Crystalline Vitamin B_{12}

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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 B_{12} CONTENTS OF COMMERCIAL LIVER EXTRACTS FOR PARENTERAL USE
(15 U.S.P. injectable units/ml)

Source	Vitamin B_{12} content (microbiological assay)			
	LLD units/ml	μg/ml	μg/U.S.P. unit	Per cent of dry weight
Company A	72,000	6.5	0.4	0.003
Company B				
(Sample 1)	13,000	1.2	0.1	0.00055
(" 2)	19,000	1.7	0.1	.....
Company C				
(Sample 1)	154,000	14.0	0.9	0.014
(" 2)	80,000	8.0	0.5	0.0065
Company D				
(Sample 1)	29,000	2.6	0.2	0.001
(" 2)	39,000	3.5	0.2	0.0014

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,[1] 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[2] 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[3] 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 B_{12}.[4] Its potency 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 potency 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.[5] 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 reticulocytes, 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 B_{12}, 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 (*1*).

It was of interest to determine the relative contents of vitamin B_{12} in several commercial liver extracts for parenteral use; the results of the assays are summarized in Table 1.

[1] Columbia University, College of Physicians and Surgeons.

[2] Department of Poultry Husbandry, Agricultural Experiment Station, University of Maryland. Dr. Briggs is now at the University of Minnesota.

[3] 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.

[4] 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 upon source or activity is undesirable. The name vitamin B_{12} 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.

[5] 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 responses 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,[6] 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 B_{12} 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 B_{12}.

Further research is in progress on the composition, structure, and biological activity of vitamin B_{12}.

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

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Activity of Vitamin B_{12} for the Growth of *Lactobacillus lactis*[1]

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A factor (LLD) required by *Lactobacillus lactis* Dorner was found (*3*) in refined liver extracts in concentrations bearing an almost linear relationship to the unit potency of the extracts 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 B_{12}, 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 B_{12} has been assayed for LLD activity by essentially the method reported previously (*3*). The growth-promoting effect of crystalline vitamin B_{12} has been compared with that of a liver concentrate used as an arbitrary standard[2] for the LLD factor assay. The standard was assigned a potency of 1,000 units/mg. On this basis, the potency of vitamin B_{12} 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 B_{12} 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 B_{12} 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 (*2*). The LLD factor activity occurs in fairly high amounts, in approximately decreasing order, in a papain digest of acid precipitate from cow manure,[3] the acid precipitate from cow manure,[3] 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

[1] Scientific Paper No. A198, Contribution No. 2113 of the Maryland Agricultural Experiment Station. This study was supported by a grant from Merck & Co., Inc., Rahway, New Jersey. The author is indebted to George M. Briggs for many valuable suggestions in connection with this study and to Margaret Karitas for technical assistance.

[2] This liver concentrate standard and the crystalline vitamin B_{12} were supplied by Merck & Co., Inc., during collaborative studies.

[3] 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.

[6] The commercial liver preparations listed in the table were found to contain the equivalent of from 0.7 to 10.6 μg/ml of pteroylglutamic acid, as measured by chicken pancreas conjugase digestion followed by *L. casei* assay, using pteroylglutamic acid as the standard.