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Studies on the Analysis of Vitamins D

4. STUDIES ON THE IRRADIATION OF ERGOSTEROL AND 7-DEHYDROCHOLESTEROL AND THE ANALYSIS OF THE PRODUCTS FOR CALCIFEROL, VITAMIN D_3, AND COMPONENT STEROLS

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The chemical analysis of vitamins D in synthetic irradiation products is of considerable importance and offers more promise of success than attempts on fish-liver oils. There are, however, a number of difficulties to be met which have rendered most chemical methods of analysis unreliable. A review of published methods of analysis has been made and the difficulties discussed in earlier papers (Parts 1, 2 and 3; Green, 1951*a*, *b*, *c*).

In the experimental work to be described, analytical methods for the determination of vitamins D and related compounds have been evolved, with consideration of three factors. First, although it is realized that the irradiation products are of widely varying composition (depending mainly on the extent of photochemical reaction), the influence of this fact on the problems of chemical analysis has usually been neglected. Thus, many methods, based on examination of highly standardized types of industrial product fail in the hands of workers investigating materials of varying composition. Adequate study of the composition of the irradiation products is a prerequisite of success in vitamin D determination. Secondly, biological assay control must be rigorously applied at all stages of evolution of a chemical method for vitamin D. Thirdly, agreement between chemical and biological assays should not necessarily be accepted as evidence for quantitative determination in the absence of recovery experiments.

The ultimate means of measurement have been the iodine trichloride titration method, described by Green (1951*a*) and the antimony trichloride-acetyl chloride colorimetric method of Nield, Russell & Zimmerli (1940).

EXPERIMENTAL

Materials and apparatus

Sterols (for specification, see Green, 1951*a*). *Calciferol and vitamin* D_3. For recovery experiments only, the International Standard preparations (see note in text). *Floridin earth.* Prepared for chromatography, British Drug House Ltd. Sieved to remove particles finer than 160 mesh/in. *Antimony trichloride.* A.R. grade. *Acetyl chloride.* A.R. grade. *Chloroform.* Purified as described by Nield *et al.* (1940). *Other reagents.* (For specification, see Green, 1951 *a*, *b*, *c*.)

ICl_3 titrations were carried out on the Spekker micro-absorptiometer, using spectrum green 604 filters. $SbCl_3$-acetyl chloride colorimetric measurements were made on the same micro-absorptiometer, using spectrum blue-green 603 filters, and on the Uvispek spectrophotometer.

The $SbCl_3$*-acetyl chloride determination*

The directions of Nield *et al.* (1940) for the preparation of the reagent have been found satisfactory, but it has not been found advisable to keep the prepared solution up to 9 weeks, as claimed to be possible. The reagent has, in the present work, been kept up to 3 weeks, during which period it is stable. Trouble with the reagent is generally caused either by moisture absorption or by the presence of impurities in the $CHCl_3$. To avoid the former, the reagent is stored in a desiccator between experiments. The $CHCl_3$, however, is the chief cause of difficulty. Old samples develop impurities which do not appear to be easily removable, and reagents prepared with them soon deteriorate. The usual symptoms are erratic colour development and general diminution of chromogenic capacity; if these symptoms occur within 3 weeks, a new preparation of $CHCl_3$ should be obtained. With these precautions, observance of cleanliness and dryness of all apparatus in contact with the reagent, and a certain amount of practice, the reagent yields results with a degree of precision within $\pm 5\%$.

Vitamin D is determined by adding the reagent to a $CHCl_3$ solution of the test material, up to a known standard volume (in these experiments 2–5 ml.). Reagent volume should always be large compared to the volume of test solution, and never less than five times as great. This eliminates the quenching effect due to dilution of the reagent on adding it to the test solution. The addition of reagent may be carried out directly in the optical cell, if large enough, or in a stoppered vessel from which a portion for measurement may be taken. During the measurement, the optical cells should be covered with ground-glass cover slips to avoid moisture absorption, which causes cloudiness and 'streakiness' on the internal glass walls, and also volatilization, which may damage the instrument. When using the reagent in the Uvispek spectrophotometer, the instrument may be protected by incorporating a removable housing, fitting into the cell mount and designed to give maximum protection from vapours or spillage.

Providing moisture ingress into the cell is small, three consecutive readings on different solutions may be made in the same cell, which need only be rinsed with $CHCl_3$ between readings. If consecutive readings are delayed, the cells should be rinsed between readings with acetone and then blown out with air until quite dry.

$E^{1\%}_{1\,cm.}$ for the reaction with pure vitamin D_3 was determined, from a large number of readings, as 1820.

Method for the determination of vitamin D in irradiation products

The method needs no special precautions beyond those customarily observed to prevent oxidation.

Saponification. If the sample is dissolved in oil, first saponify 0·5–1·0 g. Add 0·5 N ethanolic KOH, freshly prepared with 95% (v/v) ethanol (10 ml./g. of sample) and reflux, in a 25 ml. conical flask containing a cold finger condenser, for about 5 min. on a steam bath. Wash into a separator with 10 ml. water, and extract twice with 25 ml. portions of ether. Wash the ethereal extracts successively with water, 10 ml. 0·5 N aqueous KOH, and then with water until free from alkali. Make the ether solution up to volume, after filtration if necessary.

Chromatography. Take a portion of the ethereal solution that contains preferably not more than 25 mg. of sterols and evaporate at the pump to small bulk. Add a little ethanol and blow off to dryness with an inert gas stream. Dissolve the residue in 5–10 ml. light petroleum. If the original sample was not dissolved in oil, make up directly in light petroleum. With low potency material, it may be necessary to take more than 25 mg. for chromatography. Quantities of sterols up to 100 mg. may be used, but this may cause clogging of the column and inefficient elution, unless the procedure is carried out very slowly.

The column consists of a glass tube about 15 cm. long and 1·3 cm. internal diameter, constricted at the base and fitted with a 6 cm. stem 1 mm. in bore, which ensures slow flow. The constriction is plugged with glass wool.

Activate the floridin earth by boiling for 1 min. with conc. HCl (5 g. earth to 15 ml. acid) and pour the hot stirred slurry into the tube until the column is about 8 cm. long. With practice this can be done with one tipping motion to give a perfectly even column. After the acid has passed, but without allowing the top of the column to dry, wash through with 20 ml. of 90% (v/v) ethanol in small quantities, followed by 5×5 ml. of light petroleum. During the ethanol washing, add a small filter-paper disk to the top of the column and compress the column about 5 mm. with a glass plunger. Add the sample and, after its passage, wash through with 40 ml. light petroleum. Change the receiver and elute with 100 ml. of CCl_4.

