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REFERENCES

- Barnes, J. M. & Magee, P. N. (1954). *Brit. J. industr. Med.* **11**, 167.
- Birnie, J. H. & Grayson, J. (1952). *J. Physiol.* **116**, 189.
- Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C. & Udenfriend, S. (1955). *Science*, **121**, 603.
- Bruce, H. M. (1947). *J. Hyg., Camb.*, **45**, 169.
- Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 202.
- Cheng, K. K. (1951). *Brit. J. exp. Path.* **32**, 444.
- Cook, L., Toner, J. J. & Fellows, E. J. (1954). *J. Pharmacol.* **111**, 131.
- Dubois, K. P., Doull, J. & Coon, J. M. (1950). *J. Pharmacol.* **99**, 376.
- Dutton, A. H. & Heath, D. F. (1956). *Nature, Lond.*, **178**, 644.
- Heath, D. F. & Jarvis, J. A. E. (1955). *Analyst*, **80**, 613.
- Magee, P. N. & Barnes, J. M. (1956). *Brit. J. Cancer*, **10**, 114.
- Miller, J. A. & Miller, E. C. (1953). *Advanc. Cancer Res.* **1**, pp. 339-396.
- Myers, D. K., Mendel, B., Gersmann, H. R. & Ketelaar, J. A. A. (1952). *Nature, Lond.*, **170**, 805.
- Nygaard, O., Eldjarn, L. & Nakken, K. F. (1954). *Cancer Res.* **14**, 625.
- Peters, R. A. (1936). *Lancet*, **1**, 1161.
- Peters, R. A. (1952). *Proc. Roy. Soc. B*, **139**, 143.
- Popper, H., de la Hueraga, J. & Koch-Weser, D. (1954). *Ann. N.Y. Acad. Sci.* **57**, 936.
- Soberman, R. J. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 789.

The Activity for Chicks of some Vitamin B₁₂-like Compounds

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In the last few years several compounds similar to the cobalamins have been found in nature; in addition to these, new analogues of vitamin B₁₂ have been produced by guiding the biosynthetic activities of certain micro-organisms. The preparation, chemical nature and properties of these substances have been reviewed by Kon (1955); they differ from the cobalamins in the nature of the nucleotide part of the molecule. Since many of them have growth-promoting activity for micro-organisms, it was of interest to determine whether or not they were of use to higher animals. The comparatively large amount of material necessary for an animal test limited the number of samples examined to those which could be relatively easily prepared, but we have been able to test on chicks five of the benziminazole series, three purine derivatives, factor B (which has no nucleotide) and factor D (of which the chemical constitution is unknown).

EXPERIMENTAL

Substances tested

Naturally occurring vitamin B₁₂-like compounds. Factors A, B, D and pseudovitamin B₁₂ were prepared from calf faeces. Vitamin B₁₂ III, later identified as the 5-hydroxy-benziminazole analogue of vitamin B₁₂ (Robinson, Miller, McPherson & Folkers, 1955; Friedrich & Bernhauer, 1956), was isolated from sewage by Friedrich & Bernhauer (1953).

Unnatural analogues of vitamin B₁₂. These were prepared by 'shunting' the biosynthesis of vitamin B₁₂ by the addition

to the medium of the appropriate intermediate (Fantes & O'Callaghan, 1954; Ford, Holdsworth & Kon, 1954). The samples tested were the analogues containing benziminazole, 5 (or 6)-methylbenziminazole, 5:6-dichlorobenziminazole, 2:3-naphthiminazole or 2:6-diaminopurine in the nucleotide.

Preparation for test. All these substances, with the exception of factor D, were purified by repeated ionophoresis and chromatography and contained less than 0.5% of vitamin B₁₂. Factor D and some early samples of factor A were purified by chromatography only. Solutions of the analogues were prepared and standardized by measuring the absorption at 361 mμ; the $E_{1\%}^{1\text{cm}}$ value was assumed to be the same as for cyanocobalamin.

