NATURE OF THE PROVITAMIN D OF THE RIBBED MUSSEL, MODIOLUS DEMISSUS (DILLWYN)

BY H. R. ROSENBERG AND J. WADDELL

(From the Jackson Laboratory, Deepwater, New Jersey, and the Biological Laboratory, E. I. du Pont de Nemours and Company, New Brunswick, New Jersey)

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Since 1934, when it was shown that ergosterol is not the only provitamin D occurring in nature (1), the provitamin D content of the mixed sterols from many sources has been investigated (2–6). It has been found that the sterols from many of the invertebrates contain a surprisingly large amount of provitamin D as measured spectroscopically. Windaus (3) and Bock and Wetter (4) were able to isolate, in certain cases, a provitamin D fraction sufficiently pure for identification and they always obtained either ergosterol or 7-dehydrocholesterol. Boer et al. (7, 8) described the preparation of provitamin-containing sterols from several invertebrate sources and claimed that, on irradiation, these sterols yield a vitamin D which is highly efficacious when tested on chicks. These workers also described the purified provitamin sterol from selected sources, particularly the edible mussel, Mytilus edulis, and the periwinkle, Littorina littorea. The physical and chemical constants given for these provitamin fractions indicated that they were different from known sterols, but the fact was not thoroughly excluded that they were mixtures.

More recently van der Vliet (9, 10) reported a detailed study of Mytilus provitamin D and concluded that it was a mixture. While none of the components was isolated in pure form, on the basis of fractionation procedures, degradation, and biological efficacy of certain fractions he concluded that the provitamin mixture consisted of approximately 50 per cent 7-dehydrocholesterol, 17 per cent ergosterol, and nearly 33 per cent of a third provitamin tentatively identified as 22-dehydro-7-dehydrocholesterol (Δ^5, 7, 22-cholestatrien-3-ol). In addition, there was evidence of a small amount of an unidentified fourth component.

Prior to World War II the mixed sterols of M. edulis were developed in Holland as a commercial source of provitamin D. Large quantities of these sterols were imported by the du Pont company during the period 1938–40 and irradiated with ultraviolet light to yield vitamin D preparations for use particularly in poultry and animal feeds. Thus we had an opportunity of learning the provitamin D content of these sterols and, after careful irradiation, the yield and biological efficacy of the vitamin D
produced, as determined by both rat and chick assays by using the U. S. P. reference cod liver oil as the standard preparation in each test.

At the same time we carried out extensive studies on American sources of provitamin D-containing animal sterols. Following Boer et al. (7, 8), we examined a wide variety of invertebrates, among them samples of *M. edulis* from both the Atlantic and Pacific coasts. The sterols from these latter were found to possess approximately the same provitamin D content as reported by the Dutch workers. A more profitable source, however, proved to be the sterols from the ribbed mussel, *Modiolus demissus* (Dillwyn)\(^1\) (11), which occurs abundantly in the intertidal zone and the marshes of the Atlantic seaboard from New Jersey to Florida. Not only was this mussel more easily and more abundantly available than *Mytilus* but also the provitamin D content of the isolated sterols was found to be much higher. Further, it was found that, on irradiation with ultraviolet light, this provitamin D gave a greater yield of vitamin D with a higher chick-rat ratio of efficacy.

In this paper we wish to describe briefly some of our findings as to the nature of the provitamin D from *Modiolus* based on preliminary chemical and physical characterization and also on the biological efficacy of the vitamin D which it yielded on irradiation. In an accompanying paper (12) there are described the isolation and characterization of a spectroscopically pure provitamin D fraction from this same source.

**EXPERIMENTAL**

_Provitamin D Content of Mixed Sterols of M. demissus (Dillwyn)—_The method of preparing the sterol fraction from the ribbed mussel may be described briefly. Freshly gathered mussels were first steamed thoroughly and the meat was then separated by hand from the shells. Packed in well tinned cans, this meat could be held in frozen storage as long as desired. The steamed meat represented approximately 10 per cent of the weight of the mussels as gathered.

In order to hydrolyze the protein and saponify the fat in one operation, the mussel meat was treated with aqueous-alcoholic alkali for several hours at a refluxing temperature. During the latter part of this treatment some of the solvent was distilled, and then the remaining solution was diluted with more water and repeatedly extracted with ether. After washing the ether extract to neutral reaction with water, the ether was distilled off and the sterol residue was recrystallized from alcohol till sufficiently pure. The

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\(^1\) The investigation of this mussel as a source of provitamin D was first suggested by Dr. W. S. Calcott, of our Company. We are indebted to Dr. Curtis L. Newcombe, then of the Virginia Fisheries Laboratory, Jamestown, Virginia, for its identification as *Modiolus demissus* (Dillwyn).
yield of total sterols obtained in this way was of the order of 1.1 gm. per pound of mussel meat.