Chromatography is preferably carried out by gravity percolation and should not take less than 1 hr. or more than 2 hr. to complete. Quick passage may cause loss by non-adsorption, and prolonged passage causes some destruction of vitamin D. The rate of flow varies throughout the procedure, being slow when ethanol is being passed, but speeding up as the other solvents wet the column. Rates are adjusted by the conventional screw clip and rubber tube on the side arm of a filter flask. Suction should not be used during adsorption, but may assist during elution.

Solubility and digitonin separation. Evaporate the CCl_4 solution to dryness at the pump. To the residue, add by pipette 20 ml. of ethanol and swirl or shake in a glass-stoppered flask to dissolve. Add exactly 8·6 ml. water, with continuous swirling, to bring the ethanol concentration to 72% (v/v). Transfer immediately to a 50 ml. centrifuge tube and allow to stand 5 min. Should a precipitate form, remove by centrifuging, wash the residue with 5 ml. 72% ethanol and combine with the clear supernatant in a second centrifuge tube. Add 150 mg. digitonin dissolved, by warming, in 5 ml. 72% ethanol, stir, and allow to stand 1 hr. If no precipitate forms at the ethanol dilution stage or if only a turbidity occurs, add digitonin directly. Separate the digitonide by centrifuging, and filter the supernatant into a separating funnel. Extract twice with 15 ml. portions of CCl_4, and combine the extracts. Filter, evaporate the filtrate to dryness at the pump. Dissolve the residue in a standard volume of CCl_4.

Vitamin D measurement. The CCl_4 solution may be titrated directly with ICl_3. If the $SbCl_3$-acetyl chloride reaction is to be used, evaporate a measured portion and make up in $CHCl_3$, so that the solution contains 30–100 μg./ml. For estimation on the micro-absorptiometer, take 0·1–0·4 ml. (3–10 μg. of vitamin D) and make up to 2·0 ml. with reagent in a small test tube. Match at two levels after 3 min. against a blank cell containing reagent. Read off vitamin D content from a standard curve. For spectrophotometric estimation, rather larger quantities are necessary. Take readings (at two levels) after 3 min. at 500 mμ. against an internal blank

measurement at 550 mμ. Determine the standard 'intercept' to be subtracted, by measuring the 550–500 mμ. readings on a cell containing pure reagent. Calculate vitamin D (after subtraction of the 'intercept') from extinction values.

The whole analytical procedure is completed in about 6 hr.

Studies of the analytical method

Studies of the method were made by investigation of experimental irradiation products prepared from ergosterol and 7-dehydrocholesterol. The provitamins were irradiated in ether or benzene solution at varying concentrations and for varying periods of time, using unfiltered light from a Hanovia S. 500 lamp. All irradiations were carried out in fused-silica flasks at 2·5 cm. from the lamp, at the boiling point of the solvent used.

Weight of sample. Results obtained by the method are independent of the weight of sample taken, over wide limits. This is an important criterion of efficient separation of vitamin D from interfering materials. As indicated later, variations of the method which are known to cause faulty separation or recovery lead to large differences being found when the sample weight is varied. Table 1 shows the agreement between chemical determinations carried out on weights of irradiation product varying between 5 and 50 mg.

Table 1. *Determinations of vitamin D on varying weights of an ergosterol irradiation product*

Wt. of sample (mg.)	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)
5·6	5·5	—
9·4	5·6	5·1
20·3	5·3	—
49·7	5·0	—

Saponification effect. The saponification method described is satisfactory for material containing vitamin D. Experiments on the saponification of solutions of pure vitamin D_3 in arachis and olive oils showed that, as determined by the $SbCl_3$ reaction, recovery is quantitative. Attempts to study the saponification of these solutions by the use of the ICl_3 titration method, however, brought to light a peculiar effect, which is worthy of mention.

The non-saponifiable fractions of arachis and olive oils yield negligible amounts of liberated iodine with ICl_3. If, however, the non-saponifiable fractions contain added vitamin D, on titration with ICl_3 they liberate iodine in very large excess over the amount expected to be liberated by the added vitamin D alone. Substances are present in these fractions which only react with ICl_3 in the presence of a reactive substance such as vitamin D. Addition of vitamin D before or after the actual saponification yields similar results, and length of saponification time does not effect the phenomenon. The extent of the spurious iodine development, however, increases with the amount of added vitamin D. At levels of 10 mg./g. of oil, an apparent recovery of about 175 % is obtained by ICl_3 titration. At 5 mg./g. of oil, the apparent recovery is about 125 %.

The mechanism of this reaction, initiated by the presence of vitamin D, has not been elucidated nor have the substances responsible been identified. Although interfering with saponification studies by means of the ICl_3 reaction, they cause no difficulty in the full vitamin D analysis method since they are eliminated by the chromatographic procedure.

Chromatography. This stage is preferably carried out before the extraction and precipitation procedures. If it is carried out after the latter operations, losses of vitamin D occur and recovery experiments give poor results. Prior chemical treatment appears to render vitamin D susceptible to destruction by chromatography.

Dissolving the crude irradiation product in light petroleum preparatory to chromatography meets with some difficulty in two types of product. Ergosterol products which have been irradiated for short times and which contain over 80 % of residual ergosterol are not easily soluble, and leave the poorly soluble ergosterol as a residue. The procedure here is to filter off the unchanged ergosterol rather than attempt to get the whole product in solution. The solid is washed with small amounts of warm solvent to free it of vitamin D. On prolonged irradiation, ergosterol is rendered totally soluble by its photochemical reaction products and no difficulty is experienced with an insoluble residue.

It has been found that ergosterol irradiation products change in composition on storage. Irradiation to the point of about 45 % ergosterol destruction yields a product which is obtained, after solvent evaporation, as a crystalline or semi-crystalline solid. This type of product is totally soluble in light petroleum, but leaves an insoluble ergosterol residue on treatment with 72 % ethanol. After several days in ether or benzene solution, the irradiation product changes in physical consistency to yield a non-crystalline, resinous mass (similar in appearance to the resins obtained by prolonged irradiation). The crude product is now more soluble in 72 % ethanol, but contains a new substance, almost totally insoluble in light petroleum. This substance is gelatinous whilst in suspension, and, when separated, is a white amorphous powder. Similar changes occur in the crude irradiation product after it has been freed from solvent, but the rate of change is much slower. For the purpose of chromatography, the substance may be treated as described for residual ergosterol and filtered from the light petroleum extract containing vitamin D.

