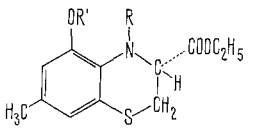
was then acidified to pH 2.5 and extracted with ether. Removal of the solvent gave 76 mg (94% yield) of an amorphous powder (VI) $C_{10}H_{11}NO_3S$ (by high resolution mass spectrometry), $[\alpha]_D$ — 33.6° (0.54% in $C_2H_5OH$; $\lambda_{max}^{EtOH}$ 306, 230 nm (log $\varepsilon$, 3.35, 4.31); $\lambda_{max}^{N\,HCl}$ 291, 283, 252, 217 nm (log $\varepsilon$, 3.57, 3.61, 3.94, 4.36).

Similar yields (96%) of compound VI were also obtained when a solution of V (0.87 g) in phosphate buffer at pH 8.2 (80 ml) was oxidized with $O_2$ for 20 min at room temperature.

*Characterization of VI.* Crystalline stable derivatives of VI were obtained as follows: a solution of VI (500 mg) in anhydrous ethanol was saturated with HCl and the mixture left at room temperature for 48 h. The crude ethyl ester (450 mg) was then treated (70 h, room temperature) with acetic anhydride (30 ml) and pyridine (20 ml). Removal of the solvents left an oil which was separated by PLC over silica ($F_{254}$, Merck) with 50% ether-chloroform to give three products (Rf 0.64, 0.54 and 0.30; located by UV-light) which were characterized as VII, VIII and IX, respectively, on the basis of the following evidence.




VII; R=H, R'=COCH$_3$
VIII; R=R'=COCH$_3$
IX; R=COCH$_3$, R'=H

Compound VII (216 mg), analyzed for $C_{14}H_{17}NO_4S$[11] ($M^+$ 295), formed prisms from ethanol, mp 76–77 °C, $[\alpha]_D$ — 49.2° (0.51% in $CHCl_3$); $\lambda_{max}^{EtOH}$ 311, 231 nm (log $\varepsilon$, 3.58, 4.36); $\nu_{max}$ ($CCl_4$) 3370 (NH), 1775 ($OCOCH_3$), 1745 ($COOC_2H_5$) cm$^{-1}$; NMR ($CCl_4$): $\delta$ 1.23 (3H, *t*, J ≃ 7, $OCH_2$–$CH_3$), 2.18 (6H, *s*, $ArCH_3$ and $OCOCH_3$), 3.0 (2H, unresolved 8-lines *m*, S–$CH_2$–CH, AB part of an ABX system), 4.10 (1H, *dd*, S–$CH_2$–CH, X part of the ABX system), 4.15 (2H, *q*, J ≃ 7, $OCH_2$–$CH_3$), 4.55 (1H, *br s*, NH), 6.55 (1H, aromatic proton), 6.58 (1H, aromatic proton).

Compound VIII (222 mg), $C_{16}H_{19}NO_5S$ ($M^+$ 337), mp 155–157 °C (from ethanol), $[\alpha]_D$ — 86.1° (0.50% in $CHCl_3$); $\lambda_{max}^{EtOH}$ 261, 232 nm (log $\varepsilon$, 4.04, 4.38); $\nu_{max}$ ($CHCl_3$) 1770 ($OCOCH_3$), 1745 ($COOC_2H_5$), 1665 ($NCOCH_3$) cm$^{-1}$; NMR ($CDCl_3$): $\delta$ 1.17 (3H, *t*, J ≃ 7, $OCH_2$–$CH_3$), 2.02 (3H, *s*, N–$COCH_3$), 2.29 (6H, *s*, $ArCH_3$ and $OCOCH_3$), 2.97 and 3.46 (2H, two *q*, $J_{AB}$ ≃ 13, $J_{AX}$ ≃ 9, $J_{BX}$ ≃ 8, S–$CH_2$–CH), 4.10 (2H, *q*, J ≃ 7, $OCH_2$–$CH_3$), 5.64 (1H, *dd*, S–$CH_2$–CH), 6.93 (1H, aromatic proton), 7.09 (1H, aromatic proton).

Compound IX (31 mg), $C_{14}H_{17}NO_4S$ ($M^+$ 295), mp 120 to 122 °C (from ethanol); $\lambda_{max}^{EtOH}$ 287 (sh), 262, 228 nm (log $\varepsilon$, 3.73, 3.99, 4.38); $\nu_{max}$ ($CHCl_3$) 3330 (OH), 1720 ($COOC_2H_5$), 1665 (N–$COCH_3$) cm$^{-1}$.

