density drum. Although this is a more restricted and lower range than generally used with this instrument, its use facilitates the determination of minimal amounts of material, gives satisfactory straight line standard curves, and precision of readings is usually within limits of ± 3 %.

(*a*) *Preparation of reagent.* The preparation of a stable fully chlorinated reagent is essential. As usually obtained, $ICl_3$ is a dry, bright-orange solid, the precise colour of which depends on its degree of purity. In air, it quickly loses chlorine to yield the dark-brown, liquid monochloride: it also loses chlorine in all solvents, at varying rates depending on the solvent. Up to 15 % decomposition of the solid reagent may occur before this state is visually noticeable by the appearance of flecks of brown liquid monochloride.

In practice the procedure adopted for obtaining a suitable reagent has been as follows. The solid (5–10 g.), ground beforehand in a mortar, is placed in a clear glass, wide-mouthed bottle, fitted with a ground glass stopper. The contents of the bottle are periodically rechlorinated by passing a stream of dry chlorine for about 10 min. or until the original bright-orange colour is regained. The bottle is kept in the dark, but, since it is of clear glass, early changes of colour in the reagent can be noticed.

(*b*) *Titration method.* The vitamin D solution is made up in $CCl_4$. For use on the micro-absorptiometer, the concentration should be 100–250 μg./ml. The solution is filled into a 5 ml. burette, and a 25 ml. accurately graduated burette is filled with pure $CCl_4$.

The reagent solution should contain about 60–80 mg. $ICl_3$/100 ml.; the exact strength is not critical. Dilute solutions of this kind are unstable and must be prepared immediately before titration, in either of the following ways. (i) Between 250 and 500 mg. is weighed, as rapidly as possible, directly into a few ml. of $CCl_4$ in a stoppered weighing bottle or small flask and dissolved in 50 ml. of $CCl_4$, the solid being always kept covered with solvent: some small insoluble yellow residue usually remains. A portion is diluted to working concentration. The final solution will give a reading of 0·150–0·200 on the absorptiometer when matched against pure $CCl_4$ in 1 cm. optical depth, using O.B.2 filters. (ii) To 10–25 ml. of $CCl_4$ in a clean dry beaker, a few crystals of the solid reagent are rapidly added and, after stirring gently with a glass rod, the supernatant is decanted into a second beaker when the desired concentration above the undissolved solid is reached. This may be estimated by a quick reading on the absorptiometer, or more conveniently, after a little practice, visually. Decantation may be followed by further dilution, if necessary, but the concentration should not be increased by adding further solid reagent. The main essential of either method of preparation is speed. Method (ii) is more convenient, takes about 30 sec. to complete, and is recommended for accurate titration.

The prepared solution is transferred to an accurately graduated 5 or 10 ml. burette, which should be filled only once with the reagent: if more reagent is needed, it must be prepared afresh by method (ii) or by sub-diluting the strong solution in method (i). All the burettes used must have fine-bore jets and well fitting taps: the latter should be lubricated with a grease resistant to organic solvents: an excellent preparation is the starch-glycerol paste described by Herrington & Starr (1942).

The titration is carried out in the 0·5 ml. cells of the micro-absorptiometer, which have an optical depth of 1 cm. Filters may be O.B.2 or spectrum green 604 (using, with the latter, heat-resisting filters of Calorex or Chance's ON. 19). Only one of the micro-mounts of the instrument is used, as there may be a slight variation in the dimensions of

aperture between the two mounts provided, and each cell is placed in turn in the same mount. The blank cell is filled with pure solvent. To the test cell is added first 0·05–0·20 ml. of the vitamin D solution (25–50 μg. vitamin D), then a measured volume of the reagent, and finally pure $CCl_4$ from the third burette to make the volume up to 0·5 ml. A small ground-glass cover slip is placed over the cell, which is inverted twice to mix the contents and then placed in the micro-mount for measurement of colour. The cell is then drained and the process repeated with increasing additions of $ICl_3$ to a constant volume of test solution, until the end point has been passed, as indicated by a fall in readings, and, visually, by re-absorption of liberated iodine.

