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Vitamin B_{12} Activity of 5,6-Dimethylbenzimidazolylcobamide Coenzyme for the Chick. (26039)

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The isolation and properties of 5,6-dimethylbenzimidazolylcobamide coenzyme (DBC coenzyme), which is structurally similar to Vit. B_{12}, have been described(1,2). This coenzyme is required for conversion of glutamate to β-methylaspartate by cell-free extracts of *Clostridium tetanomorphum*(2), and also for isomerization of methylmalonyl-CoA to succinyl-CoA(3-5). The DBC coenzyme has activity similar to that of Vit. B_{12} for supporting growth of *Ochromonas malhamensis* and the Vit. B_{12}-requiring mutant of *Escherichia coli*(2). Since presence of the coenzyme has been demonstrated in rabbit liver, it is reasonable that this compound may also be a coenzyme form of Vit. B_{12} for higher animals. In the present study, activity of the DBC coenzyme was compared with that of Vit. B_{12} for supporting growth of young chicks. A second parameter used for assessing DBC coenzyme activity was suppression of formiminoglutamic acid (FGA) excretion by Vit. B_{12}-deficient chicks. This metabolite of histidine, which is excreted in negligible quantities by normal animals, was found to be excreted in large amounts in urine of Vit. B_{12}-deficient rats(6). The same finding has been observed in Vit. B_{12}-deficient chicks (unpublished data).

Methods. Day-old female New Hampshire chicks were distributed into groups of 8 chicks each and maintained for a period of 4 weeks in electrically heated batteries with screen wire floors. The chicks were weighed at weekly intervals. The diet was fed *ad libitum.*

Diet C62, which was used throughout this study, had the same composition as Diet C47 (7) except that 200 g of the glucose was replaced by an equal weight of hydrogenated vegetable oil (Crisco) in each kg of diet. Also, the Salts A used in Diet C47 was replaced by an equal quantity of Salts N(8). Vit. B_{12} was incorporated in this diet at levels shown in Table I.

Since the DBC coenzyme is destroyed by light, it could not be given in the diet. The chicks that received either Vit. B_{12} or the DBC coenzyme by injection received no Vit. B_{12} in the diet. In each case, aqueous solutions of supplements were injected intraperitoneally. The chicks fed a suboptimal level of Vit. B_{12}, 5 μg/kg of diet, served as the reference group in determining quantity of either Vit. B_{12} or DBC coenzyme to be injected. Food intake of this group was measured 3 times weekly and the groups receiving the 5 μg level of "supplement" by injection received exactly the same amount of Vit. B_{12} or DBC coenzyme as Vit. B_{12} eaten by the reference group. For convenience, in tables and text reference is made to weights of Vit. B_{12} and

TABLE I. Growth of Chicks Receiving Graded Levels of Vit. B_{12} and DBC Coenzyme.

Supplement (μg)*	Vit. B_{12} fed		Vit. B_{12} inj.		DBC coenz. inj.		Light-treat. DBC coenz. inj.	
	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)
0	6	188 ± 7.8	—	—	—	—	—	—
1	—	—	2	213 ±11.0	2	247 ±12.4	1	254 ±11.9
2.5	2	216 ±14.4	5	250 ± 9.0	5	250 ± 8.1	—	—
5	6	260 ± 8.3	6	278 ± 8.1	4	267 ±11.0	4	276 ± 7.2
100	6	306 ± 7.6	—	—	—	—	—	—

* Quantity of vit. B_{12} fed/kg of diet. Amount of vit. B_{12} inj. was based on food intake of chicks receiving 5 μg vit. B_{12}/kg diet. DBC coenzyme was inj. in amounts equimolar to indicated weights of vit. B_{12}. See *Methods* for details.

† 8 chicks/experimental group.

DBC coenzyme. This was strictly correct only for Vit. B_{12}; the indicated quantities of coenzyme were actually equimolar to Vit. B_{12}.

Crystalline DBC coenzyme, isolated from *Propionibacterium shermanii* ATCC 9614(2), was dissolved in water. Concentration of the coenzyme was calculated from its extinction coefficient at 522 mμ and 260 mμ(2). To avoid destruction by light, the DBC coenzyme solutions were stored in dark containers and all of the precautions described by Barker *et al.*(2) were observed. To provide an additional control, a solution of DBC coenzyme was inactivated by exposure for 1 hour to a 100 watt incandescent light bulb at a distance of 6 inches. The coenzyme solution was in an open beaker in an ice bath. Ultraviolet and visible absorption spectra following this treatment were similar to those of Vit. B_{12b} (aquocobalamin).

