PRODUCTION OF CARBON MONOXIDE IN THE ENZYMATIC DEGRADATION OF RUTIN *

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Received December 16, 1959

Protocatechuic acid, phloroglucinol carboxylic acid and phloroglucinol have been isolated from stomachs of rats forcibly fed randomly labeled quercetin (Kallianos et al., 1959) while protocatechuic acid was isolated from kidney homogenates incubated with rutin (Douglass and Hogan, 1958). The molds Pullularia fermentans (Hattori and Noguchi, 1959), Aspergillus flavus and A. niger (Westlake et al., 1959) degrade rutin to rutinose, protocatechuic acid, phloroglucinol carboxylic acid and the depside of these acids. These products account for 26 of the 27 carbon atoms present in the rutin molecule. The remaining carbon (carbon-3) is here shown to be released as carbon monoxide.

A. niger was grown for 72 hours at 30° C on a rotary shaker on 100 ml of medium in 250-ml Erlenmeyer flasks. The medium contained 400 mg of rutin, 0.3 g $(NH_4)_2HPO_4$, 0.1 g KH_2PO_4, 0.4 g NZ-amine, trace metals and vitamins (Westlake et al., 1959). The mycelium was removed from the fermented medium by millipore filters (pore size 0.45 μ) and the filtrate used as the source of enzyme. For routine investigations, the reaction mixture consisted of 0.5 g of rutin, 25 ml of 0.125 M phosphate buffer, pH 6.7, and 75 ml of

* Issued as N.R.C. No. 5511

culture filtrate.

Tests for the production from rutin of carbon dioxide, formic acid, formaldehyde and methanol gave negative results. Although oxygen was required for the enzymatic reaction, consumption of oxygen or production of carbon dioxide could not be demonstrated by manometric techniques. Drs. A. C. Neish and J. E. Watkin of this laboratory generously prepared rutin-3-C^{14} by feeding buckwheat plants p-hydroxycinnamic acid labeled in the α-carbon and recovering the rutin synthesized by the plant (Underhill et al., 1957). When 18.9 mg of this rutin (47.6 mμc) was incubated at room temperature with 5 ml of culture filtrate, the mixture swept with carbon dioxide-free air and the effluent gases passed through an alkali absorption tower, no radioactivity remained in the reaction mixture or was trapped in the alkali. The gases from subsequent experiments with non-radioactive rutin gave positive tests for carbon monoxide with palladium chloride, with ammoniacal silver nitrate, and with gas phase chromatography on a molecular sieve column. (We are indebted to Dr. J. M. Roxburgh of this laboratory for the chromatographic analyses.)

Since in the manometric experiments under air neither production nor consumption of gas could be observed, the amount of carbon monoxide produced must have been equal to the oxygen consumed. This was confirmed by degrading rutin in sealed flasks (Neish, 1953) of known volume. The flasks were shaken for 4 hours at 30° C, equilibrated at 25° C, then samples of the gases withdrawn by means of a syringe and analyzed by gas phase chromatography at 25° C. The reaction mixture was assayed for residual rutin by the aluminum chloride method (Dowd, 1959). Eight hundred and seven micromoles of rutin were degraded, 703 μmoles of oxygen were consumed, and 809 μmoles of carbon monoxide produced. Thus each mole of rutin is oxidized by one mole of oxygen and one mole

of carbon monoxide is produced.

Confirmation that carbon monoxide arose from carbon-3 and that the ratio of oxygen consumed to rutin degraded is one was obtained in a manometric experiment. Rutin-3-C^{14} (11.3 mg, 18.5 μmoles, 28.5 mμc) was transferred to one side arm of a Dixon-Keilin flask whose stopcock had been bored completely through. The second side arm contained 0.4 ml of palladium catalyst (40 μmoles $PdCl_2$, 40 μmoles of HCl and 10 μmoles of $ZnSO_4$). The center well (stopcock closed) held 0.7 ml of 1 N NaOH and the main compartment 3.3 ml of enzyme and 0.1 ml of 0.125 M phosphate buffer, pH 6.7. After equilibration at 30° C the reaction was begun by tipping the enzyme into the side arm containing the rutin and washing the rutin into the main compartment. The insoluble rutin completely disappeared within 2.5 hours, but complete oxidation by the palladium catalyst of carbon monoxide to carbon dioxide required another 2.5 hours. Eighteen micromoles of oxygen was consumed. The alkali in the center well was recovered by washing through the open stopcock and the activity of the absorbed carbon dioxide found to be 27.8 mμc, i.e. 96% of the C^{14} in the original rutin.

A. niger apparently degrades rutin by first removing rutinose with a β-glycosidase, then oxidatively cleaving the double bond between carbons-2 and -3 to form an unstable intermediate from which carbon monoxide is released to yield the depside of phloroglucinol carboxylic acid and protocatechuic acid. The latter compound is subsequently hydrolyzed by an esterase to give phloroglucinol carboxylic acid and protocatechuic acid.

Carbon monoxide has been reported to be a product of respiration of the giant kelp Nereocystis luetkeana (Langdon, 1917) and of the alga Anacystis nidulans (Gafford - see Wilks, 1959). Wilks (1959) observed that plants and chlorophyll fractions from plants, when illuminated in the presence of oxygen, gave off carbon

monoxide. The present findings indicate the flavonoid pigments may be a source of carbon monoxide. Now that microorganisms, plants, and apparently animals (Metz and Sjöstrand, 1954) have been demonstrated to produce carbon monoxide, and organisms are known that catalyze the oxidation to carbon dioxide (Yagi, 1959), a carbon monoxide cycle may be said to exist in nature.

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