Table 2 summarizes these and other changes which have been observed to occur during the ageing of an ergosterol irradiation product over a period of 2 weeks.

Table 2. *Change in characteristics of irradiation product on ageing*

Reaction or description	Freshly prepared product	Product after 2 weeks in ether
Physical appearance	Crystalline	Resinous
Solubility in light petroleum	Totally soluble	Insoluble gelatinous residue
Total reactive double bonds by ICl_3 titration	2·95	2·6
Vitamin D analysis (i.u./g.):		
(*a*) by ICl_3	$5·8 \times 10^6$	$6·9 \times 10^6$
(*b*) by $SbCl_3$	$5·3 \times 10^6$	$6·3 \times 10^6$

The product which is described in Table 2, when titrated directly with ICl_3, was found to consist almost entirely of sterols containing three reactive double bonds, thus showing an absence of appreciable amounts of lumisterol or suprasterols. During ageing, the titratable double bonds decrease. Simultaneously there is an increase (not shown in Table 2) of the total $SbCl_3$-reactive material in the crude product. In addition, there is a real increase in vitamin D content as determined chemically, which has been found in many other stored samples. Part of the change in composition is almost certainly due to a slow formation of calciferol from a precursor present in the crude product, probably the precalciferol newly isolated and described by Velluz & Amiard (1949) and Velluz, Amiard & Petit (1949). The change, as shown by these authors, has been found to occur in the absence of light. However, the formation of the petrol-insoluble substance and decrease in ICl_3 titre of the total product is due to a second simultaneous reaction, which is believed to be due to the decomposition of tachysterol. It has been found that pure tachysterol, under the same conditions, forms a closely similar petrol-insoluble decomposition product. The chemical nature of the substance has not been studied in any detail. Its ultraviolet absorption spectrum shows no bands above 230 mμ., and (unlike the petrol-insoluble suprasterol to be described later) it reacts only slightly with ICl_3.

Chromatography eliminates three types of interfering material in addition to tachysterol. The first type consists of unsaturated substances (possibly hydrocarbons or unidentified sterols) which are found in the non-saponifiable fractions of vegetable oils. These substances react with ICl_3 and, in high concentration, with the $SbCl_3$ reagent. They pass into the first light petroleum filtrate from the column.

The second substance occurs in most types of vitamin D_3 product prepared by irradiation of crude concentrates of 7-dehydrocholesterol and its elimination is important. It is reactive towards $SbCl_3$ and ICl_3 reagents and is present in considerable quantities. It behaves differently from the known sterols of the photochemical sequence on chromatographic analysis, also passing into the light petroleum filtrate.

The third substance separated by chromatography is an unidentified irradiation product of calciferol, formed also during the irradiation of ergosterol; it is described in greater detail in the section on irradiation studies.

Solubility and digitonin separation. The procedure adopted finally for the solubility separation was to dissolve the sample after chromatography in ethanol and to dilute to a concentration of 72% (v/v) with water, in order to precipitate most of the provitamins and cholesterol. Many experiments were carried out on the direct extraction of the sample with 72% ethanol, but the method was found to be unsatisfactory with some products. Although the D vitamins are preferentially extracted from irradiation products by shaking with 72% ethanol, the completeness of the extraction is affected by the weight of sample taken and also its physical consistency. Extraction from resinous products was usually good, but the crystalline products obtained during the early stages of ergosterol irradiation were difficult to extract.

It was found advisable to add digitonin in considerable excess, and extraction of the solution after digitonide separation was best carried out with carbon tetrachloride. Occasionally, during this extraction, emulsification may occur, and is due to impurities in the digitonin. Pure digitonin (not readily obtainable) never gives emulsions at this stage. If emulsification is troublesome, the preparation of digitonin used should be changed.

Considerable evidence has accumulated to indicate that the digitonin separation removes from crude irradiation products, in addition to precipitable sterols, a large amount of an $SbCl_3$-reactive sterol, which is not precipitated, but forms a soluble non-reactive digitonide, extractable from aqueous ethanol by CCl_4.

In one experiment, summarized in Table 3, 52 mg. of an ergosterol-irradiation product was, without prior chromatography, dissolved in 20 ml. ethanol

and the solution, by addition of water, brought to an ethanol concentration of 72 %. After removal of the precipitate by centrifuging (stage *A*), digitonin solution was added and the digitonides removed after 1 hr. (stage *B*). The clear filtrate was extracted into CCl_4 (stage *C*) and the extracted solution evaporated to dryness at the pump. The residue was re-extracted with CCl_4 and made up to a standard volume (stage *D*). At each of the four stages, a sample (0·5–1·0 ml.) was removed and analysed directly for total reactive material with the $SbCl_3$ reagent. The results are expressed, conveniently, in terms of 'apparent calciferol'. The test samples from stages *A*, *B* and *D* were first evaporated to dryness from their ethanolic solutions, then extracted with hot CCl_4, and after evaporation of the latter solvent, made up in $CHCl_3$ for $SbCl_3$ determination. The CCl_4 solution from stage *C* was evaporated to dryness directly, and the residue dissolved in $CHCl_3$. The complete experiment was repeated, with the exception that digitonin was not added.

Table 3. *Loss of* $SbCl_3$*-reactive sterol as soluble digitonide during vitamin D analysis*

Stage of analysis	'Apparent calciferol' ($SbCl_3$ reaction) content of original product (10^6 i.u./g.)	
	Digitonin added	Without digitonin
A After dilution	8·6	8·5
B After removal of digitonin	8·9	—
C After CCl_4 extraction	5·3	8·1
D Final extracted solution	Nil	Nil

Very little loss of calciferol occurs during the procedure, and tests on all separated precipitates showed negligible quantities of $SbCl_3$-reactive material. The addition of digitonin clearly results in the elimination of a considerable amount of $SbCl_3$-reactive sterol, as soluble digitonide; this digitonide is present at stage *B* and is broken down to its constituents by extraction with hot CCl_4. The digitonide is extracted into CCl_4 and is non-reactive, as shown by the lowered analysis at stage *C* and the absence of any $SbCl_3$-reactive material at stage *D*.

Similar results are obtained if chromatography precedes the digitonin separation, but the amount of the digitonin-removable $SbCl_3$-reactive substance separated is smaller, since a part of it is removed by the floridin column. The substance responsible is not believed to be tachysterol, which has been shown not to form such a soluble digitonide.