The provitamin D content of the many batches of sterols produced as above was estimated spectroscopically by using the molecular extinction coefficients of ergosterol as the standard. The values obtained in this way were in the range from 35 to 50 per cent with an average of more than 40 per cent provitamin D. These figures are distinctly higher than those for *Mytilus* sterols (provitamin D content, 6 to 18 per cent).

**Yield and Efficacy of Vitamin D from Mussel Provitamin**—In Table I we have summarized the data which were obtained on the yield of vitamin D by the irradiation of both *Mytilus* and *Modiolus* sterols. Repeated tests with both rats and chicks were used in the assay of the different batches. In many of the chick tests (A. O. A. C. procedure (13)), values from outside laboratories in addition to those from this laboratory were included. In view of the large amounts of provitamin irradiated, the number of different batches and the replicated tests on each, the mean values reported in Table I must be regarded as being quite accurate. It is obvious that the yield of vitamin D from *Mytilus* provitamin is distinctly less than from *Modiolus* provitamin, especially when assayed by the chick method, indicating that the provitamins from the two sources are different in character. This could be due to difference in the chemical constitution or to a different ratio of components if there is a mixture of provitamins in both species of mussels.

It is also indicated by the yield values shown in Table I that *Modiolus* provitamin varies somewhat in composition, depending on the source of the

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**Table I**

<table>
<thead>
<tr>
<th>Year</th>
<th>Source</th>
<th>Activity in U. S. P. rat units per gm provitamin D</th>
<th>Chick-rat ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Activity in A. O. A. C. chick units per gm provitamin D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean, millions</td>
</tr>
<tr>
<td>1938-40</td>
<td><em>Mytilus</em>, Dutch</td>
<td>11</td>
<td>8.3-11.9</td>
</tr>
<tr>
<td>1940</td>
<td><em>Modiolus</em> (Va.)</td>
<td>14</td>
<td>11.7-17.1</td>
</tr>
<tr>
<td>1941</td>
<td>&quot; (&quot; N. C.)</td>
<td>7</td>
<td>13.1-17.0</td>
</tr>
<tr>
<td>1942</td>
<td>&quot; (N. C.)</td>
<td>11</td>
<td>11.2-17.5</td>
</tr>
<tr>
<td>1943</td>
<td>&quot; (Va., N. C.)</td>
<td>2</td>
<td>14.7-15.8</td>
</tr>
<tr>
<td>1946</td>
<td>&quot;</td>
<td>1</td>
<td>14.7</td>
</tr>
</tbody>
</table>

* On the average, each plant batch was assayed five separate times on rats and four times on chicks.
PROVITAMIN D OF RIBBED MUSSEL

mussels. In 1940 all the mussels came from Virginia; in 1941 the major-
ity came from Virginia but some were collected in North Carolina. The
entire 1942 crop originated in North Carolina. Those taken in 1943 came
from both states, while the single batch processed in 1946 came from Vir-
ginia. In addition to higher yield, it seems apparent that the provitamin
from Virginia mussels yields vitamin D with a higher chick-rat ratio than
does that from North Carolina mussels, this change in ratio being due
mainly to a difference in yield of vitamin D as assayed by chicks.

It may be mentioned that, when the vitamin D from the 1946 batch of
sterols was being assayed, we repeated the assays of one preparation from
the 1942 mussel sterols. We again found the chick-rat ratio of the latter
to be low, 0.85, while the ratio for the former was found to be 1.13. We
have no evidence as to what factor in the environment (e.g., food supply,
temperature, etc.) was responsible for the change in the sterol composi-
tion, but we believe that these data suggest that the provitamin consists
of more than one component.

Incidentally, it is difficult to understand the low yield of vitamin from
the Dutch Mytilus provitamin if, as van der Vliet suggests (10), it con-
tains approximately 50 per cent 7-dehydrocholesterol and 17 per cent ergos-
terol. Our experience would indicate that a much higher yield of vita-
min D should be obtained by irradiation of such a mixture.

Preliminary Chemical Characterization of Modiolus Provitamin D—Pre-
liminary studies on the isolation of pure provitamin D were carried out
with sterols from mussels collected during the summer of 1940. The sterol
fraction (2.5 gm.) was dissolved in light petroleum (b.p. 30-40°) and ad-
sorbed on a column of aluminum oxide. Upon elution with the same sol-
vent, to which about 1 per cent of methanol was added, sterol fractions
low in provitamin D were obtained first. At later stages of the elution
the Modiolus provitamin D was obtained in 41 per cent yield and 80 to
85 per cent purity. The material thus isolated showed a melting range
of 114-127°. A spectroscopically pure sample, described in the accom-
panying publication (12), melted at 125-127°. This low melting range
suggests either that the provitamin D is a mixture of different chemical
substances or that, if a single substance, the provitamin D is a compound
terribly different from all known, naturally occurring D provitamins.

