macrocyclic tetrapyrroles and strongly suggesting that in the natural system the cobalt is incorporated into preformed tetrapyrrole.

The present evidence indicates that uroporphyrinogen is the first macrocyclic tetrapyrrole intermediate of vitamin B₁₂. It is suggested that the next step in the formation of the corrin ring is the reopening of the macro-ring with the expulsion of the γ -methene bridge carbon as methyl group, followed by ring-closure. Mauzerall (1960) has demonstrated that ring-opening does occur with uroporphyrinogens. The new compound, a methyl-substituted corrole, would not be expected to possess physical or chemical properties described for an unsubstituted corrole (Johnson & Kay, 1965). The substitution at the α -position of the A ring prevents the formation of a fully conjugated system as in corrole. Thus the characteristic Soret band and the typical red fluorescence would not be observed. A compound of the type suggested would, however, most likely resemble a tetrahydroporphyrin, or a di- or tri-pyrrolylmethene. Since material with these absorption characteristics is sometimes found when clostridia are incubated with δ -aminolaevulic acid it is suggested that this material may represent the next biosynthetic intermediate of vitamin B₁₂.

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- Bardi, U., Boretti, G., DiMarco, A., Julita, P., Marnati, M. & Spalla, C. (1958). *Abstr. 7th int. Congr. Microbiol., Stockholm*, p. 374.
- Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E. & Wilson, M. R. (1960). *J. biol. Chem.* **235**, 181.
- Bonnett, R., Cannon, J. R., Johnson, A. W., Sutherland, I., Todd, A. R. & Smith, E. L. (1955). *Nature, Lond.*, **176**, 328.
- Bukin, V. N. & Pronyakova, G. V. (1960). *J. Biochem., Tokyo*, **47**, 781.
- Burnham, B. F. (1965). *Fed. Proc.* **24**, 223.
- Chu, T. C. & Chu, E. J. (1957). *J. biol. Chem.* **227**, 505.
- Eriksen, L. (1958). *Scand. J. clin. Lab. Invest.* **10**, 319.
- Johnson, A. W. & Kay, I. T. (1965). *Proc. chem. Soc., Lond.*, p. 89.
- Lascelles, J. (1956). *Biochem. J.* **62**, 78.
- Lascelles, J. (1964). *Tetrapyrrole Biosynthesis and its Regulation*, New York: W. A. Benjamin Inc.
- Mauzerall, D. (1960). *J. Amer. chem. Soc.* **82**, 2601.
- Mauzerall, D. & Granick, S. (1958). *J. biol. Chem.* **232**, 1141.
- Meister, A., Sober, H. A. & Tice, S. V. (1951). *J. biol. Chem.* **189**, 577.
- Porra, R. J. & Ross, B. D. (1965). *Biochem. J.* **94**, 557.
- Schwartz, S., Ikeda, K., Miller, I. M. & Watson, C. J. (1959). *Science*, **129**, 40.
- Shaposhnikov, V. N. & Finogenova, T. V. (1963). *Mikrobiologiya*, **32**, 391.
- Shemin, D., Corcoran, J. W., Rosenblum, C. & Miller, I. M. (1956). *Science*, **124**, 272.

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An Early Intermediate in the Biosynthesis of Biotin: Incorporation Studies with [³⁵S]Sulphate

By MAX A. EISENBERG

College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032, U.S.A.

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In a previous study on biotin biosynthesis, an unknown biotin vitamer, which was characterized as an amino acid, was shown to be present in the culture medium of *Phycomyces blakesleeana* (Eisenberg, 1963). A compound with a similar R_F was also reported in culture filtrates of *Escherichia coli* (Dhyse & Hertz, 1958) and *Corynebacterium xerosis* (Genghof, 1956). This compound has now been obtained in high yields from the culture medium of a biotin auxotroph of *Penicillium*

chrysogenum, and it becomes labelled when the organism is grown in the presence of [1,7-¹⁴C₂] pimelic acid (M. A. Eisenberg, unpublished work). Since pimelic acid is a direct precursor of biotin (Eisenberg, 1962; Elsford & Wright, 1962), the isotope-incorporation studies suggested the possibility of this vitamer being an intermediate in biotin synthesis. To obtain more information about the chemical composition of this unknown vitamer, the question whether sulphur is present in the

compound became of paramount importance in view of the mechanism for biotin biosynthesis proposed by Lezius, Ringelmann & Lynen (1963). The present study with [^{35}S]sulphate shows no sulphur present in this unknown vitamer.

