expected contaminants. The results based on area measurement (values not shown in figure) are consistent with the quality of the standard.

In conclusion, the HPIEC-technique described in this communication exceeds the classical separation procedures in speed of analysis and possibility of automatic operation. Besides its application in biological studies, this can be used for following the proteolytic degradation of milk proteins.

1 Swaisgood, H. E., in: Developments in Dairy Chemistry, vol. 1, Proteins, p. 1. Ed. P. F. Fox. Applied Science Publishers, London and New York 1982.
2 McKenzie, H. A., and Gordon, W. G., in: Milk Proteins, Chemistry and Molecular Biology, vol. II, pp. 87, 257 and 332. Ed. H. A. McKenzie. New York and London 1971.
3 Bican, P., J. Dairy Sci. *66* (1983) 2195.
4 Bican, P., and Blanc, B., Milchwissenschaft *37* (1982) 592.
5 Diosady, L. L., Bergen, I., and Harwalkar, V. R., Milchwissenschaft *35* (1980) 671.
6 Pearce, R. J., Aust. J. Dairy Technol. *38* (1983) 114.
7 Conti, A., Napolitano, L., and Liberatori, J., Milchwissenschaft *38* (1983) 392.
8 Sober, H. A., and Peterson, E. A., J. Am. chem. Soc. *76* (1954) 1711.
9 Regnier, F. E., Analyt. Biochem. *126* (1982) 1.
10 Zittle, C. A., and Custer, J. H., J. Dairy Sci. *46* (1963) 1183.

0014-4754/85/070958-03$1.50 + 0.20/0

Tyrosinase-cytalyzed conjugation of dopa with glutathione[1]

S. Ito[2], A. Palumbo and G. Prota

Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11 (Japan), and Stazione Zoologica di Napoli, Napoli 80121 (Italy), 19 June 1984

Summary. A convenient method is described for the preparation of 5-S- and 2-S-glutathionyldopa, based on tyrosinase oxidation of dopa in the presence of glutathione. The yields of 5-S, 2-S, and 6-S isomers produced were about 76, 12, and 5%, respectively.
Key words. S-glutathionyldopa; tyrosinase; dopa; glutathione.

In recent years, the origin and metabolism of cysteinyldopas[3], i.e. the 2-S, 5-S, and 6-S isomers, have received a good deal of attention following the extensive studies by Rorsman and his associates on the clinical use of these metabolites, as biochemical markers for early diagnosis of metastasizing melanomas[4]. Available evidence suggests that formation of cysteinyldopas in melanocytes may proceed 1) via direct addition of free cysteine to dopaquinone generated by tyrosinase-catalyzed oxidation of dopa[3], or 2) by the analogous reaction of the quinone with glutathione (GSH) followed by enzymic hydrolysis of the resulting glutathionyldopas, or 3) by both mechanisms. Which of these pathways prevails in vivo is not yet clear, but it is noteworthy that both 5-S-cysteinyldopa and 5-S-glutathionyldopa (fig. 1) have been found in melanoma tissues, which also seem to contain the hydrolytic enzymes (γ-glutamyl-transferase and peptidase) capable of converting the latter into the former[5]. This finding, coupled with the fact that GSH is generally the most abundant thiol in cells[6], points to an important role of glutathionyldopas in the biosynthesis of cysteinyldopas. As a part of our continuing studies on the role of sulphydryl compounds in pigment cell metabolism[7,8], we report here a convenient procedure for the preparation on a semi-preparative scale of 5-S- and 2-S-glutathionyldopa by tyrosinase-catalyzed conjugation of dopa with GSH.

Materials and methods. Analytical method. Conditions for high-performance liquid chromatography (HPLC) were as follows: chromatograph, Yanaco Model L-2000; detectors, Yanaco VMD-101 electrochemical detector (750 mV vs Ag/AgCl reference electrode) and Yanaco U-213 UV detector (254 nm); column, Yanaco ODS-A (4.6 × 250 mm); mobile phase, 0.1 M potassium phosphate buffer, pH 2.1–methanol, 96:4 (v/v); column temperature, 45 °C; flow rate, 0.7 ml/min. Peptide samples (ca. 1 mg) were hydrolyzed with 6 M HCl (1 ml) in evacuated, sealed tubes at 110 °C for 24 h. The hydrolysates were evaporated to dryness and analyzed with a JEOL JLC-6AH amino acid analyzer using a 4 lithium citrate buffer system.

