number of 38 000 was calculated for the purest preparation.

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REFERENCES

- Abderhalden, E. & Weber, L. E. (1910). *Ber. dtsh. chem. Ges.* **43**, 2431.
- Astrup, T. & Permin, P. M. (1947). *Nature, Lond.*, **159**, 681.
- Bergman, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
- Bergman, M., Zervas, L., Salzmann, L. & Schleich, H. (1934). *Hoppe-Seyl. Z.* **224**, 17.
- Boman, H. G. (1954). *Nature, Lond.*, **173**, 447.
- Fischer, E. (1901). *Ber. dtsh. chem. Ges.* **34**, 433.
- Fischer, E. (1903). *Ber. dtsh. chem. Ges.* **36**, 2983.
- Fischer, E. (1904). *Ber. dtsh. chem. Ges.* **37**, 2494.
- Fischer, E. (1906). *Ber. dtsh. chem. Ges.* **39**, 2931.
- Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
- Franzen, H. (1909). *Ber. dtsh. chem. Ges.* **42**, 2466.
- Grassmann, W. & Heyde, W. (1929). *Hoppe-Seyl. Z.* **183**, 32.
- Gutman, A. B. & Gutman, E. B. (1941). *Endocrinology*, **28**, 115.
- Huggins, C. B. & Neal, W. (1942). *J. exp. Med.* **76**, 527.
- Jacques, L. B. (1943). *Biochem. J.* **37**, 344.
- Kaufman, S. & Neurath, H. (1949). *J. biol. Chem.* **181**, 623.
- Kaufman, S., Neurath, H. & Schwert, G. W. (1949). *J. biol. Chem.* **177**, 793.
- Kaulla, K. N. von & Shettles, L. B. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 692.
- King, E. J. & Delory, G. E. (1939). *Biochem. J.* **33**, 1185.
- Kunkel, H. G. & Slater, R. J. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 42.
- Kunkel, H. G. & Tiselius, A. (1952). *J. gen. Physiol.* **35**, 89.
- Lassen, M. (1952). *Acta physiol. scand.* **27**, 371.
- Linderstrøm-Lang, K. (1927). *C.R. Lab. Carlsberg*, **17**, no. 4.
- Linderstrøm-Lang, K. & Holter, H. (1931). *C.R. Lab. Carlsberg*, **19**, no. 4.
- London, M. & Hudson, P. B. (1953). *Arch. Biochem. Biophys.* **46**, 141.
- Lundquist, F. (1952). *Acta physiol. scand.* **25**, 178.
- Lundquist, F. (1953). *Mammalian Germ Cells* (Ciba Symposium), p. 71. London: Churchill.
- Lundquist, F. & Buus, O. (1952). *Acta physiol. scand.* **25**, (Suppl. 89), 58.
- Lundquist, F. & Seedorff, H. H. (1952). *Nature, Lond.*, **170**, 1115.
- MacIntire, W. H., Palmer, G. & Marshall, H. L. (1945). *Industr. Engng Chem.* **37**, 164.
- Northrop, J. H., Kunitz, M. & Herriott, R. (1948). *Crystalline Enzymes*. New York: Columbia University Press.
- Oettlé, A. G. (1950). *Proc. Soc. Stud. Fertil.* **2**, 71.
- Parks, R. E. & Plaut, G. W. E. (1953). *J. biol. Chem.* **203**, 755.
- Permin, P. M. (1950). *Acta physiol. scand.* **20**, 388.
- Roche, J. & Bouchilloux, S. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 567.
- Schott, H. F., Larkin, J. B., Rockland, L. B. & Dunn, M. S. (1947). *J. org. Chem.* **12**, 490.
- Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.
- Sherry, S. & Troll, W. (1954). *J. biol. Chem.* **208**, 95.
- Smith, E. L. (1951). *Advanc. Enzymol.* **12**, 190.
- Smith, E. L. & Slonim, N. B. (1948). *J. biol. Chem.* **176**, 835.
- Swingle, S. M. & Tiselius, A. (1951). *Biochem. J.* **48**, 171.
- Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.