RESULTS

Recovery experiments

Agreement of chemical determinations with biological assays has not, without additional evidence, been accepted as a criterion during studies on the analytical method. Recovery experiments are the most critical proof that vitamin D is in fact being measured by the method. Such recovery checks have been carried out during determinations on a number of irradiation products. Some typical results are shown in Table 4. Recoveries are usually over 90 % of added vitamin D. It has been found highly important in attempting these recovery experiments with small amounts of added vitamin D to use extremely pure material. For some tests, small amounts of International Standard calciferol and vitamin D_3 were used. In others, only specimens which conformed to this high degree of purity were used. Recovery experiments with specimens of ordinary commercial purity give poor results.

Analytical studies of the irradiation of ergosterol

Irradiation method. For these studies, ergosterol was irradiated in concentrations up to 1 % (w/v) in ether and up to 12 % (w/v) in benzene. Irradiations were usually carried out on 100 ml. of solution in 250 ml. fused-silica flasks, illuminated with the Hanovia S. 500 lamp at 2·5 cm. from the flask.

The irradiation curve and comparative analyses. Ergosterol (1 g.) was irradiated in 100 ml. ether. Samples of 20 mg. were taken at intervals and assayed chemically. Additional samples at each interval were taken and, after evaporation of the solvent, dissolved in arachis oil and assayed biologically by the chick radiographic method.

Table 5 gives the results obtained by two methods of chemical determination and the bioassay figures. Fig. 1 shows the typical 'irradiation in ether' curve, where calciferol potency is plotted against irradiation time.

Agreement between the results obtained by the $SbCl_3$ determination and the bioassay is very close throughout the whole irradiation range, falling within the fiducial limits ($P = 0·05$) of the latter assay. Results from ICl_3 titration (on the same analytical sample) are, as expected, higher than the

Table 4. *Recovery of added calciferol and vitamin* D_3 *in analysis of irradiation products*

Product type	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)	Wt. of sample (mg.)	Added vitamin D (mg.)	Recovery of added vitamin D (%)
Calciferol	6·35	5·9	51·0	5·535	102
Calciferol	3·69	—	54·5	1·434	103
Vitamin D_3	1·47	1·6	43·3	2·160	102·5
	1·78	1·90	41·0	2·088	92

Table 5. *Calciferol analyses during the irradiation of ergosterol*

(1 g. irradiated)

Irradiation time (min.)	Chemical analysis (10^6 i.u./g.)		Bioassay (10^6 i.u./g.)	Limits of error of bioassay ($P=0{\cdot}05$) (%)
	ICl_3	$SbCl_3$		
0	—	<0·02	—	—
20	2·8	2·4	2·3	87–115
40	4·6	4·3	3·9	71–141
60	7·8	6·3	6·0	77–130
90	8·4	6·8	6·7	89–113
120	9·2	7·8	7·6	93–107
150	8·0	6·0	5·6	83–120
180	7·6	4·0	4·1	85–118

$SbCl_3$ figures, and these two sets of results diverge as irradiation proceeds, due to accumulation of suprasterols. Table 5 shows (and this has been confirmed on many series of analyses) that the ICl_3 figure is rarely more than 20 % higher than the true vitamin D figure as determined by $SbCl_3$ analysis, before the peak potency is reached. If irradiation is continued beyond the limits shown in the table, the ICl_3 figure remains fairly constant or falls slightly for a long period, and finally rises when suprasterol accumulation is large.

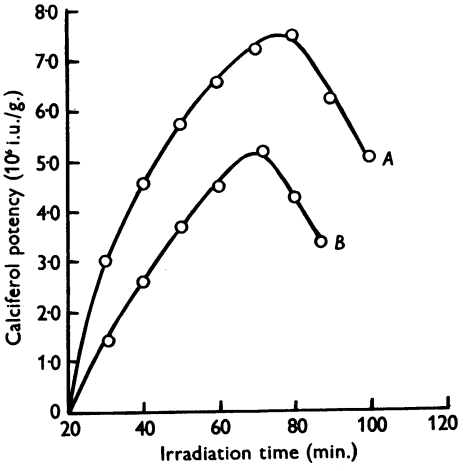


Fig. 1. Irradiation of ergosterol in ether (*A*) and benzene (*B*).

Solvent effect on irradiation. It has been recognized for many years (for example, see Bills, Honeywell & Cox, 1931) that the formation of calciferol from ergosterol takes place much faster in ether than in solvents such as benzene or alcohols. A quantitative study of the effect has been made. It was confirmed that, on irradiating similar concentrations (1 % w/v) of ergosterol in ether and benzene, calciferol formation, in the early stages of the process, was about twice as fast in the former solvent as in the latter. The typical 'irradiation in benzene' curve is shown in Fig. 1. The peak calciferol potency is also about 1·5 times to twice as great in ether as in benzene.

Ergosterol-calciferol relationships and order of reaction. During a number of experimental irradiations, the decay of ergosterol was studied kinetically, simultaneously with studies on the formation of calciferol. Residual ergosterol was determined by digitonin precipitation.

In one experiment, 5 g. of ergosterol in 100 ml. benzene were irradiated. Fig. 2 shows the ergosterol destruction and calciferol formation curves. The concentration of ergosterol does not fall off exponentially, due to the formation of a series of substances which also absorb incident light. The observed curve

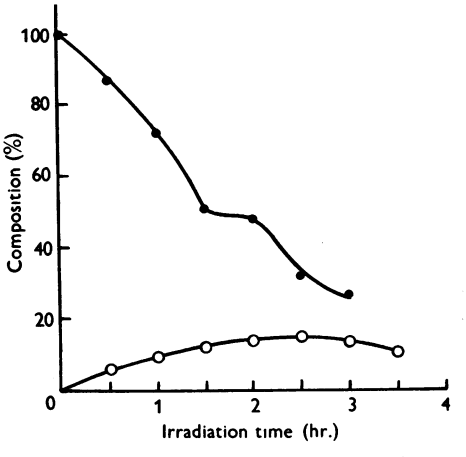


Fig. 2. Changing ergosterol-calciferol relationships during irradiation of ergosterol. ●—●, ergosterol; ○—○, calciferol.

for the rate of decay of ergosterol is quite characteristic. The initial decrease in concentration is approximately exponential, but, at about 50–60 % residual ergosterol, the curves flatten out for a considerable time during which very little ergosterol is transformed, although, at the same time, the calciferol concentration continues to increase. Finally, the ergosterol concentration falls steeply. At all initial ergosterol concentrations, and in both ether and benzene, peak calciferol potency is reached when 50–70 % transformation has occurred.