The chicks used for the FGA experiments were fed the Vit. B_{12}-free diet *ad libitum* throughout. One per cent L-histidine HCl was added to the diet to increase FGA excretion. The chicks received distilled water during collection periods. At 24 days of age the chicks were transferred from the battery to individual stainless steel cages provided with extra heat from light bulbs.

The excreta collected prior to 30 days of age served as base-line control values. Between 31 and 52 days of age some of the chicks in both experiments were injected daily with 1 μg Vit. B_{12} or an equimolar amount of the DBC coenzyme. In the second experiment *all* chicks were injected with 100 μg Vit. B_{12}/day between 53 and 59 days of age. Excretion of FGA was determined on each day's samples for several days preceding and following a change in supplementation; at other times 24 hour samples were analyzed at 2 to 3 day intervals.

The excreta were collected daily on sheets of polyethylene placed under the cages. These samples were stored at −10°C until time for assay. Each sample was suspended in distilled water in a Waring blendor, frozen (at least overnight), thawed, and centrifuged. The supernatant liquid was decolorized with 10 mg activated charcoal/ml (Darco KB) and filtered through Whatman No. 12 fluted filter paper. The pH of the extract was kept below pH 6 to avoid destruction of FGA. Concentration of FGA in these extracts was determined colorimetrically using the alkaline ferricyanide-nitroprusside reaction(9). The samples were read in an Evelyn colorimeter and a standard curve was included in each assay. A composite of extracts containing high amounts of FGA served as the standard. FGA content of the standard was determined microbiologically(10).

Results. The mean 4-week weights of chicks receiving Vit. B_{12} or DBC coenzyme are presented in Table I. Weights of chicks that received no Vit. B_{12} averaged 188 g, whereas those that received 100 μg Vit. B_{12}/kg of diet averaged 306 g. Addition of either 2.5 or 5 μg Vit. B_{12} to the diet resulted in intermediate weight gains. Vit. B_{12} was utilized to the same extent whether injected or fed. Similarly, the DBC coenzyme supported growth equal to that of Vit. B_{12}. The higher mean weight of chicks injected with the 1 μg level of DBC coenzyme as compared to that with Vit. B_{12}

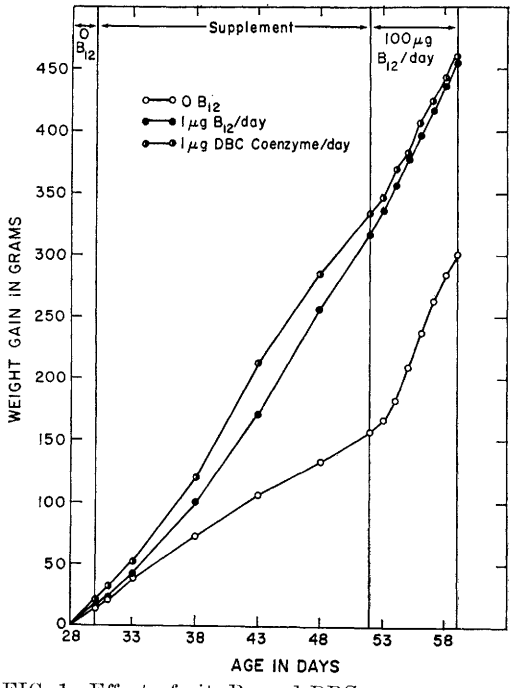

FIG. 1. Effect of vit. B_{12} and DBC coenzyme upon growth of 4-wk-old vit. B_{12}-deficient chicks (Exp. 2 of Table II).

was not statistically significant. Further, mean weights of chicks receiving 1 μg DBC coenzyme or 1 μg light-treated coenzyme were essentially the same. Therefore, the activities of Vit. B_{12} and DBC coenzyme are considered to be the same. These experiments offer no clue as to the physiologically active form of Vit. B_{12} in the chick, *i.e.*, Vit. B_{12} might be converted to the coenzyme form or the reverse might also occur.

