79. THE BEHAVIOUR OF *SCOPULARIOPSIS BREVICAULIS* TOWARDS CHOLINE

By CHARLES SIMONS

From the Organic Chemistry Department, The University, Leeds

(Received 23 April 1941)

CHOLINE (I) and its lower homologue colamine (IV) are known to occur in nature, combined, as in the phosphatides or lecithin [Schulze & Likiernik, 1891; Trier, 1911; Baumann, 1913; McLean, 1908, 1, 2]. Choline is also known to occur simply as cyclic choline sulphate, in the micro-organism *Aspergillus sydowi* [Woolley & Peterson, 1937] and also in the hepatic bile [Johnston *et al.* 1939]. In most respects choline is considered to be a vital cell ingredient. No report has been made of the natural occurrence of the two intermediate members *NN*-dimethylaminoethanol (II) and *N*-methylaminoethanol (III).

The methylation of As_2O_3 to trimethylarsine by *Scopulariopsis brevicaulis* has been discussed by Challenger *et al.* [1933; 1935; 1936]. Challenger & Higginbottom [1935] suggested that choline or betaine might be the source of the methyl group. A similar hypothesis had already been put forward by Riesser [1913] when considering the origin of the methyl group of creatine, and as an explanation of the garlic odour (assumed to be dimethyl telluride) exhaled by animals on ingestion of tellurite. Riesser's suggestion has, however, been considerably extended by the Leeds workers. It is conceivable that choline acts as a methylating agent while being converted into any or all of its three lower homologues mentioned above. Now, the amount of choline and related amino-alcohols present in a micro-organism such as a mould of the *Penicillium* or *Aspergillus* type would be small and difficult to detect. It would be advantageous therefore, to incorporate choline chloride in the arsenical medium in the hope that when detoxication (i.e. biological methylation) took place, the demethylation products would remain in the medium if the reaction were an extracellular enzymic one or diffuse into the medium if the reaction were intracellular. In this way a reasonable amount of amino-alcohols might be available for detection.

Scopulariopsis brevicaulis (sometimes called *Penicillium brevicaulis*) was grown for several weeks upon a medium incorporating inorganic salts in glucose solution containing choline chloride (series A). To some was added As_2O_3 (series B) and controls were set up on media containing neither choline nor arsenic (series D) and containing arsenic but no choline (series C). These last two were used to see the effect of medium on picrate formation.

Before investigation of the media could be carried out an ideal separation of the four aminoethanols (I, II, III, IV) had to be devised, as it is possible that demethylation could stop at any of the stages and thus produce all four.

Various compounds of the bases are described in the literature, e.g. the mercurichloride, chlorplatinite, reineckate, picrate, picrolonate, chloraurate, iodobismuthate, phosphomolybdate, phosphotungstate etc. [Guggenheim, 1920;

Kahane & Levy, 1938; Ladenburg, 1881; Knorr, 1889; Knorr & Matthes, 1898; 1901; Matthes, 1901] and it was hoped that by a judicious selection of reagents the presence of each base in a mixture could be ascertained. The similarity in properties, however, of these salts and compounds precluded this course and it was finally decided to use fractional crystallization methods on one series of salts. Trials on the picrates, picrolonates and mercurichlorides showed that the former were the best. The separation was then applied to the biological media upon which the mould had been growing. Only choline was detected.

EXPERIMENTAL

NN-Dimethylaminoethanol was prepared by the method of Ingold & Rogers [1935], *N*-methylaminoethanol by that of Slotta & Behnisch [1932] and colamine by that of Ing & Manske [1926]. Choline chloride was purchased from the British Drug Houses Ltd.

Approximately equal amounts of choline chloride and the other three bases were mixed and made acid to Congo red with HCl and reduced to a syrup on the steam bath. NaHCO₃ solution was then added in excess to make 5% excess NaHCO₃ and KI solution (153 g. I₂ and 100 g. KI in 200 ml. water) added until precipitation was complete and the mixture kept overnight. This removed most of the choline as the insoluble enneaiodide [Stanek, 1905]. The dark precipitate was filtered and sparingly washed. To the filtrate was added dilute HCl and then CuCl₂ and 'molecular' Cu and the whole boiled. This was continued until the solution had a permanent blue colour. It was then filtered and again heated and all the excess Cu precipitated as the sulphide by H₂S and again filtered. The colourless filtrate was reduced in bulk on the steam bath and NaHCO₃ added until the solution was only just acid to Congo red. The whole was then taken to dryness on the steam bath and when cold was extracted with cold methanol several times and filtered. The solution was then made to react with saturated alcoholic sodium picrate and filtered.

The significance of the recurrence of the melting point 213° would indicate a eutectic of choline and methylaminoethanol.

As it was not found possible to get a good separation of the dimethylaminoethanol from the ether extract of the picrates it was identified as follows.

Benzyl bromide and dimethylaminoethanol react in dry ether solution to give a white hygroscopic solid of the quaternary salt, M.P. 128° (cryst. alc. ether). Br, found 30.38%. Calc. for C₁₁H₁₈ONBr 30.56%. Colamine and the mono-methyl compound do not react so; hence it is possible to identify the dimethyl compound in a mixture of all three by dissolving the free bases in dry ether, adding benzyl bromide and seeding with a few crystals of the pure quaternary salt whereupon the oil crystallizes, M.P. 121°, M.M.P. 122°.

Application to biological media

Thirty-six 1 l. conical flasks, each containing the following substances, were set up and sterilized.

	Series A	Series B	Series C	Series D
No. of flasks	12	12	6	6
Czapek-Dox salt solution*	200 ml.	200 ml.	200 ml.	200 ml.
Choline chloride	2 g.	2 g.	Nil	Nil
Glucose	4 g.	4 g.	4 g.	4 g.