Two other factors are of importance in studies of the irradiation. The first of these is the effect of initial ergosterol concentration. During the first stages of photochemical change, using the mercury arc as the light source, it appears that calciferol is the main accumulating sterol, and, during this first period, it is useful to study its formation kinetically. It is then possible, by neglecting the intermediate reactions, to arrive at an experimental order of reaction for the ergosterol-calciferol transformation. Table 6 shows the results on a series of irradiations of ergosterol in benzene, using a wide range of initial concentrations. Irradiation was, in each experiment, stopped at 20 min. and samples analysed for calciferol. Total volumes irradiated were 100 and 200 ml.

Table 6. *Irradiation of ergosterol in varying concentrations*

(Time of irradiation = 20 min.)

Volume of solution irradiated	Concentration of ergosterol (g./100 ml.)	Potency of product (10^6 i.u./g.)	Total calciferol formed (10^6 i.u.)
100 ml.	0·25	5·68	1·42
	0·35	3·95	1·38
	0·5	3·39	1·69
	1·0	2·85	2·85
	2·0	1·93	3·86
	4·0	1·10	4·40
	6·0	0·72	4·32
200 ml.	0·25	3·18	1·59
	0·5	2·74	2·74
	1·0	1·82	3·64
	2·0	1·18	4·72
	4·0	0·62	4·92
	6·0	0·39	4·68

For a standard volume of solution irradiated in a given time, the calciferol potency of the product decreases, but not proportionally, as the ergosterol concentration increases up to a certain point. At this concentration—about 4 % for 100 ml. of solution and 2 % for 200 ml.—the order of reaction with respect to ergosterol is less than unity, and continuously varying. Above these threshold concentrations, however, the calciferol potency varies inversely with the initial ergosterol concentration, the total calciferol formed in a given time becomes constant (about $4{\cdot}5 \times 10^6$ i.u. in all volumes), and the reaction is therefore of zero order. Because the intensity of radiation is a constant in all experiments, the threshold concentration is different for each volume being irradiated; the critical factor is manifestly not concentration of ergosterol, but transparency of solution. When the volume is large enough or the concentration high enough to absorb all the incident radiation, the reaction is of zero order; the amount of calciferol formed is then independent of either volume or concentration and is a constant for a given intensity of radiation. The amount of ergosterol under these conditions is very large compared to the amount being transformed by the radiation, and its concentration remains almost constant. In later stages of the irradiation, of course, these considerations do not apply.

A second consideration of interest is the changing ratio, throughout the reaction, of the calciferol formed to ergosterol destroyed. Calciferol is formed most economically in the early stages, and for best yields based on ergosterol consumed, irradiation should not proceed beyond 15 % destruction, when about 5 % calciferol is formed. Later, ergosterol destruction becomes heavy in proportion to calciferol formation.

Analytical studies of 7-dehydrocholesterol irradiation

The irradiation of 7-dehydrocholesterol was studied by methods similar to those described for ergosterol. A 27 % concentrate of 7-dehydrocholesterol was used. In one experiment 2 g. of the concentrate dissolved in 100 ml. ether were irradiated over 5 hr., taking samples at hourly intervals. Table 7 shows vitamin D assays at each stage by chemical and biological methods.

Table 7. *Vitamin D_3 analyses on 7-dehydrocholesterol irradiation product*

Irradiation time (hr.)	Chemical analysis (10^6 i.u./g.) ICl_3	Chemical analysis (10^6 i.u./g.) $SbCl_3$	Bioassay (10^6 i.u./g.)	Limits of error of bioassay ($P = 0{\cdot}05$) (%)
1	1·26	1·33	1·32	87–115
2	2·26	2·02	2·14	83–120
3	1·89	1·73	2·05	81–118
4	1·91	1·60	1·88	80–125
5	1·88	1·46	1·56	89–112

The shape of the irradiation curve is similar to that obtained with ergosterol. Agreement between ICl_3 and $SbCl_3$ determination is usually close with vitamin D_3 irradiation products, before the peak potency is reached. Agreement between biological assays and $SbCl_3$ determinations is good.

The destruction of calciferol and vitamin D_3 by ultraviolet irradiation

A study of the photochemical destruction of the vitamins D is not only important for an understanding of the complexities of the ergosterol-calciferol relationship, but also yields useful information from the analytical aspect.

In initial experiments, 1 g. of calciferol in 100 ml. of ether or benzene was irradiated, and periodic samples analysed, by direct $SbCl_3$ determination, for residual calciferol. Some of the irradiation curves obtained are shown in Fig. 3. In addition, total reactive sterols were titrated with ICl_3, and the destruction curve obtained is shown in Fig. 4. The time ordinate on these graphs is arbitrary, as the rate of calciferol decomposition varies to a considerable extent with the age of the mercury lamp used.

If the curves obtained by direct $SbCl_3$ analysis are considered first, it is evident that destruction of calciferol in benzene is slower than in ether, but, in most experiments, the curves are similar in general shape. Destruction is rapid in the very early stages

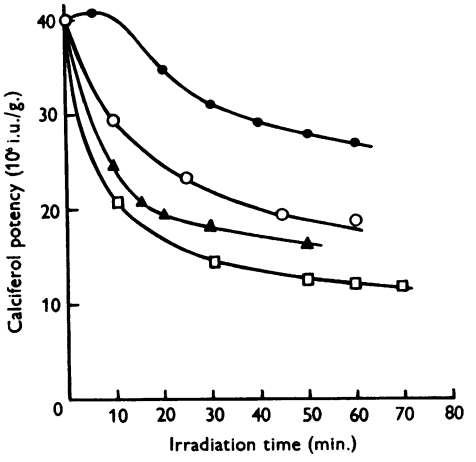


Fig. 3. Destruction of calciferol on irradiation under varying conditions. ●—●, benzene, old lamp; ○—○, ether, old lamp; ▲—▲, benzene, new lamp; □—□, ether, new lamp.

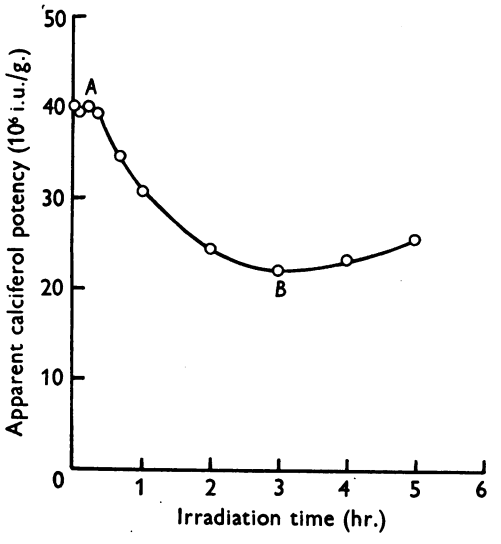


Fig. 4. Destruction of calciferol on irradiation: total reactive sterols determined by ICl_3.

of irradiation and apparently exponential. The curves then flatten out for a considerable length of irradiation time and finally descend again fairly steeply. This characteristic decomposition curve is difficult to explain.