P. chrysogenum 62078 (strain obtained from the culture collection of Dr E. Tatum) was grown in a modified Czapek-Dox medium. The sulphate concentration was decreased to the lowest permissible level for maximum incorporation of [^{35}S]sulphate by substituting MgCl_2 for MgSO_4 . To 500 ml. of the media in a Fernbach flask was added 50 μg . of D-biotin and 100 μC of $\text{Na}_2^{35}\text{SO}_4$. The $\text{Na}_2^{35}\text{SO}_4$ was purchased from New England Nuclear Corp. and had a specific activity of 1.33 mc/mg. The maximum dilution of the specific activity by the non-labelled sulphate of the medium was 30-fold. A washed spore suspension from a 2-week-old culture grown on Sabouraud's medium was used to inoculate the medium. The flask was vigorously shaken on a New Brunswick incubator-shaker at 26° for 7 days. The mycelial mat was then removed by filtration and the filtrate adjusted to pH 2.2. Portions (5.0 ml.) of the filtrate were chromatographed on the 0.9 cm. $\times$ 15 cm. or 0.9 cm. $\times$ 50 cm. columns of the Spinco model 120 amino acid analyser. The shorter column was developed with 0.35 N-sodium citrate buffer, pH 5.28, at 50° and the longer column with 0.38 N-sodium citrate buffer, pH 4.26, at 30° and 50°. The columns were connected directly to a Vanguard fraction collector and 5.0 ml. samples taken. Each sample was assayed for biological activity by a modified disk-assay procedure of Genghof, Partridge & Carpenter (1948) and for radioactivity with a Packard Tri-Carb scintillation counter.

Previous studies (M. A. Eisenberg, unpublished work) with the unknown vitamer on the columns of the amino acid analyser showed the unknown vitamer to appear between lysine and histidine on the short column at pH 5.28. On the medium column at pH 4.26, it appeared just beyond arginine. Fig. 1 shows that at pH 5.28 two biologically active peaks are obtained; the first consists primarily of dethiobiotin and the second of the unknown vitamer. Most of the radioactivity is eluted early with the neutral compounds and there is no coincidence between radioactivity and biological activity of the unknown vitamer. To further ascertain whether the small amount of radioactivity present was associated with the biologically active component, the contents of tubes 11–16 were pooled, the pH was adjusted to 2.2 and the solution was rechromatographed on the same column. Over 91% of the total biological activity and only 42% of the radioactivity was recovered, but again there was no indication of coincidence. The contents of those tubes containing biologically active material were

Fig. 1. Chromatographic separation of the unknown vitamer present in culture filtrates of *P. chrysogenum*. The column was developed with 0.35 N-sodium citrate buffer, pH 5.28, at 50°. ●—●, Radioactivity; ○—○, biological activity.

again pooled, the pH was adjusted to 2.2 and the solution was chromatographed on the medium column at pH 4.26. Under these conditions, over 80% of the biological activity was recovered and all the radioactivity lost. When the sample was placed directly on the medium column, only a few counts above background were found for each tube containing biologically active material and these values were not statistically significant.

It was previously postulated that pimelic acid may condense with a C_3 unit as the first step in the biosynthesis of biotin (Eisenberg, 1963). Of necessity, then, all subsequent intermediates would be labelled when cultures are grown in a medium containing ^{14}C -labelled pimelic acid. Evidence is now available that this unknown vitamer becomes labelled in the presence of [$1,7\text{-}^{14}\text{C}_2$]pimelic acid. In addition, when yeast cells are grown on the ^{14}C -labelled vitamer, the isotope is incorporated into biotin (M. A. Eisenberg, unpublished work). This suggests that the unknown vitamer is directly on the pathway of biotin biosynthesis. The absence of sulphur eliminates cysteine as the C_3 donor as postulated by Lezius *et al.* (1963), and indicates the incorporation of sulphur at a later stage as originally postulated by Tatum (1945). Further evidence in support of this late stage of sulphur incorporation is the finding of Pai

& Lichstein (1965) of the repression of biotin biosynthesis from dethiobiotin in resting cells of *E. coli* grown on biotin. This suggests that this reaction is directly on the biosynthetic pathway. A similar repression by biotin has been observed for the unknown vitamer and dethiobiotin in cultures of *P. chrysogenum*.

Two other C₃ donors that could possibly condense with pimelic acid are serine and alanine. The former could have readily arisen from cysteine via the cysteine-dehydratase reaction in the experiments of Lezius *et al.* (1963) and would have had a particularly high specific activity if serine synthesis via other pathways were repressed. Unfortunately, the specific activity of the serine in the proteins of the organism used was not checked. On the basis of the information presently available, the following scheme for biotin synthesis is proposed:

Serine or alanine + pimelic acid →→ unknown
vitamer →→ dethiobiotin →→ biotin

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- Dhyse, F. G. & Hertz, R. (1958). *Arch. Biochem. Biophys.* **74**, 7.
 Eisenberg, M. A. (1962). *Biochem. biophys. Res. Commun.* **8**, 437.
 Eisenberg, M. A. (1963). *J. Bact.* **86**, 673.
 Elsford, H. L. & Wright, L. D. (1962). *Fed. Proc.* **21**, 467.
 Genghof, D. S. (1956). *Arch. Biochem. Biophys.* **62**, 63.
 Genghof, D. S., Partridge, C. W. H. & Carpenter, F. H. (1948). *Arch. Biochem. Biophys.* **17**, 413.
 Lezius, A., Ringelmann, E. & Lynen, F. (1963). *Biochem. Z.* **336**, 510.
 Pai, C. H. & Lichstein, H. C. (1965). *Biochim. biophys. Acta*, **100**, 43.
 Tatum, E. (1945). *J. biol. Chem.* **160**, 455.