*Discussion.* The results reported in this study show evidence that, under the conditions of the in vitro phaeomelanogenesis, 5-S-cysteinyldopa (I) as well as 2-S-cysteinyldopa (II) are rapidly converted into the corresponding dihydrobenzothiazines III and IV, which are the first UV-detectable intermediates preceding the appearance of pigments. The assignment of the structures III and IV for these new intermediates is mainly based on their absorption spectra and on the mass spectra of the corresponding diethyl esters, because the instability of the products has precluded chemical characterization. However, indirect evidence for the proposed structures is provided by the study of the model compound (V), the cyclization product (VI) of which has been unequivocally characterized by spectral and chemical data.

Considering that the cyclization of the cysteinic residue of the model compound (V), as well as of the phaeomelanin precursors (I and II), takes place almost quantitatively[12] within a few min, a self-catalyzed reaction involving oxidation and reduction steps is likely for the formation of dihydrobenzothiazines (scheme 1). Presumably, the cyclization reaction is initiated by the formation of a catalytic amount[13] of *o*-quinone (B) which undergoes an intramolecular elimination of water producing as intermediate an *o*-quinonimine derivative (C); this may be reduced to dihydrobenzothiazine by the starting product which in turn is oxidized to the corresponding *o*-quinone and the reaction continues spontaneously.

Further experiments are now required to clarify the pathway by which the dihydrobenzothiazines III and IV, the existence of which has not been described previously, are oxidized in the subsequent steps of the in vitro phaeomelanogenesis.

*Riassunto.* Mediante esperimenti in vitro, è stato accertato che i primi stadi della biosintesi delle feomelanine conducono alla formazione di derivati diidrobenzotiazinici, ai quali sono state assegnate le strutture III e IV.

G. Prota, S. Crescenzi,
G. Misuraca and R. A. Nicolaus

*Istituto di Chimica Organica dell'Università di Napoli, Stazione Zoologica, Napoli (Italy), 27 April 1970.*

[11] Satisfactory C, H, N and S analyses of VII, VIII and IX were obtained. IR-spectra were recorded on a Perkin-Elmer Infracord (mod. 137 E) and NMR-spectra on a Varian A-60 apparatus (internal reference TMS); *s* denotes a singlet, *d* a doublet, *t* a triplet, *q* a quartet, *m* a multiplet, *dd* a doublet of doublets, *br* broad.

[12] The quantitative conversion of I (or II) into III (or IV) can be observed spectrophotometrically, but the unfavourable properties of the product precludes its isolation in very high yields.

[13] As expected, manometric experiments showed that only a catalytic amount of oxygen is required during the cyclization reaction.

## The Synthesis of Monofluoroacetic Acid by a Tissue Culture of *Acacia georginae*

Monofluoroacetic acid is a highly toxic compound to mammals and has been detected in several plant species from Australia, South Africa, and Brazil[1]. The assumption that the synthesis of this compound may be a response to high concentrations of available inorganic fluoride in the soil or water appears to be supported by the observations of Cheng et al.[2] and Lovelace et al.[2] who have reported the presence of fluoroacetate in the foliage of soybeans and common forage plants exposed to gaseous inorganic fluoride.

Peters et al.[3] and Preuss[3] have described the in vivo synthesis of fluoroacetate by *Acacia georginae* grown

under controlled conditions. PETERS et al.[4] have also reported the in vitro synthesis of fluoroacetate by extracts of *Acacia georginae* in the presence of sodium pyruvate, potassium fluoride, manganese chloride, potassium phosphate, and adenosine triphosphate. However, these systems have not facilitated the study of the biosynthesis of fluoroacetate, since individual plants of *Acacia* are extremely variable in their capacity to synthesize fluoroacetate in the presence of inorganic fluoride.

Experiments were designed to determine the capacity of a tissue culture derived from *Acacia georginae* to synthesize fluoroacetate. The tissue culture, derived from a single primary explant, and growing under carefully controlled conditions of nutrient supply, light, and temperature, provided a uniform and reliable source of material. The callus culture was derived from a stem section of a seedling of *Acacia georginae* and was grown on a medium similar to that described by MURASHIGE and SKOOG[5,6].

The cultures were grown for 10 weeks on this medium alone or after the addition of $10^{-3}M$ sodium fluoride. For purposes of comparison, a tissue culture of tomato stem was grown under similar conditions[7]. The cultures were grown in an incubator with a twelve-hour light period and 'day' and 'night' temperatures of $25.5 \pm 0.5$ °C and $21.5 \pm 0.5$ °C, respectively. After the 10-week growth period, the cultures and the media on which they were growing were extracted and analyzed to determine the presence of fluoroacetate. The procedures will be described elsewhere[8].

Fluoroacetate was identified by the retention time of the methyl ester by gas chromatography on 3 different columns (Table). The column of Tween 80-phosphoric acid gave the best separation. In addition to fluoroacetic acid, the following acids were also identified (in order of their retention time on the Tween 80 column): glycolic, oxalic, propionic, malic, and succinic. A peak as yet unidentified is eluted between malic and succinic acids.