Biological tests

Method and diet. In general, the method of Coates, Harrison & Kon (1951) was followed. Rhode Island Red × Light Sussex chicks were used, bred from hens given a diet low in vitamin B₁₂. Each experimental group consisted of about 10-15 chicks of mixed sexes. The test materials were given either by intramuscular injection twice weekly or were added to the basal diet, which had the following percentage composition: ground maize, 37.8; ground barley, 20; defatted soya grits, 35; dried grass, 3; bone meal, 1.5; limestone flour, 1; arachis oil, 1; salt mixture, 0.7 (NaCl, 0.672 part; MnSO₄·4H₂O, 0.028 part). To each 100 g. of diet were added vitamin A (680 i.u.) and vitamin D₃ (64 i.u.) dissolved in the arachis oil, and a vitamin supplement consisting of riboflavin (0.77 mg.), nicotinic acid (5.5 mg.), biotin (0.02 mg.), folic acid (0.08 mg.), thiamine hydrochloride (0.33 mg.), pyridoxine hydrochloride (0.44 mg.) and calcium pantothenate (1.65 mg.).

Exploratory tests. Each sample was submitted to a preliminary test to give some indication of its potency. One group of birds received the basal diet alone and a second was given the test substance at a level of between 5 and 20 µg./100 g. of diet, according to the amount of material available. The growth response over 4 weeks was compared with that of a similar group of chicks receiving 0.5 or 1 µg. of cyanocobalamin/100 g. of diet. A fourth group, given both cyanocobalamin and the test substance, was included in order to detect any possible synergism or antagonism between the two substances. At the end of the test the birds were killed and their livers were removed for chromatographic examination (see below).

Assays of potency. Any substance showing vitamin B₁₂ activity in the exploratory test was submitted to a full-scale biological assay. The method was similar to that of Coates *et al.* (1951) except that the chicks were kept for the first 7 days on the basal diet and their growth during this period was noted. Birds that grew abnormally fast or slowly were culled and only those that grew at a medium rate were distributed in the test groups. This preliminary culling has been found to reduce appreciably the error of the assays. Three or four graded doses of the test material were compared with three or four graded doses of cyanocobalamin, and the test period lasted 3 weeks. The final weight of the chicks was used in the calculations of potency and true fiducial limits, which were made as recommended by British Standards Institution (1940).

Preparation of liver extracts

The pooled livers from a group of chicks were homogenized with an equal weight of water. Of this homogenate 20 ml. was poured into 150 ml. of boiling 0.29M acetate buffer (pH 4.8) containing 0.01% of KCN, and the liquid was filtered. The filtrate was treated with two 2 g. portions of decolorizing charcoal (Norite, British Drug Houses Ltd.), stirred for 30 min. at 60° and centrifuged. The factors adsorbed on the charcoal were eluted by refluxing twice with 50 ml. portions of aqueous acetone (2 parts of acetone: 1 part of water containing 0.03% of KCN). The acetone extract was concentrated under reduced pressure to 2 ml.

Chromatography and ionophoresis of liver extracts. Portions (0.01 ml.) of the liver extracts were examined by chromatography (Holdsworth, in preparation) and by ionophoresis in 2N acetic acid (Holdsworth, 1953). The position of the factors on the chromatogram was revealed by a bioautographic technique with *Bacterium coli* 113-3 (Ford & Holdsworth, 1952).

RESULTS

Naturally occurring vitamin B₁₂-like factors

5-Hydroxybenziminazole analogue (vitamin B₁₂ III). Of the naturally-occurring vitamin B₁₂-like substances only the 5-hydroxybenziminazole analogue showed any activity for the chick. Its potency is given in Table 1.

Factor A. Factor A was tested several times by mouth and by injection. Occasionally, very slight activity of the order of 1% or less was detected, but it is possible that it was due to the presence of active impurities, particularly in the early samples that had not been purified by electrophoresis.

Pseudovitamin B₁₂. The mean weights of birds at 3 or 4 weeks of age in three tests on pseudovitamin B₁₂, given by mouth, are shown in Table 2. Pseudovitamin B₁₂ given alone depressed growth significantly below that of the negative controls on one of three occasions. Also, it reduced significantly the growth-promoting effect of vitamin B₁₂ when given simultaneously with it at the ratio of 100:1. No such antagonism was observed when the two substances were given by injection.

Factor B. In several tests of factor B a similar antagonism to vitamin B₁₂ was observed when the two were given by mouth but not when they were injected.