The Effect of *o*-Phenylenediamine on the Biosynthesis of Vitamin B₁₂: A New Vitamin B₁₂ Analogue

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By adding *o*-phenylenediamine or any one of a number of related substances to a synthetic medium, Dulaney & Williams (1953) claimed to have doubled or trebled vitamin B₁₂ production by *Streptomyces griseus* and some other species, as judged by the *Escherichia coli* (mutant) assay. 1:2-Diamino-4:5-dimethylbenzene, a part of the vitamin B₁₂ molecule and a postulated precursor of it (Woolley, 1950, 1951), had no such effect. These authors thought it unlikely that increased vitamin

B₁₂ was produced by incorporating phenylenediamine into the molecule. Tracer studies with an unstated level of deuterio phenylenediamine showed that not more than 20% of the vitamin was labelled. It was shown that phenylenediamine inhibited the growth of *Strep. griseus* and it was suggested that a compound antagonistic to phenylenediamine, namely vitamin B₁₂, was produced in increased amounts so as to overcome this inhibition.

We thought that the apparent increased vitamin B₁₂ titre might be caused by a different mechanism and therefore re-investigated the problem. A preliminary account of this work has already been published (Fantes & O'Callaghan, 1954).

METHODS

Medium. The synthetic medium used was the same as that described by Dulaney & Williams (1953). It was autoclaved at 15 lb./sq.in. steam pressure for 20 min. Phenylenediamine (autoclaved separately as the hydrochloride) was added to the medium just before inoculation. Varying concentrations of phenylenediamine were prepared in such a way that the addition of the same volume (1 ml.) produced the desired level of phenylenediamine in the medium.

Growth conditions. The strain of *Strep. griseus* used was derived from the same parent strain as the one used by Dulaney & Williams, but was not identical with it. The preparation of the vegetative inoculum and the fermentations were again done essentially as described by the American authors, except that 40 ml. medium were used/250 ml. flask. Instead of autoclaving the diluted samples before assaying, they were heated in a boiling-water bath for 10 min.

Microbiological assay. Two types of microbiological assay were employed: an *Esch. coli* plate assay (Harrison, Lees & Wood, 1951), but with the medium devised by Burkholder (1951), and a *Lactobacillus leichmannii* plate assay.

Chromatography and bioautographs. For the chromatographic analysis equal volumes (about 10 µl.) of the heated samples were applied to Whatman no. 4 sheets. Development was downward with butanol nearly saturated with water (98 parts of saturated and 2 parts of dry solvent). The bottom of the tank was covered with the same solvent mixture and the inside lined with gauze, which dipped into the liquid covering the bottom of the tank. A small beaker with aqueous KCN was also kept in the tank to prevent the spontaneous conversion of the cyano into the hydroxo compounds. Development was carried out at room temperature for 24–48 hr.

Bioautographs were prepared by a technique similar to the one first described by Cuthbertson & Lester Smith (1949), but making use of the improvements introduced by Ford & Holdsworth (1953). For a quantitative assessment of relative zone sizes the plates were photographed after

incubation; the white zones were then cut out from the photographs and weighed. Three or four levels of pure vitamin B₁₂ were chromatographed on the same sheet, when quantitative estimations were desired. It was found that the zone weight was proportional to the logarithm of the amount of vitamin applied to the paper. Graphs were constructed and the amount of vitamin B₁₂ in experimental samples was ascertained by means of them. (The weighing of zone photographs as a means of estimating zone size was suggested to us by Mr G. C. Ashton of these laboratories.)

RESULTS

A typical culture containing 40–480 µg. *o*-phenylenediamine base/ml. of medium was assayed on the fourth, fifth and sixth day by the *Esch. coli* and *Lb. leichmannii* plate assays. The results obtained on the fourth day are shown in Table 1; the results obtained on the subsequent days were similar.