A series of experiments was carried out, in which calciferol was irradiated in benzene at various initial concentrations. Determinations of residual calciferol were made at two intervals of 20 min. Table 8 shows that, although the total amount of calciferol destroyed in a given time increases with initial concentration, no direct proportionality exists. The reaction is of varying fractional order with respect to calciferol. Table 8 shows clearly the remarkable slowing down of destruction in the second irradiation period, becoming more marked as the initial concentration of calciferol is increased. At a concentration of 2 g./100 ml., practically no calciferol is destroyed during the second 20 min. irradiation.

Table 8. *Irradiation of calciferol in benzene*

Weight of calciferol in 100 ml. (g.)	Total calciferol destroyed (%)	
	20 min.	40 min.
0·25	52·5	76·5
0·5	45·8	51·3
1·0	35·3	39·0
2·0	26·3	26·3
1·0*	27·0	—

* 1·0 g. suprasterols added.

Attempts were made to determine whether the presence of other sterols (in particular, the accumulation of suprasterols) had a specific retarding effect on the decomposition process. Since all the products of calciferol, with the exception of the hypothetical toxisterol, are almost transparent in the range of active wavelengths, this effect, if present, would not be related to interference with light absorption. To a solution of 1 g. of calciferol in 100 ml. benzene, 1 g. of mixed suprasterols (prepared by total destructive irradiation of calciferol) was added. The solution was then irradiated and residual calciferol determined after 20 min.

As shown in Table 8, there does appear to be small specific retarding effect due to the presence of mixed suprasterols, calciferol destruction in 1 % solution being reduced from 35·3 to 27 %.

Evidence for the existence of new substances from the irradiation of vitamins D

(*a*) *Titration evidence.* If, in Fig. 4, the calciferol degradation curve as determined by ICl_3 titration is now considered, there is evidence for the existence of a number of hitherto undescribed substances produced by the photochemical destruction. During the course of the irradiation, the total titratable double bonds per molecule drops from three to a final value very close to two. The curve is not a smooth one and exhibits several points of interest. During the first few minutes of irradiation, there is a slight drop in ICl_3 titration value which is followed by a rise to the original value (point *A* in curve shown) the curve then descends to a point (*B*) where the observed double bond per molecule titration

value is about 1·5. Finally the titration value rises to about 2 and is unaffected by further irradiation. Irradiation of vitamin D_3 produces a similarly shaped curve.

At point *A* an intermediate exists which, like calciferol, has three reactive bonds, and is therefore not a known suprasterol. Spectrographic analysis of the product at this point shows only the calciferol absorption band slightly shifted to shorter wavelengths; there is no band at 248 mμ., nor has this band (due to toxisterol) ever been observed in these experiments. At *B*, there is evidence for the existence of a second intermediate which has a titre of less than two double bonds. Both these substances are photochemically labile and finally produce suprasterols.

(*b*) *Separation of new product.* In some experiments on the irradiation of pure calciferol, it was found that the crude destruction product yielded, on extraction with light petroleum, a large amount of a new insoluble substance. Suprasterols I and II, the only identified products of calciferol irradiation, are both very soluble in light petroleum. 5 g. of calciferol in 500 ml. ether were irradiated for 13 hr. in a 100 ml. fused-silica flask. After evaporation of the solvent, the resinous product was dissolved in 300 ml. light petroleum and the solution filtered through a sintered-glass filter. The washed residue was gelatinous until free from contaminant material, but dried to an amorphous, white to light-yellow powder. Yield 1·9 g.

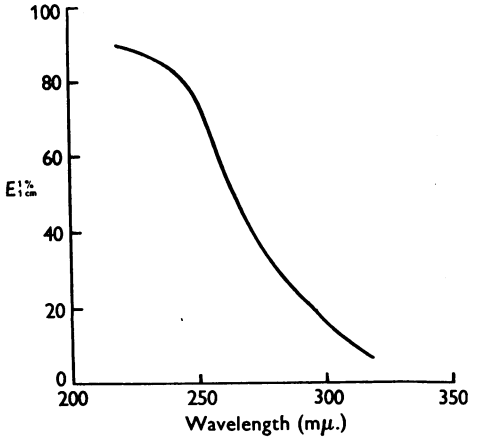


Fig. 5. Ultraviolet absorption curve of 'Suprasterol III'.

This substance, tentatively called *suprasterol III*, is soluble to the extent of about 1 % in boiling ether and is only sparingly soluble in CCl_4. Its solubility is greatly increased by admixture with crude suprasterols I and II. The substance is very soluble in ethanol and methanol and does not precipitate with digitonin. Attempts at crystallization from methanol and ethyl acetate failed. Dropwise addition of water to a methanol solution produced a gelatinous precipitation, but no crystalline material. No success met attempts to obtain a crystalline 3:5-dinitrobenzoate. Suprasterol III, dissolved in $CHCl_3$ and titrated with ICl_3, gives a figure of two double bonds. It is not affected by prolonged ultraviolet irradiation.

Formation of suprasterol III appeared to be dependent on conditions which could not be controlled. Yields were always variable and in a large number of experiments the substance was not formed. Spectrographic examination of the crude material in ethanol gave an absorption curve (Fig. 5) without bands, which is similar to that of the known suprasterols, but with greater general absorption below 260 mμ.

(*c*) *Chromatographic evidence.* If the crude product produced by total destructive irradiation of calciferol is chromatographed on floridin earth, a substance is separated which is not either residual calciferol or a known suprasterol.

In one experiment 1 g. of calciferol in 100 ml. ether was irradiated for 1 hr. After solvent evaporation, the product was dissolved in 100 ml. light petroleum and insoluble materials separated. A portion was analysed for residual calciferol by direct $SbCl_3$ estimation. The light petroleum solution (5 ml.) was then chromatographed on a standard floridin column and the two determinations were repeated on the CCl_4 eluate. The results are recorded in Table 9.