Factor D. Factor D reduced the effect of vitamin B₁₂ by mouth, and by injection on the one occasion on which it was tested; shortage of material prevented further investigation.

Summary of results. The results of all tests on the naturally occurring vitamin B₁₂-like substances are summarized in Table 1.

Unnatural analogues of vitamin B₁₂

Results of assays of unnatural analogues of vitamin B₁₂ are given in Table 3. Of those tested, all the benziminazole derivatives proved active, but the only purine derivative tested, that of diamino-purine, showed no activity.

Table 1. *Growth activity for chicks of the naturally occurring vitamin B₁₂-like factors*

Substance tested	Nucleotide base	Activity, expressed where possible as a percentage of that of vitamin B ₁₂	
		By mouth	By injection
Factor A	2-Methyladenine	<1	<1
Factor B	None	Antagonism to vitamin B ₁₂ at 40 × dose	Inactive
Factor D	Not known	Antagonism to vitamin B ₁₂ at 10 × dose	Antagonism to vitamin B ₁₂ at 10 × dose
Pseudovitamin B ₁₂	Adenine	Antagonism to vitamin B ₁₂ at 100 × dose	Inactive
Vitamin B ₁₂ III	5-Hydroxybenziminazole	3.9 (1.8 & 7.5)*	5.1 (1.4 & 9.7)*

* True fiducial limits at *P* = 0.95.

Chromatography of liver extracts

It was clear from chromatograms of liver extracts that the substances tested reached the liver. Chromatograms of extracts from livers of birds that received the benziminazole, the 5 (or 6)-methylbenziminazole, the 5:6-dichlorobenziminazole or the naphthiminazole analogues showed the presence of the corresponding compounds that were all active for chicks. There was little or no change in the

amounts of vitamin B₁₂ in the livers, suggesting that the compounds were active *per se* and not through conversion into vitamin B₁₂. A typical chromatogram from a test on one of the active analogues (that with benziminazole) is shown in Fig. 1a.

When pseudovitamin B₁₂ or factor B was given simultaneously with vitamin B₁₂ by mouth, less vitamin B₁₂ was detectable in chromatograms of the liver extracts than when the same dose of vitamin B₁₂ was given alone. An example of this type of

Table 2. *Mean weights of chicks given (by mouth) vitamin B₁₂, pseudovitamin B₁₂, or both*

Expt. no.	No. of birds/group		Vitamin B ₁₂ (μg./100 g. of diet)	Pseudo-vitamin B ₁₂ (μg./100 g. of diet)	Initial values		Final values		s.e. of mean	Least difference (g.) needed for significance at <i>P</i> = 0.05†
	Initial*	Final*			Age (days)	Wt. (g.)	Age (weeks)	Wt. (g.)		
1	13	9	0	0	1	41	3	113	6.7	20
	13	8	0	5	1	41	3	92	7.1	
	13	13	1	0	1	41	3	203	5.6	
2	8	8	0	0	1	42	4	140	9.8	29
	8	8	0	5	1	40	4	131	9.8	
	8	7	0.5	0	1	41	4	232	11.0	31
	8	7	0.5	5	1	40	4	221	11.0	
3	10	10	0	0	7	53	4	104	10.2	29
	10	10	0	100	7	53	4	119	10.2	
	10	10	1	0	7	54	4	221	10.2	29
	10	10	1	100	7	53	4	150	10.2	

* The differences in numbers of birds are due to deaths during the experiment. Statistical analyses were done on numbers of birds remaining at the end of the experiment.

† In comparisons within bracketed pairs.

Table 3. *Results of chick assays of unnatural analogues of vitamin B₁₂*

Analogue tested	Route of adminis- tration	No. of groups		No. of birds/ group	Activity expressed as a percentage of that of vitamin B ₁₂			
		Standard	Test		Value	True fiducial	Pooled	Limits of error at P = 0.95
						limits at P = 0.95		
Benziminazole	Mouth	4	3	11	27	10, 68	23	17, 32
		4	4	10	21	12, 33		
		4	4	10	25	13, 43		
	Injection	3	3	11	23	15, 33	—	—
	5 (or 6)-Methylbenzimin- azole	Mouth	3	3	11	35	22, 52	—
Injection		3	3	11	41	25, 64	—	—
5:6-Dichlorobenziminazole	Mouth	3	2	11	49	27, 121	75	56, 101
		3	2	13	82	50, 146		
		3	3	12	92	50, 163		
	Injection	3	3	11	160	90, 535	116	86, 155
		3	2	13	95	61, 168		
		3	3	12	114	75, 179		
2:3-Naphthiminazole	Mouth	3	2	10	62	17, 149	85	58, 123
		3	3	13	96	58, 156		
	Injection	3	3	12	97	70, 132	—	—
2:6-Diaminopurine	Mouth	1	1	15	No activity or antagonism			
	Injection	1	1	15	No activity or antagonism			