The table shows clearly that certain concentrations of phenylenediamine in the medium produced titres far in excess of the controls, although higher concentrations of phenylenediamine considerably inhibited growth. In another experiment the contents of some of the flasks and four levels of vitamin B₁₂ were subjected to chromatographic analysis and bioautography with both test organisms. All samples containing phenylenediamine showed the presence of a new substance with an *R_F* value just below that of vitamin B₁₂. The zone size of this new growth factor increased with increasing levels of phenylenediamine, the zones due to vitamin B₁₂ decreasing simultaneously. When the weights (78.0, 63.6, 54.2, 42.0 mg.) of the zones due to the four vitamin B₁₂ standards were plotted against the logarithms of their concentrations (0.4, 0.2, 0.1, 0.05 µg./ml.) a straight line was obtained. From the vitamin B₁₂ zone weights of the samples their true vitamin B₁₂ content could then be deduced from the graph. The results are shown in Table 2.

Although the presence of phenylenediamine in the medium gave rise to increased 'vitamin B₁₂' titres, biosynthesis of true vitamin B₁₂ was depressed.

Table 1. *The effect of o-phenylenediamine on vitamin B₁₂ titre of Streptomyces griseus fermentation liquor (day 4)*

Each figure represents the average assay value obtained from three individual shake flasks.

Concentration of <i>o</i> -phenylenediamine (µg./ml. medium)	0	40	80	160	240	320	480
µg. apparent vitamin B ₁₂ /ml. (<i>Esch. coli</i>)	0.22	0.41	0.50	0.55	0.52	0.39	0.29
µg. apparent vitamin B ₁₂ /ml. (<i>Lb. leichmannii</i>)	0.22	0.40	0.42	0.37	0.37	0.32	0.25

Table 2. *Comparison of vitamin B₁₂ content of Streptomyces griseus fermentation liquor obtained by direct assay and calculated from chromatographic zone size*

Concentration of <i>o</i> -phenylenediamine (µg./ml. medium) ...	0	80	160	320
Apparent vitamin B ₁₂ content (µg./ml., <i>Esch. coli</i> assay)	0.36	0.48	0.79	0.55
True vitamin B ₁₂ content (µg./ml., obtained by chromatography and <i>Esch. coli</i> bioautography)	0.36	0.069	0.050	0.033

To find out if the formation of the new growth factor could be suppressed by the inclusion in the medium of 1:2-diamino-4:5-dimethylbenzene, fermentations were carried out with a level of 80 μ g. phenylenediamine/ml. and various concentrations of diaminodimethylbenzene, the lowest being 2 μ g./ml. Bioautographs showed the complete absence of the new factor in these experiments. In order to determine the 'inhibition index' a further experiment was carried out with 40 μ g. phenylenediamine/ml. and 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ μ g. diaminodimethylbenzene/ml. Only vitamin B₁₂ was formed in the presence of 2, 1, $\frac{1}{2}$, $\frac{1}{4}$ μ g. diaminodimethylbenzene/ml. With $\frac{1}{8}$ μ g. diaminodimethylbenzene/ml. a small amount of the new material was produced; $\frac{1}{16}$ μ g. diaminodimethylbenzene/ml. gave zones similar to a control containing only 40 μ g. phenylenediamine/ml. The inhibition index in this system therefore lies somewhere between 160 and 320.

Isolation of new factor

As isolation of the new substance from the synthetic medium was difficult, we investigated the effect of phenylenediamine on a complex medium capable of much higher productivity. Qualitatively the picture was similar, although the total 'vitamin B₁₂' titre achieved in the presence of phenylenediamine was only slightly above that in its absence. Purification procedures normally used for isolating vitamin B₁₂ were applied to a fermentation broth that initially contained 160 μ g. phenylenediamine/ml. A red concentrate from it contained vitamin B₁₂ and the new substance. This was streaked on to Whatman 3 MM sheets and chromatographically developed in the same way as described above for bioautograph samples. Two red bands formed. The ones containing the new substance were cut out and eluted with water; the eluate was concentrated and the solute crystallized from aqueous acetone. Its spectrum strongly resembled that of vitamin B₁₂ and had a similar main peak at 361 m μ .