It may be necessary to repeat several readings around the end point, using minimal increments of reagent in order to obtain, as accurately as possible, the true maximum reading. All readings should be made rapidly, preferably without zeroing the galvanometer each time. The cells must be quite clean, and the optical surfaces should not be touched during the titration procedure. It has been found advisable, after making up the contents of the titration cell and closing it with the glass cover slip, to wipe the optical surfaces quickly with a piece of silk fabric before inserting the cell into the instrument.

(*c*) *Effect of filter.* The spectrum green 604 filters give readings about 30% higher than the O.B.2 filters, leading to a corresponding increase in sensitivity. However, owing to the narrower transmission range of the former filters, the end point is much sharper and rather more difficult to determine: using the 604 filters, therefore, the reagent, near the end point, should be added in the smallest possible increments.

### *Titration results and standard curves*

*Vitamin $D_3$.* Table 6 shows typical titration results at two levels of vitamin $D_3$, using spectrum green 604 filters, and Fig. 2 shows standard curves obtained by plotting maximum titration readings against micrograms of vitamin $D_3$, using both types of filter. The curves are linear over the range used.

Table 6. *Titration of vitamin $D_3$ on micro-absorptiometer*

(Total volume 0·5 ml.)

| Weight of $D_3$ (μg.) | $ICl_3$ added (ml.) | Reading (*E*) |
|---|---|---|
| 52·0 | 0·08 | 0·182 |
| | 0·10 | 0·200 |
| | 0·12 | 0·208 |
| | 0·14 | 0·215 |
| | 0·16 | 0·218 (peak) |
| | 0·18 | 0·200 |
| 35·3 | 0·10 | 0·126 |
| | 0·12 | 0·132 |
| | 0·13 | 0·148 (peak) |
| | 0·14 | 0·139 |
| | 0·16 | 0·134 |
| | 0·18 | 0·126 |

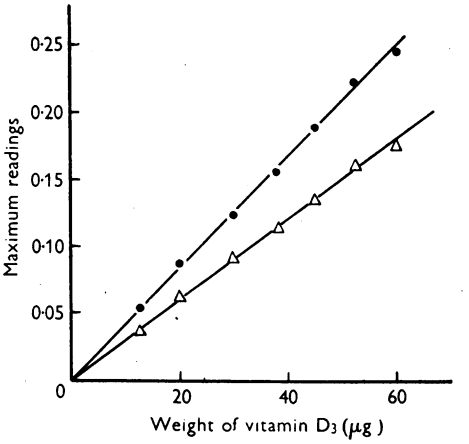


Fig. 2. Standard curves for vitamin $D_3$. Titration carried out with $ICl_3$ on the Spekker micro-absorptiometer, using two types of filter. Total volume, 0·5 ml.; optical depth, 1 cm. ●—●, spectrum green 604; △—△, O.B. 2.

*Calciferol.* As already stated, most samples of calciferol tested (at least twelve in number, with different origins) only three double bonds were titrated with $ICl_3$. With one sample, however, which was checked many times by titration on the micro-absorptiometer, a four double-bond titration was always obtained. Fig. 3 shows the two standard curves constructed by plotting maximum readings against μg. of calciferol, using O.B. 2 filters. Curve *B* corresponds to the three-bond titration and is, of course, almost identical with the vitamin $D_3$ curve.

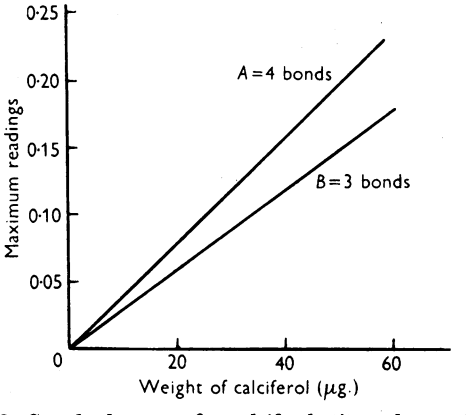


Fig. 3. Standard curves for calciferol, titrated on micro-absorptiometer; total volume, 0·5 ml.; optical depth 1 cm.; O.B.2 filters.