Table 9. *Chromatographic analysis of calciferol irradiation product*

	Residual calciferol (mg./g.)	Total suprasterols (by ICl_3) (mg./g.)
Before chromatography	395	618
After chromatography	385	125

Residual calciferol after irradiation remains practically unchanged after chromatography. The ICl_3 titration results are expressed in terms of 'total suprasterols' in mg./g. of original total product. The figure before chromatography is obtained by subtracting from the total titration value the contribution due to residual calciferol and then calculating from the known two double-bond titration value of the suprasterols. The figure after chromatography is obtained in the same manner and shows that a large amount of material is removed by chromatography. As already shown by Green (1951*b*), the suprasterols I and II are eluted from floridin with CCl_4, and therefore the loss is due to an unidentified irradiation product of calciferol which is not either of the former suprasterols.

Closely similar results have been obtained with products isolated from the irradiation of pure vitamin D_3.

Comparison of chemical and biological analysis on a number of irradiation products

A large number of vitamin D_3 irradiation products have been assayed chemically and biologically over a period of about 3 years, and are recorded in Table 10. Most of these were commercial products (of unknown history) prepared by dissolving the crude synthetic concentrates in arachis or cod liver oils, and ranged in potency from 0·5 to $2·5 \times 10^6$ i.u./g. Two samples were oil dilutions of much lower potency. The lowest potency material recorded in the table is about 10,000 i.u./g., but the method will deal satisfactorily with potencies down to 100 i.u./g. The latter order of potency is, however, not usually encountered in the absence of vitamin A.

Table 10. *Comparison of chemical and biological analyses on a number of vitamins D_3 irradiation products in oil solution*

Sample number	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)	Limits of error of biosasay ($P=0·05$) (%)
235	1·78	1·70	85–117
247	2·16	2·46	81–124
233	1·26	1·56	81–124
256	0·97	0·91	89–113
272	2·48	2·99	86–116
267	2·14	2·40	87–115
297	1·17	1·34	77–130
302	1·38	1·48	78–128
316	0·51	0·54	82–122
334	0·85	0·86	87–115
374	1·88	1·80	85–117
381	1·10	1·32	90–111
384	1·24	1·01	—
385	0·65	0·76	—
386	0·99	1·10	—
392	1·46	1·60	—
403	0·88	0·86	87–115
P. 1	1·51	1·46	82–124
P. 2	1·26	1·24	83–120
S.D. 1	49,500	48,200	85–117
S.D. 2	11,200	10,800	82–122

Dr M. D. Wright, to whom the author is indebted for the large number of biological assays required during the course of this work, has supplied the following note. 'Vitamin D_3 assays are carried out in these laboratories according to the method of Olsson (1936*a*, *b*). The tarso-metatarsal distances in the chick hock joints are measured radiographically after 4–5 weeks, using diets incorporating graded doses of vitamin D_3.'

Biological assays have been carried out against the new international standard of vitamin D_3 since its adoption. Before that date, the British Standards Institution standard preparation was used, in frequent comparison with a sample of pure crystalline vitamin D_3 (which several biological tests have shown to be equal in potency to the new international standard). The B.S.I. standard preparation was shown to have 80% of the vitamin D_3 potency of both the pure specimen and the new international standard. All the assays recorded have been brought into line and expressed in terms of vitamin D_3 standards of equivalent potency.

A scheme for full analysis of component sterols in ergosterol irradiation products

As a result of work described above and in preceding parts, a scheme suggests itself for the full analysis of the component sterols of the irradiation mixture. The sterols comprising the crude irradiation product of ergosterol can be divided into three classes (1) sterols precipitated with digitonin (ergosterol), (2) sterols reactive to ICl_3 and to the $SbCl_3$-acetyl chloride reagent (calciferol, tachysterol, pre-calciferol, and possibly an unidentified component), (3) sterols reactive to ICl_3 only (lumisterol, suprasterols and perhaps unidentified components).

The analytical scheme is shown in outline in Table 11. Ergosterol is first estimated by gravimetric separation of its digitonide. Direct estimation with the $SbCl_3$ reagent measures calciferol, tachysterol, pre-calciferol, etc. The complete analysis for calciferol is now carried out; $SbCl_3$ determination measures calciferol, and ICl_3 titration measures, in addition, lumisterol and suprasterols I and II. The latter three substances are thus obtained by difference. Tachysterol and pre-calciferol are obtained by difference from the two $SbCl_3$ determinations.

A further sample is now taken and extracted with hot light petroleum by frequent washing. The residue is determined as 'petrol-insoluble fraction', including perhaps the suprasterol III and tachysterol oxidation compound already described. The remainder of this sample, after solution in aqueous ethanol, is precipitated with digitonin to remove ergosterol, and the remainder is dissolved in dioxan and refluxed with maleic anhydride for 2 hr.

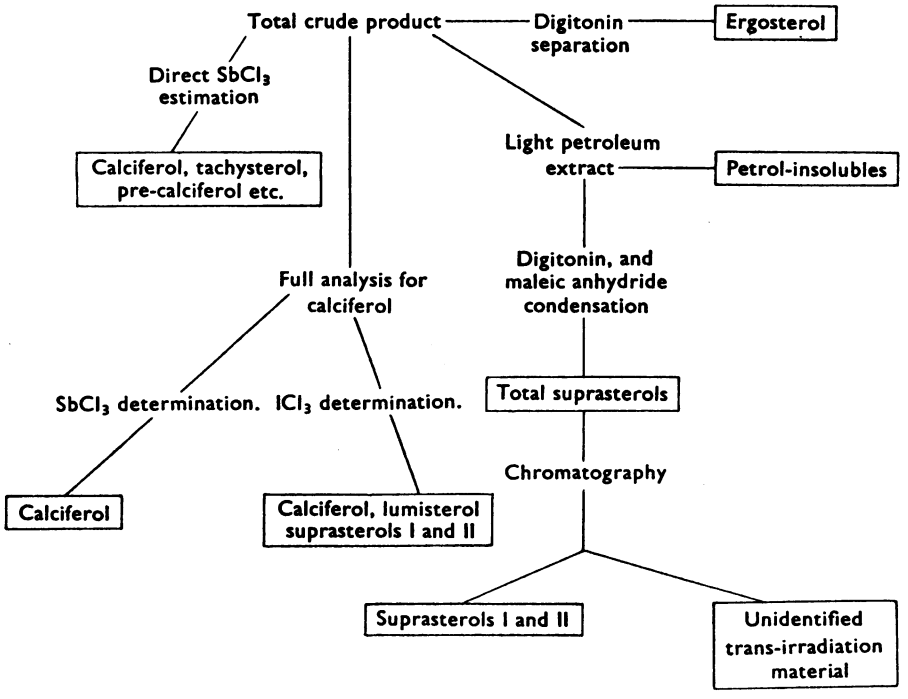
This step removes all other sterols except the total suprasterols (Green, 1951*c*), which are determined by ICl_3 titration. The suprasterol fraction may now be chromatographed on floridin, taking a CCl_4 eluate. ICl_3 titration now gives suprasterols I and II, the difference being due to unidentified over-irradiation products. Lumisterol can now be calculated by difference.