Preparation of glutathionyldopas. A solution of L-dopa (197 mg; 1 mmole) and GSH (614 mg; 2 mmoles) in 100 ml of 0.05 M sodium phosphate buffer, pH 6.8, was stirred at room temperature after the addition of mushroom tyrosinase (50 mg; 2000 units/mg from Sigma Chem. Co.). The reaction was monitored by HPLC. After 3 h when most of dopa (> 95%) had disappeared, the reaction was stopped by the addition of 6 M HCl (5 ml). The reaction mixture was passed through a column (2.0 × 8 cm) of Dowex 50W-X2 (200–400 mesh, equilibrated with water). After washing with 0.5 M HCl (100 ml), the column was eluted with 3 M HCl and 20-ml fractions were collected and monitored by HPLC. Fractions 1–7, which contained the glutathionyldopas, were evaporated to dryness in a rotary evaporator at 40 °C; each evaporation was conducted on 40 ml volume or less to minimize hydrolytic cleavage of the peptide bonds. The residue, taken up in 2 M HCl (2 ml), was chromatographed as described in figure 2. Fractions 13 and 14

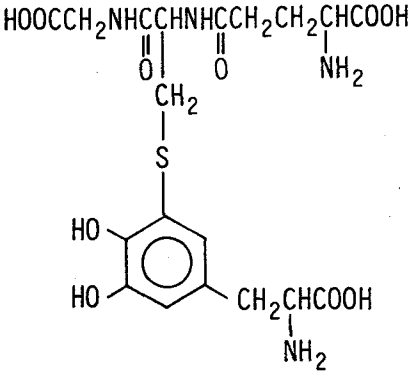

Figure 1. Structure of 5-S-glutathionyldopa.

were combined and rechromatographed on the same column. Evaporation of appropriate fractions in a rotary evaporator (40 ml volume or less for each evaporation) left 424 mg of 5-S-glutathionyldopa dihydrochloride (from fr. 23–28 in fig. 2) and 52 mg of 2-S-glutathionyldopa dihydrochloride (from fr. 12 in fig. 2 and fr. 12 and 13 on rechromatography). A mixture of 6-S- and 2-S-glutathionyldopa (53 mg) was also obtained from fraction 15 in figure 2 and fractions 14–16 on rechromatography. Crystallization from water:pyridine:ethanol gave 231 mg of 5-S-glutathionyldopa (93% purity by HPLC) and 27 mg of 2-S-glutathionyldopa (96% purity by HPLC) as colorless to pale blue, fine crystals.

The 5-S-glutathionyldopa had a m.p. ca. 230 °C (dec.). Composition was found to be: C, 43.30; H, 5.85; N, 10.00; S, 5.85%. Calculated composition for $C_{19}H_{26}N_4O_{10}S \cdot 1.5\ H_2O$: C, 43.10; H, 5.52; N, 10.58; S, 6.05%. UV (in 0.1 M HCl) λ_{max} 291 nm (ε 2630) and 255 nm (3770). Amino acid composition; glycine:glutamic acid:5-S-cysteinyldopa, 1.00:1.00:0.94. PMR (in 2 M DCl–D_2O) δ 6.86 (s), 6.77 (s).

The 2-S-glutationyldopa had a m.p. ca. 205 °C (dec.). Composition found: C, 40.28; H, 5.85; N, 9.69; S, 5.80%. Calculated for $C_{19}H_{26}N_4O_{10}S \cdot 3.5H_2O$: C, 40.35; H, 5.88; N, 9.91; S, 5.67%. UV (in 0.1 M HCl) λ_{max} 291 nm (ε 3110) and 256 nm (2940). Amino acid composition; glycine:glutamic acid:2-S-cysteinyldopa, 1.00:1.00:0.93. PMR (in 2 M DCl-D_2O) δ 6.94 and 6.81 (ABq, J = 8.1 Hz).

Results and discussion. Oxidation of dopa in the presence of GSH proceeds smoothly, giving 5-S-, 2-S-, and 6-S-glutathionyldopa and diglutathionyldopa in a quantitative total yield. Attempts to separate the mixture on boric acid gel (Affigel 601 from Bio-Rad) were unsuccessful, but the separation was achieved on Dowex 50W (fig. 2) using as the eluent 2 M HCl, under conditions similar to those used for the separation of cysteinyldopa isomers[3]. Evaporation and crystallization afforded free 5-S- and 2-S-glutathionyldopa in reasonably pure form. Care should be taken in the evaporation to minimize hydrolytic cleavage of the peptide bonds. The structures of 5-S- and 2-S-glutathionyldopa were proved by 1) the close similarity of the UV spectra to those of 5-S- and 2-S-cysteinyldopa[3,9], 2) the amino-acid compositions, and 3) the PMR spectra indicating the substitution patterns on the aromatic ring. The tyrosinase conjugation of dopa with GSH has already been described by Fehling et al.[10]; however, only the major product, 5-S-glutathionyldopa, was isolated by preparative HPLC.