Table 7. *Titration of sterols on the micro-absorptiometer*

(0·5 ml. cells. Spectrum Green 604 filters.)

| Substance | Weight titrated ($\mu$g.) | Maximum titration reading | No. of double bonds titrated |
|---|---|---|---|
| Ergosterol | 40·0 | 0·170 | 3·05 |
| Lumisterol | 47·0 | 0·126 | 1·97 |
| Lumisteryl dinitrobenzoate | 67·0 | 0·136 | 2·10 |
| Pyrocalciferyl acetate | 58·0 | 0·204 | 2·85 |
| *iso*Pyrocalciferyl acetate | 48·0 | 0·183 | 3·10 |
| Suprasterol II | 54·0 | 0·151 | 2·05 |
| Tachysteryl dinitrotoluate | 95·0 | 0·145 | 1·73 |
| Tachysterol | 67·0 | 0·122 | 1·35 |

A number of experiments were carried out to determine the reason for the existence of two calciferol curves. First, chemical purification of the samples by fractional crystallization of the free sterols and their dinitrobenzoates failed to disturb the titration results. Exhaustive drying at 0·01 mm. was similarly without effect. The possibility of peroxide catalytic reaction of the fourth double bond was considered, but titration of normal samples in the presence of benzoyl peroxide or anti-oxidants such as ethyl gallate, quinol, etc. produced no change in the reactions. At present, no explanation can be offered.

*Other sterols.* The titration of other sterols and their pure esters was carried out as described for the vitamins D. Calculation of the number of double bonds titrated was made by computation from the standard vitamin $D_3$ curve. Table 7 presents the titration data and calculations for ergosterol, lumisterol, pyrocalciferyl acetate, *iso*pyrocalciferyl acetate, tachysterol, suprasterol II and 7-dehydrocholesterol.

## DISCUSSION

Iodine trichloride in carbon tetrachloride solution undergoes two types of reaction with certain classes of unsaturated substances. The first type involves immediate and quantitative addition of the iodine trichloride molecule and is apparently limited to hydrocarbons and fatty acids which contain one or more isolated double bonds or two conjugated double bonds. The second-type reaction, which has been found to occur with substances containing systems of three or more conjugated double bonds and, in addition, a group of sterols related to the vitamins D, involves chlorination and liberation of iodine. Iodine monochloride, in general, reacts similarly: but, with this reagent, the second-type reaction is not so specific: cholesterol, for example, which only contains one unsaturated linkage liberates iodine from iodine monochloride. The two types of reaction are, for several reasons, remarkable, and the iodine liberation reaction is of a unique character. Although there is a wealth of literature dealing with the action of iodine chlorides on unsaturated substances, all the reactions hitherto described have involved iodination. The present reactions also differ from those previously described by taking place under the mildest possible conditions, in cold carbon tetrachloride solution. Finally, although occurring in pure non-polar solvent, the reactions are instantaneous and stoicheiometric.

Temperature is without effect either on the type, the velocity, or the quantitative character of the reaction, which proceeds identically at room temperature or at $-23°$ (the melting point of carbon tetrachloride). There is no peroxide effect involved in either type of reaction: their occurrence is independent of the presence of substances such as benzoyl peroxide or powerful antioxidants, such as quinol, pyrogallol, or ethyl gallate.

The type II reaction (iodine liberation) gives rise to speculation as to the mechanism involved. The reaction

$$R + 2ICl_3 \rightarrow RCl_6 + I_2,$$

where R is a molecule containing three conjugated double bonds, is one of semi-addition to R, or substitution of the $ICl_3$ molecule.

The absence of reaction between $ICl_3$ and cholesterol, in contrast to the reaction of ICl with the latter substance, indicates that the chlorination does not necessarily proceed through a first step of dissociation of the trichloride to the monochloride and free chlorine (although this dissociation, of course, always occurs). It is also improbable that, in carbon tetrachloride solution, sufficient ionization occurs to enter into the reaction mechanism.

