REFERENCES

- Bills, C. E., Honeywell, E. M. & Cox, W. M. (1931). *J. biol. Chem.* **92**, 601.
Green, J. (1951 a). *Biochem. J.* **49**, 36.
Green, J. (1951 b). *Biochem. J.* **49**, 45.
Green, J. (1951 c). *Biochem. J.* **49**, 54.
Nield, C. N., Russell, W. C. & Zimmerli, A. (1940). *J. biol. Chem.* **136**, 73.
Olsson, N. (1936 a). *Arch. Geflügelk.* **10**, 11.
Olsson, N. (1936 b). *K. fysiogr. Sällsk. Lund. Förh.* **9**, 1.
Velluz, L. & Amiard, G. (1949). *C.R. Acad. Sci., Paris*, **228**, 692.
Velluz, L. Amiard, G. & Petit, A. (1949). *Bull. Soc. chim., Paris*, **16**, 501.

Studies on the Analysis of Vitamins D

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

By J. GREEN

Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

(Received 13 January 1951)

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, 1951a,b,c) the principal methods that have been proposed by various workers on the subject have been discussed.

It has already been shown (Green, 1951a), that it is possible to separate vitamin A from vitamin D quantitatively by chromatographic analysis on acid-washed florisil 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, 1951d) 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, 1951d) 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

72 % (v/v) ethanol added. The procedure is then as previously described.

Final estimation. The total final CCl_4 solution, or a suitable portion thereof, is evaporated to dryness and the SbCl_5 -acetyl chloride reagent added directly to the dry residue. This procedure gives better results than if the test material is made up in CHCl_3 before addition of the reagent. If the latter method is used, turbidity is sometimes produced on adding the reagent. Even using the recommended method, trouble may be caused by slight turbidity in the reaction mixture; the latter must be quite clear and should be examined in a bright light before matching. Addition of a little acetyl chloride often produces clarification; alternatively, at least 10 ml. of the reaction mixture should be filtered rapidly through a small (5 cm. diameter) Whatman no. 1 filter paper previously moistened with acetyl chloride, and the last 5 ml. taken for measurement.

Extinction values were measured after exactly 3 min., on the Uvispek spectrophotometer at 500 $\text{m}\mu$., using the 500–550 $\text{m}\mu$. internal blank method of Nield, Russell & Zimmerli (1940), to eliminate residual interfering colours which have a small but almost constant absorption over this wavelength range.

Recovery experiments

Recovery experiments with pure vitamin D_3 have been carried out on fish-liver oils having a wide range of potencies. With low potency oils, especially, it is necessary to add extremely small amounts of vitamin D_3 , and the material used must be of high purity. Good recoveries have been obtained on additions of as little as 25 μg . of vitamin D_3 , using the International Standard material or a specimen of proved equivalent purity. Such commercial samples of vitamin D_3 as are available in this country are usually not suitable for use in recovery experiments.

For these experiments, a small quantity of the pure vitamin D_3 was dissolved in light petroleum at a concentration of not less than 250 $\mu\text{g}/\text{ml}$. A portion of this solution was added after saponification of the oil. The solution was always analysed colorimetrically for vitamin D_3 when the final measurement on the test substance was carried out. It is essential that the solution of vitamin D_3 should remain quite stable over the period of several hours

necessary for the analysis of the oil. Specimens of vitamin D_3 which are not stable in light petroleum over this period are unsuitable for recovery use.

Results on fish-liver oils

A number of fish liver oils of widely differing potency have been assayed chemically and biologically. For certain samples obtained from other laboratories (together with statements of biological potency), individual limits of error are not available, but the limits of error ($P=0.05$) of these assays are unlikely to be wider than 75–125 %.

Oils of high potency in vitamins A and D. Table 1 shows the results obtained on two tunny liver oils (each containing about 290,000 i.u./g. of vitamin A), and, also, the results of the recovery experiments on these oils. Samples of 100 mg. were taken for each chemical analysis. Recoveries of over 85 % of added vitamin D_3 are shown. From the replicate chemical determinations of oil 1, in Table 1, the coefficient of

Table 1. *Vitamin D analyses on two tunny liver oils*

	Bioassay (i.u./g.)	Chemical analyses (i.u./g.)	Added vitamin D_3 (μg .)	Added D_3 recovered (%)
Oil 1	19,800	24,200	—	—
		18,600	—	—
		19,800	475	86.1
		29,000	—	—
		25,600	251	88.6
		Mean 23,400	—	—
Oil 2	20,000	26,400	—	—
		24,800	310	92

variation is calculated as about 16 %. This means that in an average set of replicate assays, about two-thirds of the results will probably fall in the range 84–116 % of the mean. Biological and chemical assays show reasonable agreement.

