

## MODE OF ACTION OF VITAMIN D—A RESUME

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THE discovery of vitamin D as the specific etiological agent in rickets was an event of great significance to the science of nutrition. It led to intensive investigations on the occurrence, properties, isolation and preparation of vitamin D in the pure state. At the same time the physiological function of vitamin D and prevention and treatment of rickets were being studied by different groups of workers. After nearly thirty years of work, more than ten substances with varying vitamin D activity have been discovered, of these vitamin D<sub>2</sub> and D<sub>3</sub> are the most important. Although much valuable information on the biological side has been added during the same period, vitamin D is one of the few known vitamins about the mode of action of which there is very little definite knowledge.

Cod-liver oil had been in use for medical purposes<sup>1</sup> since 1789; its use for rickets was first reported in 1824. It was in 1909, however, that any indications of the mode of its action became available when Schabad<sup>2</sup> reported his classical experiments on the effects of cod-liver oil on calcium and phosphorus metabolism in rickets. In 1919 Mellanby<sup>3</sup> found that cod-liver oil was effective in curing rickets experimentally produced in puppies. McCollum<sup>4</sup> and others (1921) demonstrated the curative action of the oil in rat rickets; from the same laboratory came the first proof of the presence of a fat-soluble vitamin in cod-liver oil in addition to vitamin A discovered earlier.

The observation of Huldschinsky<sup>5</sup> (1919) on the therapeutic effect of ultra-violet rays in infantile rickets started another series of researches in a number of laboratories culminating in the isolation of calciferol in pure form in 1932 by Askew *et al.*<sup>6</sup> and Windaus<sup>7</sup> *et al.* with an activity of 40,000 I. U. per mgm. of the crystalline material. Calciferol or vitamin D<sub>2</sub> is a product obtained on irradiation of ergosterol. Soon it was proved that calciferol was not the naturally occurring vitamin D found in the liver oils or the one generated in the skin by the action of ultra-violet rays. An irradiated product of 7-dehydrocholesterol was identified as the natural vitamin (Bills,<sup>8</sup> 1938) and is now known as vitamin D<sub>3</sub> having an antirachitic activity for rats comparable with that of calciferol.

The progress in the knowledge about the mechanism of action of vitamin D did not, however, keep pace with that on the chemical side. Only two definite results have emerged thus far as a result of intensive studies carried out by numerous workers. The first is that in therapeutic doses, vitamin D promotes retention of calcium and phosphorus, particularly in rachitic animals and the second, that vitamin D, also in therapeutic doses, cures rickets by permitting calcification of the bone to proceed in a normal manner. An adequate supply of vitamin D to the young animal can prevent the onset of rickets. These manifestations however, are the end results of vitamin

D action. They do not throw much light on how the action is brought about. Certain hypotheses have been put forward to explain the action. They are not entirely satisfactory but are currently accepted for want of more convincing explanation. It is therefore the aim of this article to review in brief the evidence for and against these hypotheses with a view to indicating further lines of attack which may possibly lead to a better understanding of the mode of action of vitamin D. So much work on the subject has been published that it would be impossible to deal with all of it in this article. An attempt has therefore been made to cite some of the more important references which have largely contributed to the present state of our knowledge regarding vitamin D.

As mentioned earlier, Schabad was the first to demonstrate that in rachitic infants, the administration of cod-liver oil increased the retention of calcium and phosphorus. Findlay, Paton and Sharpe<sup>9</sup> (1920-22) suggested that the increased loss of calcium through the faeces was due to the fact that calcium was not properly utilised in the body of the rachitic children and hence was re-excreted in the digestive tract. Telfer<sup>10</sup> (1922-23) and Orr, Holt, Wilkins and Boone<sup>11</sup> (1923-24) also observed diminished retention of calcium and phosphorus in clinical rickets. Further metabolic studies by Telfer<sup>12</sup> (1926) using cod-liver oil and of Hottinger<sup>13</sup> (1929) using irradiated ergosterol brought out the fact that under the influence of vitamin D there was a shift from faeces to urine in the excretion of calcium and phosphorus. Watchorn<sup>14</sup> (1930) reported a decrease in the faecal calcium and phosphorus of rachitic rats after administration of irradiated ergosterol. A reduction in calcium excreted with the faeces of rachitic rats administered irradiated ergosterol was also observed by Kern, Montgomery and Still<sup>15</sup> (1931) and Harris and Innes<sup>16</sup> (1931). Such observations inevitably pointed to the conclusion that vitamin D favoured the absorption of calcium and/or phosphorus from the gut. It must be pointed out that these conclusions were based on studies in which the sum of calcium and phosphorus elimination in urine and faeces was compared with the intake of these elements. Such balance studies are expected to yield correct information only when there is no re-excretion in the gut of the substances absorbed from the upper reaches of the small intestine. When such is not the case, the interpretation of balance studies would lead to erroneous conclusions regarding the process of absorption. There is a certain amount of evidence to show that calcium and phosphorus, both are re-excreted into the intestine. This fact was taken into consideration by Harris<sup>17</sup> (1932) when he suggested the use of "net absorption" to indicate the difference between the intake of calcium and the sum of urinary and faecal calcium. But other authors were

less careful in the use of the term absorption, and it has come to be accepted that vitamin D increased specifically the absorption of calcium from the intestine.

