Crystalline Vitamin B₁₂

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A crystalline compound which in microgram quantities has produced positive hematological responses in initial tests in patients with Addisonian pernicious anemia has been isolated from liver.

The effectiveness of liver as a specific agent in the dietary treatment of pernicious anemia was described by Minot and Murphy in 1926 (2). After this discovery, many investigations were carried out upon the purification of the principle (or principles) in liver responsible for its therapeutic effectiveness. One of the difficulties in the development of purification methods was the lack of a sufficient number of available pernicious anemia patients for the testing of fractions resulting from purification attempts. Although many efforts have been made to develop a biological assay, no method has been described which has been definitely successful in guiding the fractionation work.

### TABLE 1
**APPROXIMATE VITAMIN B₁₂ CONTENTS OF COMMERCIAL LIVER EXTRACTS FOR PARENTERAL USE**

<table>
<thead>
<tr>
<th>Source</th>
<th>Vitamin B₁₂ content (microbiological assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLD units/ml</td>
</tr>
<tr>
<td>Company A</td>
<td>72,000</td>
</tr>
<tr>
<td>Company B (Sample 1)</td>
<td>13,000</td>
</tr>
<tr>
<td>( &quot; 2)</td>
<td>19,000</td>
</tr>
<tr>
<td>Company C (Sample 1)</td>
<td>154,000</td>
</tr>
<tr>
<td>( &quot; 2)</td>
<td>80,000</td>
</tr>
<tr>
<td>Company D (Sample 1)</td>
<td>29,000</td>
</tr>
<tr>
<td>( &quot; 2)</td>
<td>39,000</td>
</tr>
</tbody>
</table>

Commercial liver preparations, which are crude concentrates of the active principle, have been available for medical use for some time. The activity of these preparations is standardized by clinical tests.

Research in these laboratories in 1942, together with collaborative clinical tests conducted by Randolph West, showed that further purification of the "anti-pernicious anemia" principle in commercial liver concentrates could be effected. Subsequently, these chemical and clinical studies were extended, and more recently Mary S. Shorb and George M. Briggs collaboratively tested certain clinically highly active fractions for growth activity for *Lactobacillus lactis* Dorner and found them to be microbiologically active. This microorganism was found by Dr. Shorb to require two unidentified growth factors (3); one of them (LLD factor) appeared to be related to the activity of commercial liver preparations used in the treatment of pernicious anemia. For convenience in the testing of the fractions, use was made of an arbitrarily selected standard liver concentrate which was assigned a potency of 1,000 LLD units/mg.

Further purification of clinically active liver fractions has led to the isolation, in minute amounts, of a crystalline compound which is highly active for the growth of *L. lactis*. This compound is being called vitamin B₁₂. The purity of the fraction is about 11,000,000 LLD units/mg, and 0.000013 μg/ml of culture medium is capable of supporting half-maximal growth under the conditions used. This assay value was found by Dr. Shorb (4), using a 23-hr growth period. The compound crystallizes in the form of small red needles which, after drying, showed refractive indices of α, 1.616; β, 1.652; and γ, 1.664. On the micro-stage, the crystals darken to black at about 210–220°, but do not liquefy below 300°.

Randolph West (5) has tested this crystalline compound for activity in the clinical treatment of pernicious anemia in relapse. In one patient a single intramuscular dose of 150 μg gave a very strong hematopoietic response; in two other patients doses of 3 and 6 μg, respectively, produced a prompt increase in the circulating reticuloocytes, red cells, and hemoglobin. These results are supported by early tests conducted by Dr. West, in which three separate concentrates, containing by microbiological assay 2–5 μg of vitamin B₁₂, gave strongly positive responses in four patients.

The biological activity of the new vitamin is extremely high in terms of its activity in these tests on pernicious anemia. For example, using pteroylglutamic acid, hematopoietic responses have been obtained with doses of the order of 20,000–50,000 μg during the first 10 days of treatment (6).

It was of interest to determine the relative contents of vitamin B₁₂ in several commercial liver extracts for parenteral use; the results of the assays are summarized in Table 1.

We are indebted to Dr. Shorb for many of the initial assays. We are also indebted to Miss Muriel Caswell and her colleagues of the Merck laboratories for microbiological assays.