From preliminary experiments, it was found that the high excretion rate of FGA in the Vit. B_{12}-deficient chick decreased somewhat slowly upon Vit. B_{12} supplementation. If the DBC coenzyme were the form of Vit. B_{12} active in producing a drop in FGA excretion, it seemed reasonable that the coenzyme might cause a more rapid and perhaps greater drop than was seen with Vit. B_{12}. The chicks in the early studies received 100 μg Vit. B_{12}/kg of diet and their daily food consumption was such that they received about 2 to 3 μg Vit. B_{12}/day. For the present experiments the level of supplementation was reduced to 1 μg/day in an effort to accentuate any possible difference between Vit. B_{12} and DBC coenzyme in their effect upon FGA excretion.

Growth rates of the chicks in the second FGA experiment are presented in Fig. 1. Chicks receiving Vit. B_{12} or DBC coenzyme grew at equal rates, as would be expected from the above growth studies with day-old chicks. Supplementation with 100 μg Vit. B_{12}/day markedly increased rate of gain of the Vit. B_{12}-deficient chicks; however, it did not further accelerate rate of gain of the previously supplemented chicks.

Data on FGA excretion are presented in Table II. All chicks at beginning of experiment were excreting large amounts of FGA, a little over 40 μmoles/24 hours/100 g body weight in the first experiment and somewhat higher amounts in the second experiment. This confirms in the chick the effects observed in Vit. B_{12}-deficient rats by Silverman and

TABLE II. Effect of Vit. B_{12} and DBC Coenzyme upon Excretion of Formiminoglutamic Acid by the Chick (μmoles FGA/Day/100 g Body Wt).

Group No.	Supplement* / Age of chicks, days	None 29–30	1 μg/day 31	32	33–34	51–52	100 μg/day 53	54	55–59
	Exp. 1								
1	None	44	38	40	32	36	—	—	—
2	B_{12}	44	46	20	14	14	—	—	—
3	DBC coenz.	42	21	10	14	16			
	Exp. 2								
1	None	82	52	63	55	74	27	26	18
2	B_{12}	127	76	46	37	22	16	15	14
3	DBC coenz.	74	47	31	30	10	5	4	5

* Vit. B_{12} deficient chicks were inj. daily with 1 μg vit. B_{12} or an equimolar quantity of DBC coenzyme (Groups 2 and 3) at age of 31-52 days. On the 53rd day every chick in all groups received 100 μg vit. B_{12}/day. Diet C62 was supplemented with 1% L-histidine HCl. Five chicks/exp. group.

Pitney(6). The unsupplemented chickens continued to excrete large amounts of FGA. Both Vit. B_{12} and the DBC coenzyme caused a marked reduction in FGA excretion, which was down to minimal levels by the end of 2 days. The daily 100 μg Vit. B_{12} supplement, which was given to *all* chicks in the second experiment, caused a very marked and immediate drop in FGA excretion of Vit. B_{12}-deficient chicks that had not been previously supplemented. This very high supplement of Vit. B_{12} also caused a further reduction of FGA excretion by the 2 previously supplemented groups. Thus, by both parameters, growth and reduction of FGA excretion, Vit. B_{12} and DBC coenzyme had similar activities.

Summary. Vit. B_{12} and the 5,6-dimethylbenzimidazolylcobamide coenzyme were equally active in (1) supporting growth of chicks up to 4 weeks of age and in (2) overcoming the impaired metabolism of formiminoglutamic acid that occurs in Vit. B_{12}-deficient chicks.

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Fragility and Extractable Collagen in the Lathyritic Chick Embryo. An Assay for Lathyrogenic Agents.* (26040)

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The lathyrogenic substances, β-aminopropionitrile and semicarbazide, when introduced into the chick embryo induce a loosening of the intermolecular structure within collagen fibrils allowing them to dissolve in cold neutral saline(1). This intrafibrillar alteration is associated with marked increase in fragility of the entire organism. Both phenomena are demonstrable between one and 3 hours following a single injection and increase steadily for at least 72 hours. Both were also shown to be dosage dependent. Analytical studies(1) plus histologic and electron microscope examination of thin sections of normal and lathyritic skin before and after extraction(2) led to the conclusion that insoluble fibrils, formed prior to administration of the agent, were rendered soluble in cold saline. Skin, bone, aorta and tendon were all involved.

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