In 1926, Bergeim<sup>18</sup> introduced  $\text{Fe}_2\text{O}_3$  together with calcium salts in the intestine and determined the ratio of Fe to Ca at various levels of the small intestine. He came to the conclusion that deficiency of vitamin D did not adversely affect the absorption of calcium. Taylor and Weld<sup>19</sup> (1932) also reported that vitamin D had no influence on calcium absorption. Nicolaysen<sup>20</sup> (1937) was the first to seek direct proof of the effect of vitamin D on absorption of calcium from the small intestine. He used the isolated loop technique of Verzar<sup>21</sup> (1936) and showed that in rachitic rats, the rate of absorption of calcium was slower than in the animals protected against rickets by vitamin D. Nicolaysen's results found wide and uncritical acceptance. When Patwardhan and Chitre<sup>22</sup> (1942) studied the absorption of calcium in three groups of rats which were (a) rendered rachitic, (b) protected against rickets by dosage with vitamin D and (c) rendered hypervitaminotic by massive dosage with vitamin D respectively, they could find no significant difference in the rates of absorption of calcium from the small intestine among the rats belonging to these groups.

Thus, it cannot be denied that in spite of clear-cut evidence that in therapeutic doses vitamin D increases calcium and phosphorus retention, no direct evidence of its effect on absorption from the intestine has yet been forthcoming [Wolbach<sup>23</sup> (1947)]. Hence no definite conclusion can be reached with regard to the mechanism by which vitamin D increases the retention of calcium and phosphorus.

It will be of interest now to discuss other manifestations of vitamin D action, particularly those affecting blood and the bone. A deficiency of vitamin D is responsible for infantile rickets (Hess<sup>27</sup>; Eliot and Park, *loc. cit.*). It is also responsible for experimentally produced rickets in animals (Mellanby, 1919, *loc. cit.*, and McCollum, *et al.*, 1921, *loc. cit.*). The levels of calcium and inorganic phosphorus in the serum of normal children are roughly between 10-12 mg. per cent. and 4 to 6 mg. per cent. respectively (Howland and Kramer,<sup>24</sup> 1923; Patwardhan, Chitre and Sukhatankar,<sup>25</sup> 1944). In rickets, Ca or inorganic  $\text{P}^{2+}$  or both may fall below normal levels. Eliot and Park<sup>26</sup> (1942) state that in rickets serum calcium remains at approximately the normal level, but the inorganic phosphorus decreases considerably. They do mention the possibility, however, that calcium and not the inorganic phosphorus may be decreased. Patwardhan, Chitre and Sukhatankar (*loc. cit.*) found a greater frequency of low calcium levels than of low inorganic phosphorus in radiologically diagnosed rickets studied by them in a hospital in Bombay. What the predisposing conditions are which determine the lowering of calcium or phosphorus or of both is not yet quite clear. Whatever the condition, the administration of vitamin D in therapeutic doses, brings about a return to the normal, both in clinical as well as in experimental rickets. These changes in

the blood are, however, reflected in the composition of bone. The fact that rachitic bone is poorly calcified is too well known to require elaborate description. The response of rachitic animals to vitamin D resulting in increased calcification has been the basis of several methods of vitamin D assay.

There are reasons to believe that there is nothing inherently wrong with the rachitic bone. Shipley, Kramer and Howland<sup>28</sup> (1926) showed that slices of tibiae from rachitic rats calcified when immersed in an inorganic solution of known composition containing Ca and inorganic P. The product of Ca and P determined whether calcification would take place or not. They also demonstrated that rachitic bone slices would not calcify in the serum of rachitic animals whereas they would if immersed in the serum of non-rachitic animals. Robison and his colleagues [Robison and Soames<sup>29</sup> (1930), Robison, McLeod and Rosenheim<sup>30</sup> (1930)] demonstrated that in the presence of organic phosphoric esters, rachitic bone would calcify *in vitro* with lower concentration of inorganic P. It is permissible to assume therefore that in rickets, bone does not calcify because the fluid environment in the immediate vicinity of the zone of provisional calcification is not suitable for promoting bone formation. Since the interstitial fluid should be in equilibrium with blood, the defect must primarily be looked for in the blood itself.

