(2) *Production of amylase.* The rate of amylase production of *Aspergillus oryzae* is considerably higher in deep-culture with the Vibro Mixer than in either stationary or shaken cultures; this property is obviously for the greater part due to the fact that amylase production is correlated with mycelium formation. A further characteristic of the Vibro Mix cultures is the increase in amylase titre during the phase of decreasing mycelium content as can be seen in Figure 2. It should be remarked that stationary or shaken cultures never show as marked an autolysis as Vibro Mix cultures.

A property which corresponds strikingly to the increased excretion of organic nitrogen is the markedly higher rate of amylase production of small-inoculum Vibro Mix cultures as compared with large inocula. This seems to indicate that the stimulated excretion of organic nitrogen during the phases of increase and decrease of mycelium content is not due simply to autolysis, i.e. dissolution of the cell contents, but represents also a change in the pattern of the synthesizing activity. To complete the picture it is necessary to indicate that stationary or shaken cultures from small inocula show neither increased rate of amylase production nor increased maximum yield of the enzyme when compared with large-inoculum cultures.

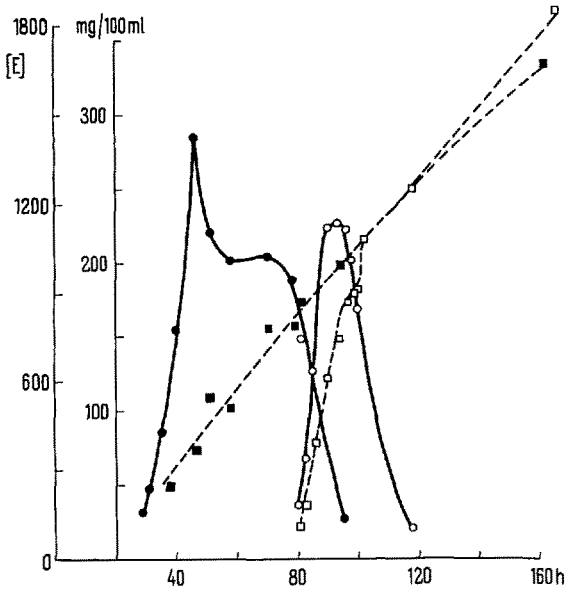

Fig. 2. Production of dextrinogenic amylase (E) (■, □) by *Aspergillus oryzae* in Vibro Mix cultures during the phases of increase and decrease of mycelium formation (●, ○) with large (■, ●) and small (□, ○) inocula.

(3) *Propagation of various micro-organisms.* Filamentous growth was obtained with various species of *Penicillium*, *Aspergillus* and *Byssochlamis*. Yeasts and acetic acid bacteria could be grown easily. Recently we have been using the apparatus advantageously for the propagation of organisms oxidizing long-chain hydrocarbons. A distinctive feature of the Vibro Mixer is the production of a fine emulsion, even with waxy components, in the three-phase system: air-water-oil.

Prospective applications. As a result of a very intimate mixing of air and water (thus allowing a low air-flow rate) the apparatus may find further applications in those cases where volatile products or substrate compounds are involved as in vinegar manufacture. In view of the homogeneous growth obtained with filamentous fungi, and as only little mycelium is attached to the vessel surface above and below the level of the substrate, the method should be suitable for continuous propagation of filamentous fungi on the laboratory or pilot plant scale[4].

Zusammenfassung. In Vibro-Mischer-Kulturen erfolgt die Amylasebildung bei *Aspergillus oryzae* in der linearen Wachstumsphase im Gegensatz zu Ruhe- und Schüttelkulturen schneller mit kleiner als mit grosser Impfung. Während der Phase der raschen Abnahme der Mycelkonzentration läuft die Amylasebildung noch weiter.

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Department of Applied Microbiology and Biology, The Royal College of Science and Technology, Glasgow (Scotland), December 19th, 1963.

[4] The experiments described in this paper were carried out at the Department of Agricultural Bacteriology and Fermentation, Swiss Federal Institute of Technology, Zürich, where careful technical assistance has been provided by Miss A. MOHLER.

Reticulol, a New Metabolic Isocoumarin

While examining the metabolic products of a strain of *Streptomyces rubrireticulae* a previously unknown, colorless, crystalline phenol, $C_{11}H_{10}O_5$, was isolated. The compound, m.p. 193–193.5°, λ^{max}_{MeOH} 245, 278, and 330 mμ (log ε 4.68, 3.86 and 3.76 respectively), was named reticulol for convenience and its structure was established as Ia by degradative and physical studies.

Microanalysis detected the presence of an O-methyl group and the formation of a diacetate (ν_{max} 1795, sh. 1782, 1200 cm^{-1}) established that two of the other oxygens were phenolic hydroxyls. The remaining oxygens were shown to be present in a hydrogen bonded aromatic ester grouping (ν_{max} 1680 cm^{-1} shifting to 1740 cm^{-1} on acetylation and 1710 cm^{-1} on methylation). No reaction was obtained between reticulol and dichlorodiphenylmethane thus eliminating the possibility that the two phenol groups are *ortho* to one another[1]. The ester function in dimethylreticulol (Ib) was shown to be cyclic by virtue of its acid stability and the ease with which it was hydrolyzed by aqueous base to the corresponding carboxylic acid (IIa) without loss of carbon. The same acid

[1] L. JURD, J. org. Chem. *27*, 872 (1962).

was obtained when the saponification was carried out in the presence of dimethyl sulfate. The lack of introduction of a new, base stable methoxyl group during this latter treatment ruled out the possibility that reticulol might have a chromone or coumarin skeleton. Further, the derived acid (IIa) formed a 2,4-dinitrophenylhydrazine derivative (IIc). Since neither reticulol nor dimethylreticulol form ketone derivatives, this function must have been generated during saponification; behavior consistent with the presence of the comparatively rare isocoumarin nucleus in reticulol. This was confirmed when ozonization of dimethylreticulol yielded 3,4,5-trimethoxy-phthalic acid[2]. This fact, together with the demonstration of hydrogen bonding between one of the phenolic hydroxyls and the isocoumarin carbonyl, suggests a 6,7,8-trioxygenated aromatic ring with the two free hydroxyls separated by the O-methyl group, as required by the lack of reaction with dichlorodiphenylmethane.