It is believed that the most important indication of the reaction mechanism may be given by the observed reaction with $\beta$-carotene. The transient greenish-blue colour formed at the point of contact with the reagent is almost certainly due to an initial mesomeric change in the $\beta$-carotene molecule under the influence of an iodine trichloride molecule or a $Cl^-$ or $[Cl_3]^-$ ion: this is akin to the type of mesomeric change (with resultant strong colour formation) produced by the action of antimony trichloride on carotenoid structures and vitamin D. The transient colour reaction of carotene with iodine trichloride is at once followed by addition of the

reagent, iodine being liberated only just before the end point. The conjugated chain is therefore almost completely saturated with iodine and chlorine atoms at first (no free chlorine is detectable spectroscopically at any point of the reaction before the end point), and the halogenated compound formed is, in the case of β-carotene, relatively stable before the iodine-liberation threshold is reached. Iodine liberation then takes place perhaps by chlorine substitution by excess reagent or by intramolecular arrangement to a more stable structure, with loss of iodine. It is possible that some sequence of events such as this, but very much quicker, occurs in the reaction with vitamins D and other reactive substances which liberate iodine, in these cases the first-formed iodine-chlorine complex being extremely unstable. This hypothesis of a two-stage reaction mechanism may be extended to cover the type I reaction. Thus, in the reaction with *cyclo*hexene, if the reacted solution is allowed to stand for 24 hr., iodine is slowly liberated: this occurs even in the absence of excess reagent and it is therefore difficult to see how the second-stage reaction, in the case of *cyclo*hexene, occurs without intermolecular rearrangement, since the products of the reaction are presumably dichlorohexahydrobenzene and iodochlorohexahydrobenzene.

It is tentatively suggested, however, that the two types of $ICl_3$ reaction are basically of the same character, the apparent difference being due to the extremely varying stabilities of the first-formed iodine-chlorine addition compounds. The preferential iodine-liberation reaction which takes place with systems of more than three conjugated double bonds and the series of sterols derived photochemically from ergosterol indicates that a steric factor determines these stabilities: accommodation of the large iodine atom in their molecular structures is probably too difficult for certain types of unsaturated compounds.

These hypotheses reconcile the observed reaction with theoretical requirements concerning the known polarizability of iodine chloride.

The quantitative titration of sterols on the micro-absorptiometer (Table 7) gives some interesting results. In all cases, with the exception of tachysterol, an integral number of bonds is titrated (within experimental error). Although, in the case of calciferol only three bonds usually react and the side-chain double bond is preferentially unreactive, in ergosterol, its provitamin, which contains one unconjugated and two conjugated double bonds, it is found that all three bonds react, and no anomalous behaviour has been discovered. The side-chain double bond, which is preferentially non-reactive in calciferol, is reactive in ergosterol. Even more curiously, lumisterol, which is an ergosterol diastereomer, reacts to the extent of only two double bonds, as do the acetates of pyrocalciferol and *iso*pyrocalciferol (free sterols not tested). The suprasterols have not so far been assigned a definite molecular structure: according to Müller (1935), suprasterol I contains three double bonds, but these are not conjugated. Suprasterol II also appears to have three unconjugated double bonds. Although the suprasterols are isomers of ergosterol, they do not contain the sterol skeleton. They react to the extent of two double bonds with iodine trichloride, and, therefore, if the tentative suggestions as to their structure are correct, are the only pure substances so far tested which liberate iodine from iodine trichloride and which do not possess a conjugated system.

7-Dehydrocholesterol contains two conjugated double bonds and is the only substance with less than a total of three double bonds which liberates iodine from iodine trichloride.

The titration of the purest obtainable specimen of tachysterol dinitrotoluate gave a figure of only 1·73 bonds titrated. Tachysterol itself, isolated from the dinitrotoluate by alkaline hydrolysis, was found to be very unstable, and the maximum bond titration value obtained was 1·35. Tachysterol contains, like the vitamins D, a system of three conjugated double bonds, and would be expected to show a bond titration figure of about 3. The discrepancy appears to be too large to be entirely accounted for by the known instability of tachysterol, especially in view of the very low result obtained by titration of the dinitrotoluate.