Microbiological activity of new factor

The microbiological potency of the new substance was compared with that of vitamin B₁₂. Solutions of the two materials with equal optical densities at 361 m μ . were prepared and appropriate dilutions were assayed on *Esch. coli* and *Lb. leichmannii* plates. Under these conditions the new substance was approximately 1.8 times as active as vitamin B₁₂ for *Lb. leichmannii* and 2.4 times as active for *Esch. coli*.

Partial identification of the new factor

The crystalline material (3.0 mg.) dissolved in 6N-HCl (1.5 ml.) was heated at 150° in a sealed tube for 7.5 hr. (Buchanan, Johnson, Mills & Todd,

1950). The yellow-brown solution was freed from a little insoluble material by filtration and from excess hydrochloric acid by concentration under reduced pressure. The residue, dissolved in a few ml. water, was passed through an 'Amberlite' IR-120 (H⁺) resin column (6 $\times$ 1 cm.), which was then washed with water (100 ml.). 2N-NH₃ (100 ml.) was next passed through the column, the eluate was taken to dryness *in vacuo* and the residue dissolved in very little 0.1N-HCl; several spots of it were applied to a filter paper sheet (Whatman no. 4). Benziminazole and 5:6-dimethylbenziminazole were used as standards; chromatography and detection of fluorescent spots was carried out as described by Buchanan *et al.* The hydrolysate and benziminazole gave spots with an R_f of 0.70; the R_f of the dimethylbenziminazole was 0.74. The hydrolysate showed a further small spot with an R_f of 0.82. The hydrolysate spots were cut out and eluted with 0.1N-HCl and the absorption spectra between 200 and 300 m μ . were determined. The slower moving substance revealed two maxima (273.5 and 267 m μ .) in the same positions as those of benziminazole (5:6-dimethylbenziminazole has maxima at 283 and 275 m μ .). The spectrum of the faster moving substance showed no maximum between 250 and 300 m μ . and only general absorption at wavelengths below 250 m μ . This spot could perhaps have arisen from an impurity.

DISCUSSION

The increased apparent vitamin B₁₂ titre claimed by Dulaney & Williams (1953) when *o*-phenylenediamine was added to the medium of *Strep. griseus* fermentations is not due to a higher production of this vitamin itself, but to the formation of a vitamin B₁₂ analogue. This probably differs from the former only in lacking the two methyl groups of the benziminazole portion. The new material is microbiologically more active than vitamin B₁₂ (in plate assays). Its enhanced activity is probably the only reason for the observed titre increases in fermentations containing phenylenediamine, though it is not impossible that phenylenediamine may also have stimulated somewhat the synthesis of the main cobalt containing portion (factor B, Ford & Porter, 1952; Gant, Lester Smith & Parker, 1954), common to both substances. If such a stimulation does take place, then a mechanism similar to the one suggested by Dulaney & Williams (1953) may well be operative though the biosynthesis of a portion only of the vitamin B₁₂ molecule, and not of the whole, would be involved.

The increasing levels of the new substance at the cost of vitamin B₁₂ with increasing levels of phenylenediamine in the medium, and the complete absence of biosynthesis of the new substance in the

simultaneous presence of phenylenediamine and relatively low levels of diaminodimethylbenzene, makes it most probable that a mechanism of competition is operative. Whether the actual synthesis of diaminodimethylbenzene (or a more complex derivative of it) is suppressed by phenylenediamine, or whether diaminodimethylbenzene is synthesized at its normal level irrespective of the presence of phenylenediamine, but not incorporated into the vitamin B₁₂ molecule if excess phenylenediamine is present is not known.