The yields of glutathionyldopa isomers in the reaction mixture were estimated by UV spectrophotometry and HPLC of the eluates (fig. 2) to be approximately 76, 12, and 5% for 5-S-, 2-S-, and 6-S-glutathionyldopa, respectively. This ratio of about 6:1 was close to the approximately 5:1 ratio of 5-S- to 2-S-cysteinyldopa. On the other hand, the yield of 6-S-glutathionyldopa was much higher than that of 6-S-cysteinyldopa (ca. 1%). The yield of diglutathionyldopa (most likely 2,5-S,S isomer) was quite low (ca. 2%) in this experiment; however, the yield became much higher (> 10%) when the reaction time was extended to 4 h. Morishima et al.[11] analyzed the ratio of cysteinyldopa isomers in the urine and melanoma tissues from melanoma patients. The ratios of 5-S-, 2-S-, and 6-S-cysteinyldopa were in the range of 80:9.4–11:2.3–3.3. The proportions of cysteinyldopa isomers in vivo may reflect not only the biosynthetic rates but also the relative ease of subsequent metabolism[12,13] of each isomer and its incorporation into pheomelanin[14]. It is thus difficult to speculate about which of the glutathionyldopa and cysteinyldopa pathways prevails in vivo. However, the present study suggests that more attention should be paid to the role of 6-S-glutathionyldopa (hence 6-S-cysteinyldopa) in pheomelanogenesis.

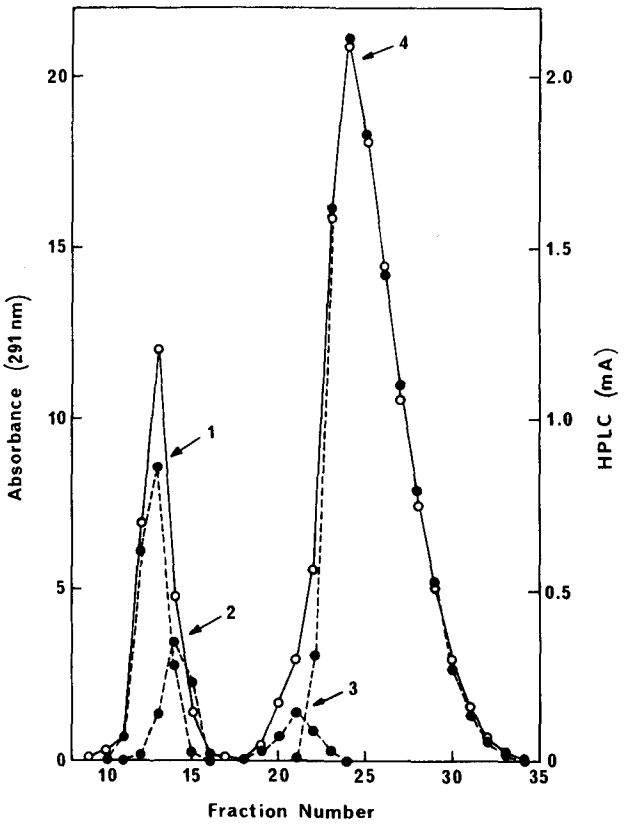

Figure 2. Chromatography of the glutathionyldopa mixture on a column (2.0 × 23 cm) of Dowex 50W-X2 (200–400 mesh; equilibrated with 2 M HCl). The column was eluted with 2 M HCl and fractions of 20 ml were collected and monitored by UV spectrophotometry at 291 nm (○——○) and HPLC with electrochemical detection (●---●). For HPLC analysis, 1 µl of the eluates was directly injected. 5-S-, 2-S-, and 6-S-glutathionyldopa and diglutathionyldopa appeared in the peaks 4, 1, 2, and 3, respectively.

1 One of the authors (G.P.) thanks the C.N.R. (P.F. Chimica Fine e Secondaria) for partial financial support.

2 To whom reprint requests should be addressed at Fujita-Gakuen University.

3 Ito, S., and Prota, G., Experientia *33* (1977) 1118, and references therein.
4 Rorsman, H., Agrup, G., Hansson, C., and Rosengren, E., in: Pigment Cell, vol. 6, p. 93. Ed. R. M. MacKie. Karger, Basel 1983.
5 Agrup, G., Falck, B., Rorsman, H., Rosengren, A.-M., and Rosengren, E., Acta derm.-vener., Stockh. *57* (1977) 221.
6 Jocelyn, P. C., Biochemistry of the SH group. Academic Press, London 1972.
7 Benedetto, J.-P., Ortonne, J.-P., Voulet, C., Khtchadourian, C., Prota, G., and Thivolet, J., J. invest. Derm. *79* (1982) 422.
8 Ito, S., Homma, K., Kiyota, M., Fujita, K., and Jimbow, K., J. invest. Derm. *80* (1983) 207.
9 Ito, S., Inoue, S., Yamamoto, Y., and Fujita, K., J. med. Chem. *24* (1981) 673.
10 Fehling, C., Hansson, C., Poulsen, J., Rorsman, H., and Rosengren, E., Acta derm.-vener., Stockh. *61* (1981) 339.
11 Morishima, T., Tatsumi, F., Fukada, E., Saito, M., Fujita, M., Nagashima, N., and Hanawa, S., Arch. derm. Res. *275* (1983) 76.
12 Ito, S., Teradaira, R., and Fujita, K., Biochem. Pharmac. *21* (1980) 3277.
13 Graff, V., and Paul, E., Br. J. Derm. *106* (1982) 53.
14 Agrup, G., Hansson, C., Rorsman, H., and Rosengren, E., Arch. derm. Res. *272* (1982) 103.

0014-4754/85/070960-02$1.50 + 0.20/0