The reasons for this designation are as follows: The long expression "anti-pernicious anemia principle" is not advisable, largely because the biological role of this new compound in the treatment of pernicious anemia or other disease is yet to be learned. The term "LLD factor" implies a biological limitation and was used originally to name an activity. The biological activities of crude materials are frequently found later to be due to several chemically related entities, as exemplified in the vitamin and antibiotic fields. A trivial name based on source or activity is undesirable. The name vitamin B₁₂ has not been used in the B series and connotes only nutritional significance. We may in the future wish to designate a name based upon chemical structure.

We wish to thank Charles Rosenblum and his associates for the determination of the optical properties.
One U.S.P. unit is defined (6) as that amount of liver extract required daily to produce satisfactory clinical and hematological response in addisonian pernicious anemia. If it is assumed that this new crystalline compound is the only substance present in these preparations which is therapeutically active, it is evident that clinical response should be obtained from the parenteral administration of approximately 1 µg of the new vitamin/day. The clinical responses obtained with single 3- and 6-µg doses of crystalline vitamin B₁₂ are not inconsistent with the approximate equivalence of 1 µg of the vitamin and 1 U.S.P. injectable unit. It should be pointed out, however, that it is customary to administer 20-60 U.S.P. units of liver extract during the first two or three days to start remission of pernicious anemia in relapse. This dose range is equivalent to not more than about 20-60 µg of vitamin B₁₂.

Further research is in progress on the composition, structure, and biological activity of vitamin B₁₂.

During this research, we have had the pleasure and benefit of the interest and advice of Henry D. Dakin.

References
4. SHORB, M. S. Science, 1948, 107, 397.

Activity of Vitamin B₁₂ for the Growth of Lactobacillus lactis

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A factor (LLD) required by Lactobacillus lactis Doerner was found (2) in refined liver extract in concentrations bearing an almost linear relationship to the unit potency of the extract used in the treatment of pernicious anemia. It was suggested that the LLD factor might be the therapeutically active principle in these extracts. A crystalline compound, vitamin B₁₂, has been isolated from liver (1) and has been shown to be highly active hematopoietically in initial tests upon cases of pernicious anemia (4).

Vitamin B₁₂ has been assayed for LLD activity by essentially the method reported previously (5). The growth-promoting effect of crystalline vitamin B₁₂ has been compared with that of a liver concentrate used as an arbitrary standard for the LLD factor assay. The standard was assigned a potency of 1,000 units/mg. On this basis, the potency of vitamin B₁₂ was found to be about 11,000,000 units/mg when a 23-hr growth period was employed and about 17,000,000 units/mg with a 42-hr growth period. These assay results illustrate the variation which may occur under varying test conditions. The conditions under which the culture is maintained may also affect the reproducibility of the assay values.

These results show that vitamin B₁₂ is either wholly or partially responsible for the LLD growth activity observed for liver extracts. It is conceivable that two or more closely related principles may be present which are responsible for the activity observed. The multiple chemical nature of certain vitamins is now well known. The minute amount of this compound required for growth places vitamin B₁₂ among the most potent microbiologically active compounds.

The liver concentrate standard is free from the other factor, TJ, required by L. lactis, at levels as high as 500 µg/tube. L. casei, L. fermenti, L. arabinosus, Streptococcus faecalis R, and Escherichia coli grow readily on the amino acid medium without supplementation with tomato juice. The addition of the liver concentrate, as a source of LLD factor, has little or no effect on their growth. The TJ factor(s) has been found to be synthesized by E. coli, L. arabinosus, L. casei, L. fermenti, and Str. faecalis R when they are grown on the amino acid medium.