The titration of many samples of calciferol and vitamin $D_3$ on the micro-absorptiometer has a high degree of reproducibility, and the overall accuracy is within limits of ± 5 %. This is considered very satisfactory in view of the errors involved in reading a restricted range of the instrument drum, inherent slight drift of readings, troubles caused by volatility in the cell, the measurement of the small quantities of solutions, and inter-sample variation. The titration of a single solution of vitamin D is reproducible within limits of ± 3 %. Standard curves should be checked on at least two independent samples of vitamin $D_3$ and calciferol.

The limiting amounts of vitamin D that can be estimated by direct titration are governed solely by the difficulties of estimating the small amounts of iodine liberated by the reaction.

## SUMMARY

1. Iodine trichloride, in carbon tetrachloride, reacts quantitatively with the vitamins D, the irradiation sterols, vitamin A, β-carotene, and some other substances, to liberate iodine. The chemistry and stoicheiometry of the reaction have been examined and discussed, and a theory of the reaction mechanism proposed.

2. A method for the quantitative estimation of the vitamins D has been developed. The determination has an overall accuracy of ± 5 %.

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# Studies on the Analysis of Vitamins D

## 2. THE ANALYTICAL PURIFICATION OF VITAMIN D BY DIFFERENTIAL SOLUBILITY, PRECIPITATION REACTIONS, AND CHROMATOGRAPHY

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Attempts to analyse synthetic irradiation products and fish-liver oils for calciferol and vitamin $D_3$ are fraught with unusual difficulties. These arise, in the main, from the very high biological potency of vitamin D, the wide range of potency in materials to be assayed and the low specificity of known chemical reactions. The difficulties have not been generally appreciated, and the literature shows that, with few exceptions, published methods are empirical. There have thus been few attempts to deal fundamentally with the analytical separation of the vitamins D from known interfering substances.

In fish-liver oils, the chief interfering substance is vitamin A, the removal of which is the main problem to be solved. Vitamin A may be present, weight for weight, in at least a hundred times the concentration of vitamin D, which it masks in all known reactions. Milas, Heggie & Reynolds (1941) attempted to remove vitamin A and carotenoids by preliminary treatment of concentrates with maleic anhydride in dioxan. Ewing, Kingsley, Brown & Emmett (1943) developed chromatographic methods for the elimination of vitamin A and claimed good results. Müller (1947) also described a complicated chromatographic procedure.

The analysis of vitamins D in irradiation products appears at first sight to be easier and advances have been made, particularly by Ewing, Powell, Brown & Emmett (1948). It is often assumed that most of the sterols in an irradiation product of ergosterol or 7-dehydrocholesterol do not interfere with the analysis of vitamin D by the best known colorimetric methods. Experience has shown, however, that without the best attainable purification of vitamin D by removal of sterols, such methods do not give results agreeing with biological assays, when applied to widely varying products. This is so for the method of Ewing *et al.* (1948).

In the present work, two reactions have been used to study the analytical behaviour of calciferol and vitamin $D_3$. Since the two substances can differ in behaviour, they have been studied independently, and conclusions drawn from study of one have not been, without test, accepted for the other. The two reactions are the antimony trichloride-acetyl chloride reaction of Nield, Russell & Zimmerli (1940) and the iodine trichloride reaction described by Green (1951).

The antimony trichloride reaction is the more specific for vitamins D, but is interfered with, to varying degrees, by the presence of cholesterol, 7-dehydrocholesterol, tachysterol (and possibly protachysterol), vitamin A and carotenoids. A disadvantage in its use is the lack of reproducibility of the reagent, which makes it difficult to obtain precise results even with fairly pure vitamin $D_3$. The iodine trichloride reagent is less specific, but gives a high degree of precision, and is additionally useful in analytical studies of the sterols produced by irradiation which do not react with the antimony trichloride reagent.