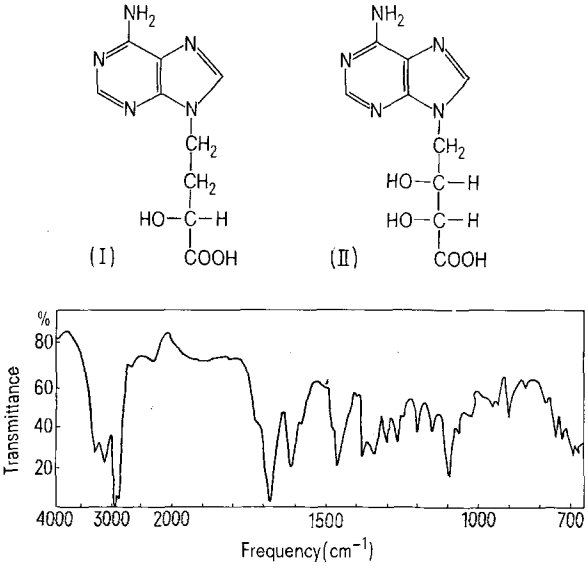
Isolation of Intermediate in Biosynthesis of Eritadenine from Adenine

A new hypocholesterolemic substance, eritadenine[1], was isolated from an edible mushroom 'Shiitake' (*Lentinus edodes*), and its chemical structure was confirmed in our laboratory[2] to be 4-(6-amino-9H-purine-9-yl)-2,3-dihydroxybutyric acid (II). Eritadenine is the first example of the adenine 9-substituted with hydroxy acid in natural products and its biosynthesis is of special interest in our investigation. In view of the structural relationship of eritadenine to adenine, the latter substance is presumed to be a possible precursor of eritadenine. To prove this hypothesis, adenine-8-C^{14} was infused into a Shiitake. From the distribution of C^{14} it was suggested that eritadenine was synthesized from adenine via the intermediate, substance A. This intermediate was obtained as crystalline powder and identified as 4-(6-amino-9H-purine-9-yl)-3-hydroxybutyric acid (I). SAITO et al.[3] and TOKITA et al.[4] isolated the same substance, but its biological role in Shiitake has not been discussed.



IR-spectrum of substance A (in Nujol).

In the cap of growing Shiitake on trunks of 'Kunugi' (*Quercus acutissima*), a small hole was made with corkborer. 20 μCi of adenine-8-C^{14} dissolved in a small volume of 0.9% sodium chloride solution was gradually infused into the hole and then the hole was blocked up. The Shiitake was harvested at 6 and 24 h after infusion and extracted with 80% ethanol. The extract was fractionated on an amino acid analyzer, Hitachi KLA-3, specially equipped UV-detector. The effluent peaks having absorption at 254 nm were collected. Radioactivity of the respective peaks was determined on a Beckman liquid scintillation spectrometer.

In the Table are shown the radioactivities of major peaks. The most radioactive substance A in 6 h sample was eluted in the fraction between 208 and 212 ml. At 24 h after infusion, the radioactivity of substance A decreased and that of eritadenine increased. Therefore, substance A could be expected to be one of the intermediates in the sequence of the biosynthesis of eritadenine from adenine.

Isolation of the substance from dried Shiitake was made by preparative amino acid analyzer in a manner similar to that previously employed for the isolation of eritadenine[2]. Approximately 70 mg were obtained from 2 kg of dried Shiitake, Anal. C, 45.20; H, 4.62; N, 29.25. Calcd. for $C_9H_{11}O_3N_5$: C, 45.57; H, 4.67; N, 29.53, mp 271-5° (dec.); $[\alpha]_D^{20}$ +17.5° (C, 0.5 in 0.1 *N* NaOH). The substance exhibits a characteristic UV-absorption of 9-substituted adenine $\lambda_{max}^{0.1\,N\,HCl}$ 261 nm (ε, 12824), $\lambda_{max}^{0.1\,N\,NaOH}$ 262 nm (ε, 14038). The IR-spectrum is shown in the Figure. The NMR-spectrum of the sodium salt had the following signals: (60 MHz in D_2O) multiplet at δ (ppm) 2.5 (2 protons), broad triplet at 4.3 (1 proton), triplet at 4.5 (2 protons), and singlet at 8.3 (2 protons). On the basis of these data, the structure of substance A was proposed as 4-(6-amino-9H-purine-9-yl)-3-hydroxybutyric acid.

Radioactivities of fractions isolated by an amino acid analyzer[a]

Peaks	Effluent (ml)	Radioactivity (cpm × 1000) Time of culture after infusion 6 h	24 h
Eritadenine	178–185	39.8	64.6
Substance A	208–212	52.7	30.5
B	245–252	29.9	20.8
Adenine	361–375	33.7	18.8

Adenine-8-C^{14} (22 mCi/m mol) was purchased from Daiichi Pure Chemical Company (Japan). [a] The column contained 0.9 × 50 cm of resin (Amberlite CG-120). It was operated at 50° using buffer flow rate of 30 ml/h. The buffer change (PH 3.25 to 4.25) was made after 2.5 h, and the change to pH 5.28 after 5 h.

Zusammenfassung. Zur Biosynthese von Eritadenin in einem Speisepilz «Shiitake» wurde 4-(6-amino-9H-purine-9-yl)-3-hydroxybuttersäure als ein Zwischenprodukt isoliert und identifiziert.