chromatogram (pseudovitamin B₁₂) is shown in Fig. 1b. Factor B differed slightly from pseudovitamin B₁₂ in that, although it appeared to decrease absorption of vitamin B₁₂, only traces of factor B itself were deposited in the liver.

DISCUSSION

Only relatively few of the possible analogues of vitamin B₁₂ have been tested on chicks and it is therefore not permissible to draw any general conclusions on the relationship between chemical constitution and biological activity. However, within the limits of these experiments certain facts emerge. Of the ten analogues tested, five showed vitamin B₁₂ activity and each of these contained benziminazole in the nucleotide. The benziminazole analogue has been reported by Briggs & Spivey Fox (1955) to have 9% of the activity of vitamin B₁₂, but our own result is somewhat higher (23%). Introduction of one methyl group into the 5 (or 6) position or of two into the 5 and 6 positions of the benziminazole nucleotide gave a progressive increase of vitamin

activity, vitamin B₁₂ itself being the dimethylbenziminazole derivative. Similarly, chlorine groups in the 5 and 6 positions increased activity so that the dichlorobenziminazole analogue was almost as active as vitamin B₁₂ when both were given by mouth, and even slightly more active than vitamin B₁₂ when both were given by injection. The size of the group introduced at positions 5 and 6 seems to bear no relation to biological activity, since addition of another aromatic ring, giving the naphthiminazole compound, also increased the activity nearly to that of vitamin B₁₂. The only member of the series to show less activity than the benziminazole analogue was the 5-hydroxy derivative (vitamin B₁₂ III). Since all the active analogues appeared in quantity in the liver and did not apparently increase liver stores of vitamin B₁₂, it is likely that they had vitamin B₁₂ activity *per se* and did not become converted into vitamin B₁₂ in the tissues. The higher activity of both the dichlorobenziminazole and naphthiminazole analogues when given by injection than when given by mouth, although not quite significant by accepted statistical standards

Fig. 1. Bio-autographs of extracts of livers from chicks given (by mouth) analogues of vitamin B₁₂ with and without vitamin B₁₂. (a) Benziminazole analogue; (b) pseudovitamin B₁₂.

suggests that they might be less efficiently absorbed from the gut than is vitamin B₁₂.

Most of the naturally occurring vitamin B₁₂-like factors contain purine nucleotides; two of them, factor A and pseudovitamin B₁₂, constitute about 70 % of the vitamin B₁₂-like substances in rumen contents and faeces (Ford, Kon & Porter, 1952). Neither was active for the chick, and in fact, when given by mouth, pseudovitamin B₁₂ appeared to compete with vitamin B₁₂ in the absorption process. No such antagonistic effect was observed with factor A (the 2-methyladenine analogue). It seemed possible that the antagonism was connected with the presence of the amino group and was mitigated by the methyl group in the 2 position. In such event the introduction of a second amino group might increase antagonism, especially since 2:6-diaminopurine is a known antagonist of nucleic acid metabolism. However, the diaminopurine analogue proved to have neither antagonism nor activity. Since neither pseudovitamin B₁₂ nor factor B was antagonistic to vitamin B₁₂ by injection it would seem that neither is a true antimetabolite to that vitamin. It is more likely that when given in sufficient quantity they interfere with the uptake of vitamin B₁₂, possibly by competing for 'intrinsic factor' or some other essential component in the mechanism of absorption of vitamin B₁₂ from the gut. This hypothesis is supported by our finding less liver storage of vitamin B₁₂ in the birds given pseudovitamin B₁₂ or factor B. In contrast to these two factors, factor D was antagonistic to vitamin B₁₂ by mouth and by injection, but as there was only sufficient for one trial and no liver chromatograms were made it would be unwise to place much reliance on this one result alone.