Tests have been made for the presence of the LLD and TJ factor activities in those source materials and fractions derived therefrom which have been reported to contain unidentified factors for chicken nutrition (5). The LLD factor activity occurs in fairly high amounts, in approximately decreasing order, in a papain digest of acid precipitate from cow manure, the acid precipitate from cow manure, fish meal, pancreatin, papain, egg white, egg yolk, and in lower amounts in alcoholic extract of whey, potassium permanganate-oxidized alcoholic extract of whey, soybean oil meal, gelatin, zein and Mylase P. enzyme. The TJ factor activity is also found, in approximately decreasing order, in the papain digest of acid precipitate from cow manure, the acid precipitate of cow manure, egg yolk, papain, and pancreatin, but in much lower amounts in fish meal, alcoholic extract of whey, soybean oil meal, crude casein, egg white, zein, Mylase P. enzyme, potassium permanganate-oxidized alcoholic extract of whey, and gelatin. The distribution of

*This liver concentrate standard and the crystalline vitamin B₃ were supplied by Merck & Co., Inc., during collaborative studies.

The acid precipitate and the papain digest of the acid precipitate from cow manure were kindly supplied by H. R. Bird, Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Maryland.

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the LLD and TJ factor activities in these materials suggests that they may be involved in chicken nutrition.

References


Activity of Vitamin B₁₂ in Addisonian Pernicious Anemia

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Crystalline vitamin B₁₂ has shown positive hematological activity in three cases of addisonian pernicious anemia.

Collaborative clinical studies were begun in 1942 with chemical investigators in the Merck Research Laboratories on the further purification of liver concentrates which are effective in the treatment of pernicious anemia.

Results from the last case receiving amorphous material and the three receiving crystalline vitamin B₁₂ are summarized in Tables 1 and 2. One mg of crystalline vitamin B₁₂ is equivalent to about 11 × 10⁴ LLD units; 1 µg, to about 11,000 units.

Case 88-58-92 went home on the 45th day and has received no treatment aside from that recorded. Case 9178-48 had eaten some liver before entering the hospital and had a reticulocyte count of 8% a week before day 0, by which time the count had fallen to 3.8%.

It is evident that there has been a rise in reticulocytes, red cell count, and hemoglobin, but it is still too early to say whether the blood picture will return to normal without further treatment in the last three cases. The rise in white count in case 9007 is striking; the platelets have risen from 120,000 to 340,000 in case 9178-48.

Table 2

<table>
<thead>
<tr>
<th>Case</th>
<th>Kings County 5007</th>
<th>Bellevue 8900-48</th>
<th>Bellevue 91178-48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>RBC × 10⁴</td>
<td>Hgb (gm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 5 14 23</td>
<td>0 5 14</td>
<td>0 5 9</td>
</tr>
<tr>
<td>RBC</td>
<td>1.5 2.6 3.4</td>
<td>1.5 2.6</td>
<td></td>
</tr>
<tr>
<td>Hgb</td>
<td>4.5 7.0 9.0</td>
<td>7.8 10.0</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.5 2.0 0.5</td>
<td>2.8 3.1</td>
<td></td>
</tr>
<tr>
<td>Hot (%)</td>
<td>14.0 25.0 29.0</td>
<td>17.0 31</td>
<td></td>
</tr>
<tr>
<td>WBC x 10⁴</td>
<td>9.0 9.0 9.0</td>
<td>6.6 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg)</td>
<td>150</td>
<td>3.3 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>LLD units</td>
<td>1.6 × 10⁴</td>
<td>5.5 × 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

Recently, a correlation was found between clinical activity and the activity of concentrates for the growth of Lactobacillus lactis Dorner. Shorb (3) has reported a relationship of the unit potency of liver extracts used in the treatment of pernicious anemia to a factor (LLD) required by L. lactis. These researches led to the isolation from liver of a crystalline compound designated vitamin B₁₂ (2, 4), which is very potent for the growth of L. lactis (2, 4). Vitamin B₁₂ has produced a positive hematological response in three patients following single intramuscular injections of 2, 6, and 150 µg, respectively.

Four patients receiving single injections of impure amorphous concentrates containing 20,000-40,000 LLD units gave strong or maximal hematological responses, while three receiving 10,000 LLD units or less gave weak or negative responses.

It is of interest that Preparation #31 of Dakin, Ungley, and West (1), which was clinically positive in 1936, has been shown by the microbiological assay to contain about 30,000 LLD units (3 µg B₁₂)/(25 mg, the dose used at that time.

Our thanks are due to Paul Reznikoff, William Dook, Dickson W. Richards, Francis Blake, W. B. Castle, and E. H. Reisner, Jr., who have received material from this group for clinical trial.

References