The new substance is almost certainly identical with the one obtained independently by Ford, Holdsworth & Kon (1954), who incubated *Esch. coli* and factor B in the presence of benziminazole and then demonstrated by chromatography the formation of a new compound with an *R_F* value just less than that of vitamin B₁₂.

SUMMARY

1. Fermentations of *Streptomyces griseus* on a complex or a synthetic medium in the presence of *o*-phenylenediamine gave rise to the production of a new analogue of vitamin B₁₂.

2. The new substance has been isolated in the form of red needles.

3. Its absorption spectrum is practically identical with that of vitamin B₁₂.

4. Benziminazole has been identified as a hydrolytic product.

5. The new material is microbiologically (plate assay) more active than vitamin B₁₂: the greater activity probably explains the apparently increased

'vitamin B₁₂' titres obtained by Dulaney & Williams (1953).

6. The biosynthesis of true vitamin B₁₂ is competitively inhibited by phenylenediamine.

7. The biosynthesis of the new material in the presence of phenylenediamine is competitively inhibited by 1:2-diamino-4:5-dimethylbenzene. The inhibition index lies somewhere between 160 and 320.

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Note added in proof. Clinical trials kindly carried out by Professor L. J. Witts and Dr Sheila Callendar at the Radcliffe Infirmary, Oxford, showed that the new material was fully active in three pernicious anaemia patients.

REFERENCES

- Buchanan, J. G., Johnson, A. W., Mills, J. A. & Todd, R. A. (1950). *J. chem. Soc.* p. 2845.
 Burkholder, P. R. (1951). *Science*, **114**, 459.
 Cuthbertson, W. F. J. & Lester Smith, E. (1949). *Biochem. J.* **44**, v.
 Dulaney, E. L. & Williams, P. L. (1953). *Mycologia*, **XLV**, 3, 345.
 Fantes, K. H. & O'Callaghan, C. H. (1954). *Biochem. J.* **58**, xxi.
 Ford, J. E. & Holdsworth, E. S. (1953). *Biochem. J.* **53**, xxii.
 Ford, J. E., Holdsworth, E. S. & Kon, S. K. (1954). *Biochem. J.* **58**, xxiv.
 Ford, J. E. & Porter, J. W. G. (1952). *Biochem. J.* **51**, v.
 Gant, D. E., Lester Smith, E. & Parker, L. F. J. (1954). *Biochem. J.* **56**, xxxiv.
 Harrison, E., Lees, K. A. & Wood, F. (1951). *Analyst*, **76**, 696.
 Woolley, D. W. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 745.
 Woolley, D. W. (1951). *J. exp. Med.* **93**, 13.

The Vitamin B₁₂ Group. Presence of 2-Methyl Purines in Factors A and H and Isolation of New Factors

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The main cobalt-containing substance in the rumen content and faeces of calves has been called 'factor A' by Ford & Porter (1952, 1953). The absorption spectrum and microbiological activity suggested its close relation to vitamin B₁₂. Preliminary reports showed that in fact the difference lies only in replacement of the 5:6-dimethylbenziminazole of vitamin B₁₂ by 2-methyladenine, a purine not previously found in nature (Brown & Lester Smith, 1954; Gant, Lester Smith & Parker, 1954). Dion, Calkins & Piffner (1954) have independently characterized as 2-methyladenine the base present in factor A, which they call pseudo (*ψ*)-vitamin B_{12a}.

By extracting portions totalling 170 kg. of pig manure and about 180 kg. of calf manure, we were able to isolate not only factor A and vitamin B₁₂ itself but also small amounts of other factors, namely, *ψ*-vitamin B₁₂ (Dion, Calkins & Piffner, 1952), factors B and C (Ford & Porter, 1952) and several new red substances that we call factors D-I. Some of them were separated only by employing both chromatography and electrophoresis on paper, successively but in either order. Factors G and H could not be separated except by taking advantage of slight differences in the behaviour of their purple dicyano complexes. Factor I appears identical