The peak corresponding to methyl fluoroacetate was obtained only from extracts of *Acacia* tissue culture grown on the medium containing sodium fluoride and from an extract of the medium itself. All other extracts of *Acacia* and tomato tissue cultures, and the media on which they were grown, gave no detectable fluoroacetate.

These results demonstrate that a stem tissue culture of *Acacia georginae* grown on a medium with added inorganic fluoride can synthesize the carbon-fluorine bond in the form of fluoroacetate. Previous work has suggested that the root is the probable site of synthesis of fluoroacetate in intact plants of *Acacia*[3]. The present results suggest that synthesis of fluoroacetate is not restricted only to the root of the intact plant or that the capacity to synthesize fluoroacetate is latent in the stem tissue of the plant.

It has not yet been determined if the synthesis of fluoroacetate in plants is catalyzed by enzyme systems, although preliminary experiments on in vitro synthesis indicate that this is the case. Nothing is known at present about the nature of the precursor of the two-carbon fragment of fluoroacetate, even though several suggestions have been made[4]. The plant tissue culture described is a uniform and reproducible biological system that grows and multiplies under defined conditions, is capable of synthesizing fluoroacetate, and may provide a useful tool for the study of this problem[9].

*Zusammenfassung.* Gewebekulturen von *Acacia georginae* vermögen Fluorid-Ionen aus dem Kulturmedium in Fluoressigsäure umzuwandeln.

P. W. PREUSS, L. COLAVITO[10] and L. H. WEINSTEIN[10]

*Department of Organic Chemistry, Hebrew University, Jerusalem (Israel), and Boyce Thompson Institute for Plant Research, Yonkers (New York, USA), 14 April 1970.*

Operating conditions for gas chromatographic analysis of methyl fluoroacetate

| | | | |
|---|---|---|---|
| Gas chromatograph | Packard Model 7620 | | |
| Detector | Flame ionization | | |
| Column | Glass | | |
| | *Dinonyl phthalate* | *Tween 80* | *Poropak Q* |
| Column dimensions | $^1/_4'' \times 6'$ | $^1/_4'' \times 6'$ | $^1/_8'' \times 6'$ |
| Column temperature | 50 °C | 88 °C | 150 °C |
| Detector temperature | 110 °C | 115 °C | 175 °C |
| Injector temperature | 110 °C | 110 °C | 175 °C |
| Solid support | Acid washed, DMSS treated chromosorb | Acid washed firebrick | Poropak Q |
| Liquid phase | 20% dinonyl phthalate | 10% tween 80-$H_3PO_4$ (9:1) | None |
| Carrier gas flow rate | 50 cm³/min | 50 cm³/min | 17 cm³/min |
| Carrier gas | Argon | Argon | Argon |

[1] J. S. C. MARAIS, Onderstepoort J. vet. Sci. Anim. Ind. *20*, 67 (1944); *18*, 203 (1943). – P. B. OELRICHS and T. MCEWAN, Nature *190*, 808 (1961). – T. MCEWAN, Nature *201*, 827 (1964). – M. M. OLIVEIRA, Experientia *19*, 586 (1963).

[2] J. LOVELACE, G. W. MILLER and G. W. WELKIE, Atmosphere Envir. *2*, 187 (1968). – J. CHENG, M. YU, G. W. MILLER and G. W. WELKIE, Envir. Sci. Technol. *2*, 367 (1968).

[3] R. A. PETERS, L. R. MURRAY and M. SHORTHOUSE, Biochem. J. *95*, 724 (1965). – P. W. PREUSS, Doctoral Dissertation, Columbia University (1967).

[4] R. A. PETERS, M. SHORTHOUSE and P. F. V. WARD, Life Sci. *4*, 749 (1965).

[5] T. MURASHIGE and F. SKOOG, Physiologia Pl. *15*, 473 (1962).

[6] Indole acetic acid was replaced by 0.05 mg/l 2,4-Dichlorophenoxyacetic acid, Edamine by 1.0 g/l Bacto-Tryptone (Difco Laboratories, Detroit, Michigan, USA). Kinetin was used at a concentration of 0.32 mg/l, and agar at 9.0 g/l.

[7] This culture was obtained from Dr. WALTER TULECKE, Antioch College, Yellow Springs, Ohio.

[8] Israel Journal of Botany, in press (1970).

[9] This investigation was supported in part by Training Fellowship No. 3 F3 AP-36053 and Research Grant No. AP 00189 from the National Air Pollution Control Administration, Consumer Protection and Environmental Health Service, US Public Health Service.

[10] Present address: Boyce Thompson Institute for Plant Research, Yonkers (New York, USA).