* Czapek-Dox salt solution [cf. Chalmers, 1935]:

NaNO ₃	2 g.
KH ₂ PO ₄	1 g.
KCl	0.5 g.
MgSO ₄	0.5 g.
FeSO ₄	0.01 g.
Water to 1 l.	

All 36 flasks were inoculated from Czapek-Dox-sucrose agar slopes of *Scopulariopsis brevicaulis* (strain B) [Challenger *et al.* 1933], incubated for 3 days at 32° and then allowed to stand at room temperature.

After 21 days, to each of series B and C were added 25 ml. of a sterile 1.2% As₂O₃ solution and the flasks again kept for some time. Series A and D were

filtered after 91 days and series B and C after 147 days. All filtrates were concentrated, it being noticed that those from series A and B smelt strongly of what was considered to be trimethylamine.

1. The filtrate from series A was made alkaline with NaOH and extracted with CHCl_3 , but very little was taken out by the solvent which was removed and the residue treated with picric acid. No picrate was obtained.

2. 10–20 ml. of series C filtrate were mixed with 0.5 g. choline chloride and made to react with 20–30 ml. of saturated alcoholic sodium picrate and then taken to dryness on the steam bath. Cryst. from MeOH m.p. 228° .

3. Repeated above for series D filtrate with similar results giving cryst. m.p. 231° .

4. Series A filtrate was acidified to congo red with HCl and taken to dryness. The residue was dissolved in alcohol and made to react with alcoholic sodium picrate and then reduced in bulk and filtered from colourless inorganic salts. It was washed with methanol, concentrated and filtered:

5. Some of the filtrate from series B was made acid to Congo red and taken to dryness. Aqueous sodium picrate (saturated) was added without filtering and the whole taken to dryness. It was heated with norite in MeOH and filtered hot giving a filtrate which deposited yellow crystals on cooling.

Residues A, B, C, E, F, G, H, J were collected, dissolved in water, acidified with HCl and extracted with ether to remove picric acid. The aqueous layer was boiled free from ether and made alkaline with NaHCO_3 . The choline was then removed as the enneaiodide and the filtrate treated with the usual reagents to remove the excess I_2 and KI, Cu etc. and finally the solution was made just acid to Congo red.

It was then reduced to dryness on the steam bath. The residue was extracted with cold MeOH three times and the liquid decomposed with saturated alcoholic sodium picrate. The residue was mainly sodium picrate. The liquid was then reduced in bulk and ether added.

Colourless ppt. Burns like glucose; is hygroscopic and reduces Fehling's solution and forms an osazone

Filtrate taken to dryness leaving an oil. This reduced Fehling's solution. It did not form a picrate and was soluble in moist ether. With dry ether it gave a ppt. The filtrate of this did not react with benzyl bromide.

The conclusions that may be drawn therefore from the foregoing are that choline is not broken down completely by the organism and that, if any is broken down, it is converted either into ammonium salts, trimethylamine or some other nitrogenous product, or that the compounds for which search was made are bound up in the mycelial portion as an essential cell constituent, possibly combined, for example, as a phosphatide.

SUMMARY

1. A possible mechanism of the biological methylation of As_2O_3 by fungi such as *Scopulariopsis brevicaulis* has been suggested with choline as the methylating substance.
2. A means of identifying the possible products formed has been worked out, utilizing a picrate separation and the formation of a quaternary ammonium salt.
3. Application of this separation has shown that demethylation of choline to its lower homologues does not occur in the medium.

The author wishes to thank the Institute of Chemistry for the award of the Pedler Scholarship by which means this research was carried out and also Prof. F. Challenger for his advice and guidance.

REFERENCES

- Baumann (1913). *Biochem. Z.* **54**, 30.
Challenger & Higginbottom (1935). *Biochem. J.* **29**, 1757.
—— & Ellis (1933). *J. chem. Soc.* p. 95.
—— & Rawlings (1936). *J. chem. Soc.* p. 264.
Chalmers (1935). *Bacteria in relation to Milk Supply*, p. 168. London: Arnold.
Guggenheim (1920). *Die Biogenen Amine*. Berlin: Springer.
Ing & Manske (1926). *J. chem. Soc.* p. 2348.
Ingold & Rogers (1935). *J. chem. Soc.* p. 723.
Johnston, Irvin & Walton (1939). *J. biol. Chem.* **131**, 425.
Kahane & Levey (1938). *Biochimie de la choline et de ses dérivées*. Paris: Hermann et Cie.
Knorr (1889). *Ber. dtsh. chem. Ges.* **22**, 2088.
—— & Matthes (1898). *Ber. dtsh. chem. Ges.* **31**, 1070.
—— (1901). *Ber. dtsh. chem. Ges.* **34**, 3484.
Ladenburg (1881). *Ber. dtsh. chem. Ges.* **14**, 2408.
McLean (1908, 1). *Hoppe-Seyl. Z.* **55**, 360.
—— (1908, 2). *Hoppe-Seyl. Z.* **57**, 296.
Matthes (1901). *Liebigs Ann.* **315**, 110.
Riesser (1913). *Hoppe-Seyl. Z.* **86**, 440.
Schulze & Likiernik (1891). *Hoppe-Seyl. Z.* **15**, 405.
Stanek (1905). *Hoppe-Seyl. Z.* **46**, 280.
Slotta & Behnisch (1932). *J. prakt. Chem.* (2), **135**, 225.
Trier (1911). *Hoppe-Seyl. Z.* **73**, 383.
Woolley & Peterson (1937). *J. biol. Chem.* **122**, 213.