Medium and low potency oils. Table 2 shows results on a halibut oil and a number of cod liver oils. Halibut liver oil (500 mg.) and 5 g. of each of the cod liver oils were taken for analysis. Recovery on these

Table 2. *Vitamin D analyses on halibut and cod liver oils*

Oil used	Bioassay (i.u./g.)	Limits of error of bioassay ($P=0.05$) (%)	Chemical analysis (i.u./g.)	Added vitamin D_3 (μg .)	Added D_3 recovered (%)
Halibut liver oil	1950		2400	82	86
			2160	94	95
			1780	—	—
			2300	—	—
			Mean 2,160	—	—
Cod liver oil 1	160	86–116	180	23.1	104
2	80	81–124	125	—	—
3	148	85–117	174	28.2	96
4	94	82–122	117	—	—

oils was generally very good, and chemical and biological assays were in reasonable agreement. From the four chemical determinations of the halibut oil shown in Table 2, the coefficient of variation is calculated as about 12 %.

Further modifications of the chromatographic separation

The use of longer columns. Separation of vitamin D from interfering substances can be enhanced by using longer columns. Columns up to 15 cm. in length have been used with success, and vitamin D₃ recovery from these columns is good. Table 3 lists

Table 3. *Effect of longer columns during vitamin D analysis of fish-liver oils*

Oil used	Bioassay (i.u./g.)	Limits of error of bioassay ($P=0.05$) (%)	Chemical analysis (i.u./g.)	
			7.5 cm. column	15 cm. column
Halibut oil	2100	—	2680	2200
Cod liver oil	150	79-127	210	180
Cod liver oil	114	85-117	160	135

a series of chemical analyses on some fish liver oils, comparing the effect of the normal 7.5 cm. with that of 15 cm. columns. Too few experiments have so far been made with these longer columns to decide as to their routine use, but they may give more promising results than columns of normal length.

Double chromatography. The effect of including a second chromatographic separation on floridin has been investigated. The usual method of analysis was followed until the final CCl₄ extract from 72 % ethanol was obtained, and then, after evaporation of the CCl₄, the residue was dissolved in light petroleum and the chromatography was repeated. Providing great care was taken during the analysis, it was possible to recover 90-95 % of pure vitamin D₃ from this treatment. However, in recovery experiments on fish-liver oils recoveries were only about 70 %. Further work is necessary to decide whether the inclusion of a second chromatogram is warranted for routine use. The method is, however, effective in removing a great deal of residual interference and has been found particularly useful in attempts to determine the vitamin D content of high-potency vitamin A concentrates which are very low in vitamin D. These concentrates are difficult to analyse even by biological methods because of the high doses of vitamin A involved. Table 4 shows analyses of a high-potency fish-liver oil, a 'vitamin A concentrate', and a 'marine oil concentrate' of commercial origin. The vitamin A concentrate contained 227,000 i.u./g. of vitamin A and approximately 250 i.u./g. of vitamin D, and was assayed by the method described including a second chromatogram. The marine oil concentrate, a yellow semi-solid

substance, contained 126,000 i.u./g. of vitamin A and about 100 i.u./g. of vitamin D. For its analysis, 2 g. were saponified and yielded about 1.2 g. of non-saponifiable matter. Because of the difficulties on putting this amount of material on the column, the non-saponifiable portion was taken up in methanol,

Table 4. *Analysis of very high potency vitamin A materials for vitamin D by a method including double chromatography*

Oil used	Bioassay (i.u./g.)	Chemical analysis (i.u./g.)
Tunny liver oil	19,500	14,500
Vitamin A concentrate	Approx. 250	213
Marine oil concentrate	Approx. 100	165

cooled to -15° , and the solution filtered through sintered glass at the same temperature. After evaporation of the filtrate, the analysis was continued, with a second chromatogram. As the results show, the method makes it possible to eliminate even the very high amounts of vitamin A used here, and gives results in reasonable agreement with the bioassays.

SUMMARY

1. The analysis of vitamin D in vitamin A-containing oils and concentrates by the method described by Green (1951*d*) yields results in reasonable agreement with biological assays. Recovery experiments show that, whilst total elimination of vitamin A is obtainable, very little loss of vitamin D occurs. A small amount of residual interference with the final antimony trichloride-acetyl chloride estimation remains, but is largely eliminated by the 'internal blank' method of measurement.

2. The method is applicable to fish-liver oils of a wide range of potency, but oils containing about 100 i.u. of vitamin D/g. fall near the lower limit of applicability. The detailed steps necessary for the analysis of a vitamin A-containing oil have generally to be decided for each particular sample. Thus factors such as the vitamin A : vitamin D ratio and percentage of non-saponifiable matter have to be considered.