Although vitamin B₁₂ deficiency in the chick is not accompanied by any haematological change and has little in common with human pernicious anaemia, a marked similarity can be observed between the results of chick-growth tests and the response of patients with pernicious anaemia. For instance, factors A and B and pseudovitamin B₁₂ are ineffective in pernicious anaemia (Bernhauer & Friedrich, 1954) and are similarly inactive for chicks. (We understand from Dr J. N. Marshall Chalmers that in tests with factor A, believed to be a pure sample, he obtained a small response in pernicious anaemia. This finding would agree with the occasional slight growth activity of factor A for chicks.)

Of the analogues active for chick growth, four have been clinically tested and all produced a haematological response in pernicious anaemia. These were the 5-hydroxybenziminazole compound (Bernhauer, Blumberger & Petrides, 1955; Brown, Cain, Gant, Parker & Smith, 1955), the benziminazole derivative (Fantes & O'Callaghan, 1954, 1955)

and the dichlorobenziminazole and naphthiminazole derivatives (Fantes & O'Callaghan, 1956). If some of the biochemical reactions in which vitamin B₁₂ is involved are common to haematopoiesis in man and to growth in young animals, the chick assay may be a useful indication of the possible activity in pernicious anaemia of a vitamin B₁₂-like substance.

SUMMARY

1. Ten analogues of vitamin B₁₂ have been tested for vitamin B₁₂ activity in chick growth.

2. The only naturally occurring analogue to show activity was the 5-hydroxybenziminazole derivative (vitamin B_{12III}), which had 4-5 % of the activity of vitamin B₁₂.

3. Four analogues containing benziminazole in the nucleotide also had vitamin B₁₂ activity; they were the analogues with benziminazole (23 %), monomethylbenziminazole (35-41 %), dichlorobenziminazole and naphthiminazole (the last two almost as active as vitamin B₁₂ itself).

4. Pseudovitamin B₁₂ and factor B in large doses by mouth, but not by injection, reduced the effect of vitamin B₁₂ given simultaneously. In a single test factor D showed similar antagonism when given by mouth or by injection.

5. Factor A and the diaminopurine derivative were inactive, but not antagonistic to vitamin B₁₂.

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REFERENCES

- Bernhauer, K., Blumberger, K. & Petrides, P. (1955). *Arzneim. Forsch.* **5**, 442.
- Bernhauer, K. & Friedrich, W. (1954). *Angew. Chem.* **66**, 776.
- Briggs, G. M. & Spivey Fox, M. R. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 318.
- British Standards Institution (1940). *Specification no. 911*.
- Brown, F. B., Cain, J. C., Gant, D. E., Parker, L. F. J. & Smith, E. L. (1955). *Biochem. J.* **59**, 82.
- Coates, M. E., Harrison, G. F. & Kon, S. K. (1951). *Analyst*, **76**, 146.
- Fantes, K. H. & O'Callaghan, C. H. (1954). *Biochem. J.* **58**, xxi.
- Fantes, K. H. & O'Callaghan, C. H. (1955). *Biochem. J.* **59**, 79.
- Fantes, K. H. & O'Callaghan, C. H. (1956). *Biochem. J.* **63**, 10p.
- Ford, J. E. & Holdsworth, E. S. (1952). *Biochem. J.* **53**, xxii.
- Ford, J. E., Holdsworth, E. S. & Kon, S. K. (1954). *Biochem. J.* **58**, xxiv.
- Ford, J. E., Kon, S. K. & Porter, J. W. G. (1952). *Chem. & Ind.* p. 495.
- Friedrich, W. & Bernhauer, K. (1953). *Angew. Chem.* **65**, 627.
- Friedrich, W. & Bernhauer, K. (1956). *Z. Naturf. B*, **11**, 68.
- Holdsworth, E. S. (1953). *Nature, Lond.*, **171**, 148.
- Kon, S. K. (1955). *Symp. biochem. Soc.* no. 13, 17.
- Robinson, F. M., Miller, I. M., McPherson, J. F. & Folkers, K. (1955). *J. Amer. chem. Soc.* **77**, 5192.