The assessment of vitamin D status

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During the past forty years, continuous attempts have been made to develop a sensitive method for the estimation of vitamin D (cholecalciferol). Several methods were developed (see Kodicek & Lawson, 1967; Sheppard, Prosser & Hubbard, 1972), and in general they can be divided into two types; (1) physico-chemical methods and (2) biological assays.

At present there is no suitable physico-chemical method for estimating the amounts of vitamin D found in animal tissues. Furthermore, the methods involve laborious separation procedures to eliminate interference by accompanying substances with similar physico-chemical properties, e.g. retinol and cholesterol. As a result, biological assays were used routinely in many laboratories for the estimation of vitamin D in tissues and also in animal foodstuffs, fish oils and pharmacological preparations. The main disadvantage of these methods, apart from low sensitivity, is the cost, labour and time required for the bioassay.

It is now known that vitamin D, which is derived either from ultraviolet irradiation of the provitamin in skin, or is obtained from the diet, is further metabolized to 25-hydroxycholecalciferol (25-HCC) and then to 1,25-dihydroxycholecalciferol (1,25-DHCC) which is believed to be the metabolically active form of the vitamin. In most species, including man (Mawer, Lumb, Schaefer & Stanbury, 1971) the principle circulating metabolite in blood is 25-HCC, while cholecalciferol accounts for only a small proportion of the total anti-rachitic activity and 1,25-DHCC contributes less than 5% (Lawson, Pelc, Bell, Wilson & Kodicek, 1971).

In the light of these findings, there has also been a renewed attempt to characterize the binding proteins for vitamin D and its metabolites in plasma and target organs (see Edelstein, 1973), and one practical result of these studies has been the development of a competitive protein-binding (CPB) assay for vitamin D and its 25-hydroxymetabolite.

The first reported CPB-assay described the estimation of cholecalciferol and its 25-hydroxymetabolite in plasma, using a specific vitamin D-binding protein from rat serum (Belsey, DeLuca & Potts, 1971). β-lipoprotein from human plasma was used as the carrier for the steroids in the assay system to overcome the problem of the limited solubility of these steroids in water, but introduced an important disadvantage which is the time factor; several days were required for equilibration and displacement. This disadvantage prevented the acceptance of the method for the routine estimation of vitamin D and its 25-hydroxymetabolite.

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Subsequently, another CPB-assay for 25-HCC was reported (Haddad & Chyu, 1971), this used a specific binding protein for 25-HCC from kidney. The solubility problem was overcome by including absolute ethanol (70 ml/l) in the assay system, reducing the time required to reach equilibrium displacement to 60 min. The free steroid was separated from the bound steroid using charcoal coated with dextran. This method provides the basis for a simple and sensitive method for the routine estimation of 25-HCC levels in peripheral blood. The method can be used to estimate levels of 25-HCC as low as 4 ng/ml plasma.

However, in these methods chromatography on silicic acid columns was used to separate 25-HCC from other vitamin D metabolites present in plasma. This technique, we find, interferes with the CPB-assay and produces erratic blank values. These are the result of substances produced during chromatography which are derived from impurities in the silicic acid produced by the interaction of the solvents with the silicic acid (Murphy, 1971).

Using small Sephadex LH-20 columns for the separation of the 25-HCC metabolite, and a partially-purified vitamin D-binding protein from rat serum as the assay protein, we were able to develop a CPB-assay for 25-HCC which eliminates these interfering substances and has several additional advantages (Edelstein, Charman, Lawson & Kodicek, 1973). The assay method is shown diagramatically in Fig. 1.

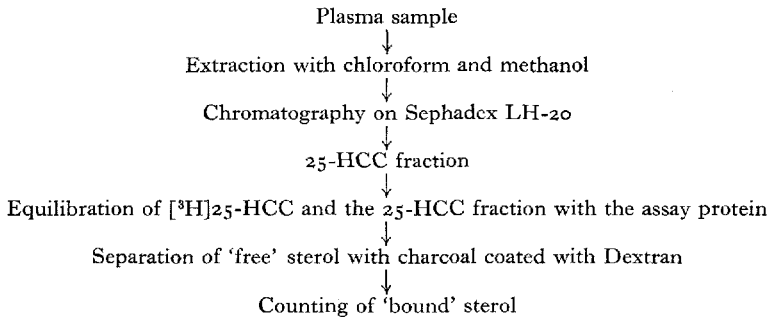

Fig. 1. Outline of the competitive protein-binding assay for the estimation of 25-hydroxycholecaliferol (25-HCC).