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Effects of Fruit on Ribulosediphosphate Carboxylase Activity in *Citrus madurensis* Leaves

Previous measurements of photosynthetic rates of *Citrus madurensis* Loureiro (Tanaka, 1954) cuttings showed intensified CO_2-uptake in fruiting as compared with non-fruiting plants[1], and other workers have reported stimulatory effects of various 'metabolic sinks' on CO_2-uptake[2,3]. WAREING et al.[2], also suggested that hormones derived from these sinks may stimulate formation of carboxylating enzymes in neighbouring leaves. In

this communication we have examined the effect of fruit on ribulosediphosphate carboxylase activity and soluble protein content in citrus leaves.

Methods. Cuttings consisting of a lamina, petiole, axillary bud and a 2 cm piece of stem were taken from mature twigs of *C. madurensis* growing in the tropical plant collection at the Faculty of Agriculture, University of Malaya. The cuttings were rooted in a propagation box, and transplanted into a plastic container holding 8 l of coarse river sand. All plants were watered twice daily with a modified Hoagland solution. 4 plants were selected for the experiment on the basis of uniform growth. Flowers developed at the same time in the 4th growth cycle, and were repeatedly removed from 2 plants, while fruit set was allowed on the other two. Leaves were sampled from this 4th growth cycle on both sets of plants 56 days after fruit set, when the developing fruits had reached the phase of cell enlargement.

Ribulosediphosphate carboxylase (EC 4.1.1.39) activity was assayed as follows: leaves were cut into 1 cm strips, and homogenized in pH 8.3 *Tris*-glycine buffer[4] (ratio of tissue to buffer – 1.2 g/10 ml). The final volume of the extract was made to 6 ml, and enzyme activity assayed in a final volume of 0.4 ml: 0.1 ml of leaf homogenate (after filtration through 2 layers of cheese cloth) was added to 0.2 ml of a reaction mixture containing 25 µmoles *Tris* (adjusted to pH 8.0 with HCl), 10 µmoles $MgCl_2$, and 0.26 µmoles EDTA. To this was added 0.05 ml mercaptoethanol (0.31 µmoles), and 0.1 ml $Na^{14}CO_3$/$NaHCO_3$ solution (10 µCi–0.17 µmole/22.3 µmoles). This mixture was incubated at 30 °C for 20 min, and the reaction initiated by adding 0.1 ml of ribulose-1.5-diphospahte (1.5 µmoles). Samples were withdrawn from the reaction mixture at 2 min intervals for 10 min, and the reaction terminated by pipetting 50 µl portions into vials containing 5 ml of an ethanol (95% v/v) – formic acid (90% v/v) mixture (1:1 v/v). Counting was performed in a dioxane based fluor at 53% efficiency.

Results and discussion. Ribulosediphosphate carboxylase activity – expressed as amount of CO_2-fixed per g leaf fresh weight per min – is presented in the Table. Results are means of duplicate determinations performed on separate lots of paired plants, and standard errors are included. Both enzyme activity and soluble protein contents are higher in the leaves of fruiting than in non-fruiting plants. These differences are significant at the 99% confidence level (*t*-test).

Effect of fruit on the amount of $^{14}CO_2$ fixed per unit fresh weight

Sample	Amount (µmoles) of $^{14}CO_2$ fixed/g fresh weight/min.	Soluble protein (mg/g fresh weight)
Fruiting plants	0.39 ± 0.05	22.3 ± 1.2
Non-fruiting plants	0.18 ± 0.02	17.2 ± 1.3

The possibility exists that the observed differences in enzyme levels are the result of a hormone imbalance caused by the removal of fruit. Auxins, cytokinins, ethylene, and gibberellins have all been detected in citrus fruits[5], and various workers have shown that ribulosediphosphate carboxylase activity can be hormonally regulated[2,6,7]. However, the possibility should not be excluded that the flux of carbohydrate from leaves to fruits has a regulatory effect on enzyme activity for LENZ and KÜNTZEL[8] have reported considerable variation in sugar and starch contents between leaves of fruiting and non-fruiting plants.

Increases in soluble protein, though significant, do not parallel the increases in enzyme activity. It is therefore suggested, that both the amount and specific activity of ribulosediphosphate carboxylase increases in the leaves of fruiting plants, and further investigations are necessary to determine the relative importance of each process.

Zusammenfassung. An fruchtenden und entblüteten Stecklingen von *Citrus madurensis* wurde an vergleichbar inserierten Blättern die Aktivität von Ribulose-Diphosphat Carboxylase und der Gehalt an löslichen Proteinen bestimmt. Sowohl die Aktivität von RuD Carboxylase als auch der Gehalt an löslichen Proteinen waren in Plättern fruchtender höher als in solchen nicht fruchtender Planzen.

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Inhibition of Protein Synthesis in Ehrlich Ascites Tumor Cells by Irradiation (365 nm) in the Presence of Skin-Photosensitizing Furocoumarins

The Ehrlich ascite tumor cells lose their in vivo ability to induce tumors after irradiation with long wavelength UV- light (365 nm) in the presence of skin-photosensitizing furocoumarins[1]. The tumor cells photoinactivated as above behave as the untreated control cells with regard to the exclusion of vital dye and oxygen uptake[2]; on the contrary their nucleic acids synthesis is strongly inhibited[3].

These results are related to the capacity of the furocoumarins to photoreact by a C_4-cycloaddition with the pyrimidine bases of nucleic acids, DNA and RNA[4–7]; the formation of covalent linkages between furocoumarins and DNA by irradiation of Ehrlich ascite cells in the presence of ^{3}H-psoralen was previously demonstrated[8].

This paper deals with the protein synthesis after photosensibilization by furocoumarins using the Ehrlich ascite cells as a model.

The cells ($2–3 \times 10^6$/0.1 ml) suspended in saline solution containing the furocoumarin were irradiated in Petri dishes with a Philips HPW 125 lamp (365 nm; irradiation