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Isomeric methoxyindolyl glucosiduronic acids in melanotic urine

It has been known for more than a century that urine from some patients with malignant melanoma contains precursors of melanin[1]. THORMÄHLEN[2,3] found that treatment of melanotic urine with sodium nitroprusside followed by sodium hydroxide gave a violet colour which changed to blue with acetic acid. LINNELL AND RAPER[4] suggested that the melanogens responsible for the Thormählen reaction of melanotic urine might be sulphate or glucosiduronic acid conjugates of 5,6-dihydroxyindole and LEONHARDI[5] obtained some evidence that one of the urinary melanogens was a conjugate of glutamic acid and 5,6-dihydroxyindole. This communication reports the occurrence of 6-methoxyindol-5-yl glucosiduronic acid and, to a lesser extent, of 5-methoxyindol-6-yl glucosiduronic acid in urine from a patient with malignant melanoma. AXELROD AND LERNER[6] found that enzymes from rat liver and hamster melanoma catalysed methylation of 5,6-dihydroxyindole by *S*-adenosylmethionine; with the liver preparation they obtained 6-hydroxy-5-methoxyindole and 5-hydroxy-6-methoxyindole in the ratio 0.45:1.

In the work described here the material in a sample of melanotic urine was separated by paper chromatography in isobutanol–water (13:1, v/v) into two fractions (R_F 0.02 and R_F 0.14) which gave positive Thormählen reactions. After treatment in anaerobic conditions with molluscan β-glucuronidase (EC 3.2.1.31) in 0.1 M acetate (pH 4.3) or with bacterial β-glucuronidase (Sigma Chemical Co., Type V) in 0.1 M phosphate (pH 6.8) the reactive material in the first fraction was converted into derivatives which could be extracted into ether. This conversion was almost completely inhibited in the presence of 5 mM saccharate. The saccharate had been freshly boiled to give about 30 % conversion into saccharo-1,4-lactone, a competitive inhibitor of β-glucuronidase[7]. It may therefore be concluded that the material in the ether extract which gave Thormählen's reaction had been liberated from one or more β-glucosiduronic acid conjugates. On chromatography in the same isobutanol–water system the ether extract gave a single spot with the same R_F (0.8) as the isomeric 5 (or 6) -hydroxy-6 (or 5) -methoxyindoles[8]. The final colour with Thormählen's reagents more closely resembled the bright blue given by the 6-methoxy isomer than the purple-grey given by the 5-methoxy isomer. 5,6-Dihydroxyindole[8] (R_F 0.65) was not detected in the ether extract. Following the observation by AXELROD AND LERNER[6] that the isomeric hydroxymethoxyindoles could be separated by thin-layer chromatography, the ether extract obtained after hydrolysis of the urinary melanogens with bacterial β-glucuronidase was re-examined by chromatography on layers of Kieselgel-G (E. Merck AG.) in CCl_4–pyridine (7:2, v/v). Samples were applied alone or in admixture with one or other of the isomeric hydroxymethoxyindoles. The unknown was separated into a major component (R_F 0.36 alone or with 5-hydroxy-6-methoxyindole; with the Thormählen reagents both unknown and reference compound gave a bright blue colour) and a minor component (R_F 0.55 alone or with 6-hydroxy-5-methoxyindole; the colour given by both unknown and reference compound was purple-grey). In twelve experiments the ratios of the areas of the minor and major components was $(0.18 \pm 0.03):1$. Examination of a 20-fold range of concentrations showed that the R_F of the back edges of the spots was more nearly constant than that of the centroids or front edges in this system. R_F values varied

considerably with size of tank, dryness of the adsorbent and duration of chromatography, but comparison with authentic materials on the one plate was easily achieved.

The ether extract contained unidentified compounds with lower R_F values (0–0.25 in the CCl_4–pyridine system) which darkened in air but did not give Thormählen's reaction. Only those indoles which have no substituent at N-1, C-2 or C-3 have been found to give this reaction[9] so that conjugates of other potential precursors of melanin such as 5,6-dihydroxydihydroindole-2-carboxylic acid would not have been detected in this investigation.

The material which had R_F 0.14 in isobutanol–water and resisted hydrolysis by β-glucuronidase was not identified. Unlike the glucosiduronic acids described here it had marked anionic mobility in 2 % (v/v) formic acid and could not be distinguished from a melanogen which was present in a crude lead salt isolated from melanotic urine by Dr. G. LEONHARDI (Universitäts-Hautklinik, Frankfurt). Neither of the methoxyindolyl glucosiduronic acids described here could be detected in this sample.

Further evidence for methylation of melanin precursors in patients with malignant melanoma was provided by analysis of melanin prepared from the melanotic urine by oxidation with persulphate. (Found: C, 52.4; H, 4.3; N, 4.5; O, 29.6; S, 2.25; OMe, 3.0; Cl, nil; ash (800°), 6.5; loss in weight at 100°, 10.05 %.) The analysis showed the presence of 0.30 alkoxyl groups (calculated as methoxyl) per nitrogen atom in the melanin.

The identity and relative proportions of the melanogens described here would not be unexpected if the reaction described by AXELROD AND LERNER[6] takes place in human tissue. Perhaps of greater significance is the small proportion of patients with malignant melanoma who show detectable melanuria. If excretion of methylated indoles depends on the distribution of malignant melanoma cells in the body its detection may be of interest in clinical examination.

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