The assay was used to estimate serum 25-HCC levels in eighteen adult male and female volunteers. The mean value for serum 25-HCC levels was found to be $15{\cdot}2 \pm 5{\cdot}6$ ($\pm$SD) ng/ml, but Fig. 2 shows that 60% of the volunteers had 25-HCC levels lower than the mean value. The mean value was similar to that found by Stamp, Round, Rowe & Haddad (1972) for volunteers in the United Kingdon but lower than the mean value of $27{\cdot}3 \pm 11{\cdot}8$ ng/ml found for volunteers in the USA. (Haddad & Chyu, 1971). This is probably the result of differences in dietary intake of vitamin D and to the higher exposure to sunshine in the USA.

Levels of 25-HCC in blood of four male volunteers maintained for a minimum of 2 months on a diet supplemented with 10 μg/d cholecalciferol were found to be significantly higher, $35{\cdot}9 \pm 15{\cdot}0$ ng/ml. This indicates that the estimation of

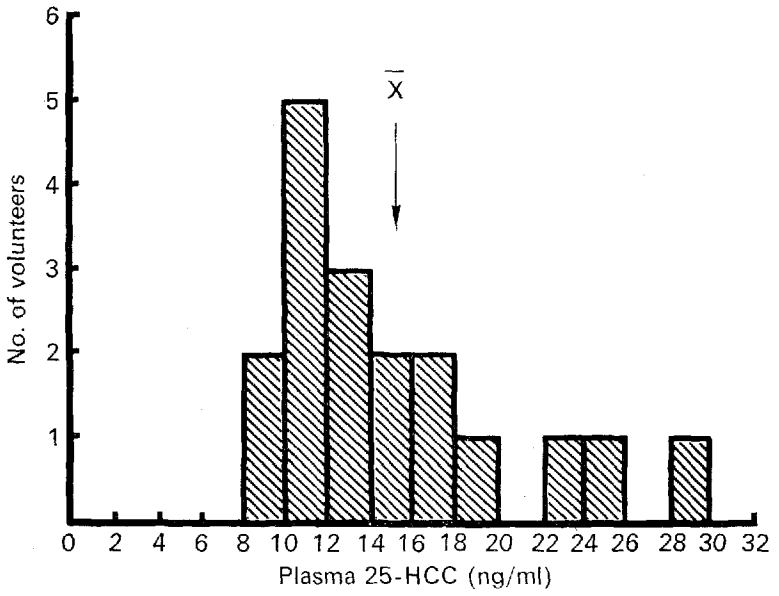

Fig. 2 Plasma 25-hydroxycholecalciferol levels (25-HCC) for eighteen men and women estimated using the competitive protein-binding assay. $\bar{X}$, mean value for plasma 25-HCC.

the plasma concentration of the 25-hydroxylated metabolite of vitamin D is a reliable method of assessing nutritional status.

Although the CPB and biological assays differ in their sensitivity and specificity, there is a high correlation between values obtained by the two methods (Table 1). This correlation was maintained even when the values obtained by the CPB-assay were expressed as 'cholecalciferol-equivalent' (e.g. ng 25-HCC × 1·4) (The Royal Society, 1972). These results also confirm that the main metabolite of vitamin D in the blood is the 25-hydroxylated metabolite.

Attempts to develop a similar CPB-assay for cholecalciferol have so far been unsuccessful. Although specific binding proteins for cholecalciferol exist (Edelstein, Lawson & Kodicek, 1972, 1973; Edelstein, 1973) the limited solubility of this steroid in water prevents the occurrence of competitive displacement.

Table 1. *Comparison of plasma 25-hydroxycholecalciferol (25-HCC) levels of men and women estimated using competitive protein-binding (CPB) assay with values estimated using a biological assay*

(Mean values and standard deviations for nine individuals for CPB assay and total vitamin D activity for bioassay)

25-HCC (ng/ml)		'Cholecalciferol equivalents' (ng/ml)
CPB assay	Bioassay	
15·3±9·0*	13·9±11·3*†	21·5±12·7†

*Correlation coefficient 0·85.
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The development of a method for estimating 1,25-DHCC will have to wait until further information is obtained about the properties of the specific binding proteins for this hormone.

However, as the main metabolite of vitamin D in blood is the 25-hydroxylated metabolite, the estimation of this steroid by a CPB-assay can serve as a useful and reliable method for the assessment of the nutritional status of vitamin D in man.

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