Placement of the remaining C-methyl group at position 3 rather than 4 follows from the production of a diacid on ozonization and also from the ultraviolet spectrum of the 2,4-dinitrophenylhydrazone (IIc), which was more typical of a saturated ketone than of an aldehyde (365 mμ rather than 360 mμ)[3].

Confirmation of this position for the C-methyl group was obtained from the p.m.r. spectrum of reticulol and the derived methyl ester (IIb). The spectrum of reticulol clearly demonstrated the presence of a vinylogously coupled C-methyl group ($\tau = 7.78$; $J = 1$ cps) while that of the methyl ester showed a sharp singlet at 7.82 τ indicative of an uncoupled methyl attached to carbon and another sharp singlet, less intense, at 6.30 τ due to the isolated methylene group.

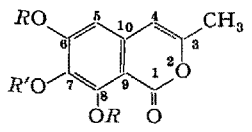
The p.m.r. spectrum of reticulol, measured in deuterated dimethylsulphoxide, provided additional evidence for the hydrogen bonding of one of the hydroxyl groups to the ester carbonyl; one hydroxyl hydrogen appearing at 6.60 τ the other being shifted to -1.13 τ.

Reticulol thus appears to belong to the small, but expanding, class of naturally occurring isocoumarins and, in common with those so far isolated, fits readily into the well known acetate-malonate biogenetic scheme[4].

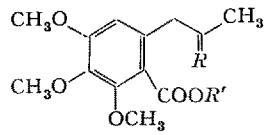
Zusammenfassung. Die Struktur des aus *Streptomyces rubreticulae* isolierten neuen Isocumarins, Reticulol, ist als 3-Methyl-6,8-dihydroxy-7-methoxyisocumarin ermittelt worden.

L. A. Mitscher, W. W. Andres, and W. McCrae

Biochemical Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River (New York USA), February 24, 1964.



Ia: $R = H$, $R' = CH_3$
Ib: $R = R' = CH_3$
Ic: $R = COCH_3$, $R' = CH_3$
Id: $R = R' = H$



IIa: $R = O$, $R' = H$
IIb: $R = O$, $R' = CH_3$
IIc: $R = N\text{-}NH\text{-}C_6H_3(NO_2)_2$, $R' = H$

[2] O. T. Schmidt and K. Bernauer, Liebigs Ann. *588*, 211 (1954).
[3] J. P. Phillips, J. org. Chem. *27*, 1443 (1962).
[4] R. Bentley, *Annual Review of Biochemistry* (Annual Reviews Inc., Palo Alto, Calif. 1962), vol. 31, p. 589.

The Nature of the Solitary Active Cells of the Central Nervous System

Isolated cells with a high content of $NADPH_2$-tetrazolium reductase (TPN-diaphorase) were reported in the cortex, corpus striatum and pallidum of rats and man by Thomas and Pearse[1,2]. From their shape and from the arrangement of their processes it was deduced that the cells were neurones. Actual proof of this was lacking; however, this communication serves to substantiate the original supposition.

Counter-staining of the enzyme preparation with a silver method (Duckett[3]) can be carried out without loss of the formazan deposit which represents the enzyme activity (Figure).

Method. The method of staining for the TPN diaphorase is given by Thomas and Pearse[1]. The method of counterstaining is carried on from the stage of fixation of the enzymatic preparation in a 15% formol solution. (1) Leave in the 15% formol solution for at least 1 h at room temperature. (2) Wash carefully in three stages of distilled water. (3) Place the section in the silver-ethylamino-oxalate solution for 30 to 60 min at 37°C. (4) Wash quickly in distilled water. (5) Place in a 1% formol solution for 1 min. (6) Wash and mount in glycerine jelly.

The silver-ethylamino-oxalate solution is composed as follows: potassium oxalate 5% solution, 20 cm³; silver nitrate 10% solution, 5 cm³. A white precipitate appears which is dissolved by adding drop by drop a 35% solution of ethylamine, until only a faint deposit is left. Usually about 2 cm³ are needed. Add 10 to 12 drops of absolute alcohol, and make up to 75 cm³ with distilled water. You may add one drop of pyridine to every 5 cm³ of the finished solution.

The section is left untoned and the cells and their nuclei should be a golden yellow, so that the black formazan deposits stand out sharply. The parasite silver deposits are excluded by careful washing in distilled water between steps 1 and 2. As a last resort, if deposits of reduced silver are still troublesome, wash the section at step 2 in a very weak ammonia solution – one drop of ammonia in 10 cm³ of distilled water.

[1] E. Thomas and A. G. E. Pearse, Sonderdruck aus Z. Zellforschung und mikroskopische Anatomie, Abteilung Histochemie *2*, 266 (Springer-Verlag, Berlin 1961).
[2] E. Thomas and A. G. E. Pearse, Acta Neuropathologica, *3*, 238 (1964).
[3] S. Duckett, Acta neuropathol., in press.