The interrelation between the mineral composition of bone and the electrolyte composition of plasma has been the basis of much work and certain hypotheses have been put forward to explain the normal process of calcification. In the main, these hypotheses postulate that when the plasma is in a state of supersaturation with respect to  $\text{Ca}_3(\text{PO}_4)_2$  and/or  $\text{CaHPO}_4$  the bone salt is laid down. The latter has the composition  $n \text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaX}$  where X may be  $\text{F}^-$ ,  $\text{OH}^-$ , or  $\text{CO}_3^{=}$  and the value of  $n$  may vary between 2 and 3. Holt, Lamer and Chown<sup>31</sup> (1925) suggested that  $\text{Ca}_3(\text{PO}_4)_2$  was laid down when the concentration of  $[\text{Ca}^{++}]^3$  and  $[\text{PO}_4^{=}]^2$  in serum exceeded the solubility product. Wendt and Clarke<sup>32</sup> (1923) had made a suggestion that  $\text{CaHPO}_4$  and not  $\text{Ca}_3(\text{PO}_4)_2$  was the salt first precipitated, a hypothesis which received support from Shear and Kramer<sup>33</sup> (1928). Logan<sup>34</sup> (1940) reviewed the evidence in support of both these hypotheses including the work done by him and his colleagues and came to the conclusion that the salt first precipitated probably had the composition of  $\text{CaHPO}_4$ ; the alteration in composition to that more commonly known was achieved by the exchange of ions. Additional evidence in favour of this hypothesis was given by Freeman and McLean<sup>35</sup> (1941) who found that in rachitic puppies, the blood was undersaturated with respect to  $\text{CaHPO}_4$  although it was not necessarily so with respect to  $\text{Ca}_3(\text{PO}_4)_2$ . Patwardhan, Chitre and Sukhatankar<sup>36</sup> (1945) also observed a similar state of affairs in experimental rickets in puppies as well as in clinical rickets in Indian infants and children. Patwardhan and Dikshit<sup>37</sup> (1946) followed up these observations by determining the changes

in the ionic concentration of  $\text{Ca}^{++}$ ,  $\text{HPO}_4^-$  and  $\text{PO}_4^{=}$  during the onset, progress and healing of rickets in puppies. They found that the process of healing indicated by radiological examination was simultaneous with or most probably preceded by a shift to the state of supersaturation with respect to  $\text{CaHPO}_4$  and  $\text{Ca}_3(\text{PO}_4)_2$  both. Whichever the salt that is precipitated to start with (a matter not unimportant in itself, but immaterial for the purposes of this article) it is clear that in a deficiency of vitamin D, the blood is undersaturated with respect to  $\text{CaHPO}_4$  and probably also  $\text{Ca}_3(\text{PO}_4)_2$ , and on administration of adequate amounts of vitamin D, the serum becomes supersaturated initiating the healing of the rachitic lesion. Here again one sees the result, but the mechanism by which it is brought about remains obscure.

The curative effect on rickets shown by vitamin D is accompanied by an increase in the serum Ca or inorganic P or both. But this cannot be the only consideration in initiating repair of a rachitic bone. Nicholay-sen<sup>38</sup> (1939) has reported that increasing the serum concentrations of Ca and inorganic P by intravenous injections did not lead to normal bone formation in rachitic animals in absence of vitamin D. It appears therefore that vitamin D influences bone formation in some other way as yet unknown. Besides, the supersaturation theory does not explain all the known facts. Firstly, bone is continually undergoing change in which the processes of demineralisation and mineralisation presumably alternate. During the growth period, it is the latter process which predominates and the bone finally assumes the shape found in the adult. During senility, it is presumably the demineralisation that assumes the upper hand, and bone in old age tends to become osteoporotic. It will not be possible to explain adequately these phenomena unless one assumes locally produced alternate stages of undersaturation and supersaturation. That bone is in a dynamic state has been beautifully demonstrated by Chiewitz and Hevesy<sup>39</sup> (1935) by the use of  $\text{P}^{32}$ .

The action of vitamin D when administered in massive doses is also difficult to explain. Hypervitaminosis D is a condition in which there is first hypercalcaemia and hyperphosphatemia accompanied by increased excretions of Ca and P in urine and decreased faecal excretion of these elements. In the early stages of hypervitaminosis D, there is thus increased retention of Ca and P. When the condition becomes severe, the retention of these elements may actually decrease. It has been suggested by Harris and Innes<sup>16</sup> (1931) that in severe hypervitaminosis the gut function fails and hence there is decreased absorption of calcium from the intestine. Patwardhan and Chitre<sup>22</sup> (1942) however found, as mentioned before, that in animals with severe induced hypervitaminosis D showing decreased retention, there was no significant difference in the absorption of calcium from the intestinal loops as compared with the absorption in normal animals.