Another sample of the mixed sterols of Modiolus was acetylated by
heating to a reflux with acetic anhydride. The acetates, showing a pro-
vitamin D content of 30 to 32 per cent and a melting range of 108-116°,
were subjected to selective adsorption on aluminum oxide and elution with
the same technique as described above for the free sterols. One pass
through the column gave a total of fourteen fractions, one of which, weigh-
ing 0.441 gm. and containing the provitamin D acetate in a concentration
of 75 per cent, corresponded to a 44 per cent recovery of the provitamin D charged. This provitamin D acetate fraction melted over the range, 98–130°, indicating that, when purified, it probably would melt above this temperature. This finding is of interest since it shows that the acetate of the *Modiolus* provitamin D melts at a higher point than the provitamin D itself, while, for example, the acetate of 7-dehydrocholesterol melts considerably below the free 7-dehydrocholesterol. It should also be noted that the melting point of the acetate of the *Modiolus* provitamin D, even in impure form, is higher than the melting point of the acetate of 7-dehydrocholesterol (128–129°).

In order further to characterize the *Modiolus* provitamin D and to distinguish it more sharply from known compounds, we exposed an alcoholic solution of the sterol fraction (5.6 gm. containing 41 per cent provitamin D) to sunlight in the presence of eosin in a mixture of 90 per cent alcohol and 10 per cent benzene. As Windaus (14) has shown, under such conditions, the non-provitamin sterols remained unchanged, while the provitamin molecule was dehydrogenated with the formation of a sparingly soluble bimolecular compound. This was isolated and separated from the regular sterols since it precipitated from the solution. This bimolecular compound from the *Modiolus* provitamin D (m.p. 194–195°) was obtained in a yield of 40.2 per cent based on the spectroscopic evidence for the amount of provitamin initially used. In another experiment the corresponding bimolecular compound of 7-dehydrocholesterol was prepared (yield 50.4 per cent) and showed a melting point (194–195°) very similar to that of *Modiolus* provitamin D, but, when mixed, a depression of the melting point was obtained (184–185°), thus ruling out the identity of the *Modiolus* provitamin D with 7-dehydrocholesterol.

**DISCUSSION**

The mixed sterols of *Modiolus* were found to contain an unusually high concentration of provitamin D, which on irradiation yielded a highly effective vitamin D preparation. Our biological results and our preliminary chemical investigations showed that this provitamin was not identical with any of the provitamins which have been described. It is not excluded that the provitamin fraction is a mixture; in fact, the variation found in the biological efficacy of the vitamin D produced by the irradiation of sterols from *Modiolus* collected at different locations and at different times suggests this rather strongly. In the accompanying paper (12) it will be shown, however, that there is some evidence that a totally new provitamin sterol is involved.

In any event our results differ considerably from those obtained by Windaus and Bock and Wetter, who were able to identify either ergosterol or
7-dehydrocholesterol as the sole provitamin D occurring in those natural sources which they investigated. Our findings also show that the provitamin D from *M. demissus* (Dillwyn) is not identical with the provitamin D from *M. edulis*. This difference may be due to a different quantitative distribution of the same provitamin D mixture or may be caused by the presence of different individual provitamins D.

The concentration of provitamin D in invertebrates in general and in *Modiolus* specifically is so high that one may raise the question as to the function that these compounds perform in the metabolism. In this connection it may be significant also that we have found very little preformed vitamin D in the non-saponifiable fraction of *Modiolus*.

We wish to thank T. Parsons, Jr., S. G. Turnbull, Jr., E. L. Rohdenburg, and G. H. Kennedy for assistance in obtaining some of the data presented.

**SUMMARY**

1. The ribbed mussel, *Modiolus demissus* (Dillwyn), collected from the tide-waters of Virginia and North Carolina, has been found to contain unusually large amounts of provitamin D. The isolated total sterols from this mussel contain 35 to 50 per cent provitamin D measured spectrophotically.

2. This provitamin D on irradiation yielded a greater amount of vitamin D with a higher chick-rat ratio of efficacy than did the provitamin D from a related species, the edible mussel, *Mytilus edulis*, cultivated in Holland.

3. Preliminary chemical evidence together with the biological data indicates that the provitamin D from *M. demissus* (Dillwyn) is different from all known provitamins. The variation in biological response of vitamin D prepared from mussels collected at different times and at different localities suggests that the provitamin D present in the mussels is not a single compound but a mixture.

**BIBLIOGRAPHY**

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