It is recognized that such a scheme as this can only be tentative and an approximation at present. The reactions on which it is based, however, are all clearly defined and the outline appears to rest on a logical basis.

DISCUSSION

In the analytical method for vitamins D which has been described, an attempt has been made to overcome the main difficulties present in the analysis of complex irradiation products. The two stages of separation used have been shown to eliminate all interfering material, with over 90% recovery of vitamin D. The $SbCl_3$-acetyl chloride colorimetric

Table 11. *Schematic outline of full analysis of ergosterol irradiation product*



method of final measurement gives a true vitamin D assay. However, the degree of separation is such that ICl_3 titration of the final test solution yields results usually not more than 20% higher than $SbCl_3$-acetyl chloride results on samples of product which have not been over-irradiated. In most industrial types of vitamin D_3 irradiation product, it has been found that ICl_3 and $SbCl_3$-acetyl chloride results may agree to within 10%.

One of the most critical tests of the method is felt to be the close agreement between chemical and biological assays on a range of widely varying experimental irradiation products (prepared by step-wise irradiation of the provitamins, and varying in provitamin content between 10 and 100%).

Of the twenty-one samples of industrial irradiation product analysed and recorded in Table 10, in only three cases does the chemical assay fall outside the limits of error ($P = 0.05$) of the bioassay (limits of error unavailable for four samples).

Analytical study of the irradiation process has produced several interesting results. It appears certain that the crude irradiation product is more complex than has been hitherto inferred and contains a number of unidentified substances. Pre-calciferol has been detected by the analytical behaviour of ageing products; whether any of the other substances indicated play a part in the direct photochemical sequence or whether they are formed in subsidiary reactions remains to be seen.

With the aid of the ICl_3 and $SbCl_3$-acetyl chloride reagents it is possible to evolve a schematic procedure for the determination of most of the individual sterols. A great deal of additional information on the composition of sterol irradiation products can probably be obtained by studies made with these two reagents.

SUMMARY

1. A method is given for the chemical determination of vitamins D in irradiation products of ergosterol and 7-dehydrocholesterol.

2. Results on a range of experimental products are in close agreement with biological assay figures.

3. Chemical and biological analyses for vitamin D_3 on twenty-one commercial irradiation products are recorded and show good agreement.

4. A study of the ultraviolet irradiation of ergosterol, 7-dehydrocholesterol, calciferol and vitamin D_3 has been made. Conditions are given for maximum calciferol formation, and factors influencing the process have been investigated.

5. The existence of unidentified substances occurring in the irradiation product has been indicated and, in some experiments, a new substance, suprasterol III, isolated.

6. A scheme is suggested for the total analysis of the components of an irradiation product.

The author wishes to thank Mr D. J. Outred and Mr D. O. Singleton for valuable assistance with the experimental work.

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Studies on the Analysis of Vitamins D

5. THE CHEMICAL DETERMINATION OF VITAMIN D IN FISH-LIVER OILS AND OTHER MATERIALS CONTAINING VITAMIN A

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The quantitative elimination of vitamin A is the most serious of all difficulties that have to be overcome in chemical methods for the analysis of vitamin D, and, up to the present, it is not generally considered that the vitamin D content of fish-liver oils can be determined by such methods with any useful degree of accuracy. In preceding papers (Green, 1951*a*,*b*,*c*) the principal methods that have been proposed by various workers on the subject have been discussed.

It has already been shown (Green, 1951*a*), that it is possible to separate vitamin A from vitamin D quantitatively by chromatographic analysis on acid-washed floridin earth. Under suitable conditions, a single liquid chromatogram can be used to free fish-liver oils from their vitamin A content without adversely affecting the vitamin D.

The present paper deals with the application of these methods to the determination of vitamin D in materials containing vitamin A.

EXPERIMENTAL AND RESULTS

Analytical method for fish liver oils

The method to be described is, with a few modifications, similar to that already described (Green, 1951*d*) for the analysis of irradiation products of ergosterol and 7-dehydrocholesterol.

Reagents. For the analysis of materials with a vitamin D potency of over 500 i.u./g., the reagents used conform to the specifications given in previous parts. Oils of lower potency (such as cod liver oils) need special precautions. The amounts of vitamin D to be measured in these oils are so small that three of the reagents (ethanol, light petroleum and digitonin) need additional purification to avoid accumulation of spurious reactive material in the final matching solutions.

Ethanol. Distilled in an all-glass apparatus over a mixture of $AgNO_3$ and NaOH. This solvent should be used for all purposes during the analysis, particularly (in 95% (v/v) concentration) for the preparation of the ethanolic KOH for saponification.

Light petroleum. Distilled from a mixture of $AgNO_3$ and NaOH and a little water. Dried over P_2O_5 and redistilled.

Digitonin. Purified from the commercial product by the method of Windaus (1925).

Weight of sample. Not less than 0·5 g. should be taken for saponification. For low potency oils, up to 5 g. may be taken.

Saponification. It may be necessary to saponify for lengths of time up to 15 min., depending on the nature of the material to be tested. Incomplete saponification may cause loss of vitamin D during chromatography.

Chromatography. This stage is as already described (Green, 1951*d*) for irradiation products. Wherever possible, no more than 20,000 i.u. of vitamin A should be passed through the column and, to this end, a suitable portion after saponification should be taken. Provided enough vitamin D is present for adequate final estimation, the amount of vitamin A passed should be as small as possible. Under these conditions, CCl_4 eluates are quite colourless; pale-yellow eluates signify over-saturation of the column with vitamin A (and possibly other substances) or incomplete washing out of the 90% ethanol used for pretreatment. With concentrates very high in vitamin A content and low in vitamin D, it may be necessary to pass as much as 200,000 i.u. of vitamin A through the column. Even using these large amounts, chromatography is usually satisfactory and eluates are almost colourless to pale yellow.

Solubility and digitonin separation. The procedure is modified for vitamin A-containing materials. After elution, the total CCl_4 eluate is evaporated to dryness at the pump in a flask fitted with a ground-glass joint. To the residue is then added 25 ml. of 72% (v/v) ethanol, and the flask stoppered and shaken vigorously for 10 min. The solution is filtered through a Whatman no. 5 paper into a 50 ml. centrifuge tube and a solution of 50–100 mg. digitonin in