So far as bone is concerned, intense calcification is observed in early stages of hyper-

vitaminosis D. When the condition becomes severe, demineralisation of bone takes place (Brown and Shohl,<sup>40</sup> 1930; Harris and Innes,<sup>16</sup> 1931; Patwardhan and Chitre,<sup>41</sup> 1938). Strangely enough this withdrawal of calcium from bone occurs with or without dietary calcium and results in a high concentration of Ca and inorganic P in blood. This latter condition may lead to metastatic calcification of soft tissues.

The observations of Ham and Lewis<sup>42</sup> (1934) are particularly interesting; they found that when massive doses of vitamin D were administered to young rats, a condition in epiphyseal cartilage resembling that found in low calcium rickets was produced. Thus here is an example where excess of vitamin D actually prevents calcification of the growing bone. The toxic effects of vitamin D on bone are exactly opposite of what they are when the vitamin is administered in therapeutic doses.

It must be pointed out that vitamin D has no local action (Robison and Rosenheim,<sup>43</sup> 1934) at the seat of calcification. Its systemic action consists, in addition to what has been described above, in a reduction of plasma phosphatase if it had increased due to rachitic lesion (Bodansky and Jaffe,<sup>44</sup> 1934) and an increase to normal of certain phosphoric esters of the red blood cell (Rapoport and Guest,<sup>45</sup> 1938). No other changes in blood have been reported either in rickets or in hypervitaminosis D which could be ascribed to the latter.

The delay in the manifestation of vitamin D action after its administration raises the question whether the vitamin undergoes any change in the body before exerting its characteristic actions. There is some circumstantial evidence in support of this concept, enough to warrant further exploration. It is well known that in a rachitic animal, the healing effect of vitamin D can be demonstrated only 24 to 48 hours after administration. Irving<sup>46</sup> (1944) found teeth more sensitive to vitamin D action than long bones; but in teeth too, the action was manifest only after 24 hours. Morgareidge and Manley<sup>47</sup> (1939) reported that the amount of  $\text{P}^{32}$  in the metaphyses of rachitic rats increased after 54 hours after the administration of  $\text{P}^{32}$  and vitamin D and that this increase coincided with the appearance of the healing line in the epiphyseal cartilage. In certain experiments carried out by the author (unpublished) it was found that even intravenous administration of 4,000,000 I.U. of vitamin D<sub>2</sub> to dogs of 11 to 12 kgm. weight caused a rise in serum calcium only after 24 hours. Against this can be mentioned the fact that the hypercalcaemic action of parathyroid hormone is manifest within a few hours and the blood calcium may return to normal within 24 hours. The delay in vitamin D action has been attributed to the possible intervention of parathyroids, for it has been suggested that vitamin D acts by stimulating parathyroids to greater activity, a suggestion which still remains to be proved. Vitamin D can act in absence of or in case of hypofunction of parathyroids and can actually relieve parathyroprivic tetany (McLean,<sup>48</sup> 1941; Drake and Sulkowitch,<sup>49</sup> 1938).

The idea that vitamin D may undergo some change in the body before exhibiting its activity

is not so far fetched as it seems. The functions which some other vitamins perform in the body are carried out in combination with proteins and certain other substances. Vitamin A, thiamine, riboflavin and nicotinic acid are the best examples of this type. Even if the above suggestion proved to be correct, there still would remain the need to find out the way in which the hypothetical derivative or compound of vitamin D would exert its action. Thus far then, there is no clue to the mode of action of vitamin D. Recent work with the radioisotopes of Ca and P with or without vitamin D has confirmed some of the earlier findings without adding anything fundamentally novel. It is a permissible conjecture that labelling of vitamin D itself, if that were possible, would yield much more valuable information on the subject.

It is difficult, however, to predict the direction which future work on vitamin D would take. There appear several possibilities which could be explored, e.g., (1) search for the hypothetical vitamin D compound, (2) more precise information about the fate of vitamin D in the body and (3) preparation of simpler compounds possessing vitamin D activity.

Investigations along the lines suggested above are already in progress in some laboratories in India and abroad, and it is expected that, if successful, they will yield much needed information on the mode of action of vitamin D.

Work on vitamin D has been very much hampered by the lack of suitable physico-chemical or chemical methods of assay. Biological methods are the only methods available at present which can be relied upon to give reasonably accurate results. These are tedious, time-consuming and require large numbers of animals. Colorimetric methods have been suggested from time to time, but they fail because they are either non-specific or require large concentrations of vitamin D to be effective. Unless this obstacle is removed from the path of investigators, further progress is bound to be slow.

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