3. When the ratio of vitamin A to vitamin D is very high, it is always necessary to use a second chromatogram, but, with normal fish-liver oils, the use of this second chromatographic separation requires caution and is not recommended. The use of 15 cm. floridin columns has been investigated and appears to be quite safe. Good results have been obtained with such columns, and their adoption may be advantageous in many analyses.

The author wishes to thank Mr D. J. Outred and Mr D. O. Singleton for their assistance with the experimental work, and Crookes Laboratories Ltd., London, for providing samples of fish-liver oils for analytical purposes.

REFERENCES

- Green, J. (1951a). *Biochem. J.* **49**, 36.
Green, J. (1951b). *Biochem. J.* **49**, 45.
Green, J. (1951c). *Biochem. J.* **49**, 54.
Green, J. (1951d). *Biochem. J.* **49**, 232.
Nield, C. N., Russell, W. C. & Zimmerli, A. (1940). *J. biol. Chem.* **136**, 73.
Windaus, A. (1925). *Hoppe-Seyl Z.* **150**, 205.

Spectrophotometric Assay of Ascorbic Acid in Pure Solutions, Blood Plasma and Urine with *peri*Naphthindanetrione Hydrate

By M. S. EL RIDI, R. MOUBASHER AND Z. F. HASSAN

*Kasr El Aini Faculty of Medicine, Biochemistry Department and Faculty of Science,
Chemistry Department Fouad I University, Cairo, Egypt*

(Received 7 September 1950)

Chemical methods of determining ascorbic acid have replaced almost entirely the earlier biological methods. Most chemical methods are based on the reducing capacity of ascorbic acid. Titration with iodine has proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances and since the colour of such products interferes with the determination of the end point of titration. Oxidation of ascorbic acid with the dye 2:6-dichlorophenolindophenol originally suggested by Tillmans (1927), has found extensive use, but in some materials the presence of reducing substances, such as 'reductones' and 'reductic acid' interferes. Several procedures have been devised to minimize these interferences. The specificity of such methods for determination of ascorbic acid is, however, not well established (Snow & Zilva, 1944). Visual titration methods by this dye are not applicable to the study of products containing water or acid-soluble pigments or reducing agents other than ascorbic acid which react with the dye at a rate sufficiently rapid to influence the end point of titration. Photometric methods (Bessey, 1938; Nochberg, Melnick & Oser, 1943) based on the 2:6-dichlorophenolindophenol reagent avoid certain of the limitations of the visual titration techniques. The fact that this dye is readily soluble in xylene makes it possible to use a xylene extraction technique (Pepkowitz, 1943) to eliminate interference from acid-soluble pigments and turbid solutions.

Reaction of derivatives of ascorbic acid with 2:4-dinitrophenylhydrazine permits the determination of ascorbic acid by methods not based upon oxidation-reduction properties. A technique described by Roe & Oesterling (1944) for total and dehydro-ascorbic acid, appears to avoid interference from certain types of reducing materials. Although osazones result from the reaction of sugars with

2:4-dinitrophenylhydrazine, their properties and rates of formation are sufficiently different from those of ascorbic acid derivatives to prevent their interference with the estimation. This method does not prevent interference from 'reductones' and 'reductic acid', but a means of correcting the results due to these substances has been reported (Penney & Zilva, 1945).

In the present work the authors propose a new spectrophotometric method for the estimation of ascorbic acid in pure solutions, blood plasma and urine. This method is based on the colour reaction obtained by the action of *perinaphthindanetrione* hydrate (IIa) and its derivative 2-nitro*perinaphthindanetrione* hydrate (IIb) with ascorbic acid. Preliminary spectroscopic examination of these two reagents has been carried out. Further study in this field is in progress, and details will be published later.

Nature of the colour reaction. Moubasher (1948) and Moubasher & Sina (1950) found that when solutions of *perinaphthindanetrione* hydrate (IIa) (cf. Errera, 1913, 1914) or 2-nitro*perinaphthindanetrione* hydrate (IIb) (cf. Calderaro, 1916) and ascorbic acid (I), are mixed at room temperature, a deep red colour rapidly develops due to the formation of dihydroxy*perinaphthindenone* (IVa) or dihydroxy-2-nitro*perinaphthindenone* (IVb) respectively. In higher concentration these products can be isolated in silky red crystalline needles, which may be identified by their melting points and mixed melting point determinations with authentic samples. The red compounds are similar to those prepared by Errera and Calderaro.

The reagents (IIa) and (IIb) are not only easily prepared, inexpensive and stable, but also have the following advantages:

(a) *Stability of the colour.* The characteristic red colour obtained is stable for about 24 hr.