THE METABOLIC PRODUCTION OF 4-METHYLUMBELLIFERONE AND ITS β-D-GLUCURONIDE FROM THE CORRESPONDING β-D-XYLOSIDE BY FIBROBLASTS IN CULTURE

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1. Introduction

Following observations that simple β-D-xylopyranosides have the power to uncouple in vivo glycosaminoglycan synthesis from that of the (normally-associated) proteins [1,2], β-D-xylopyranosides are now commonly added to cultures of cells in the course of studies of proteoglycan metabolism [3]. 4-Methylumbelliferyl β-D-xylopyranoside is prominent among the aglycone variants used in such studies, but the emphasis has hitherto been on the isolation and characterization of the high M_r, 4-methylumbelliferyl glycosaminoglycan derivatives secreted into the tissue culture media [4]. During similar studies we have observed that low-M_r fluorescent substances are also produced from the xyloside by cultured fibroblasts and are released into the medium. The characterization of two of these substances is the subject of this paper. This appears to be the first demonstration of 'de-toxicative' glucuronidation by such cells, and may be significant for the metabolism of connective-tissue-targetted substances, e.g., drugs.

2. Materials and methods

4-Methylumbelliferone (MU) was purified as in [5] whereas the corresponding β-D-glucoside (MU-Glc), β-D-glucuronide (MU-Glc.U) and β-D-xyloside (MU-Xyl) were from Koch-Light Labs Ltd (Colnbrook, Bucks). Blue dextran and Sephadex G-10 were from Pharmacia (GB) Ltd.

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Mouse 3T6 fibroblasts were cultured precisely as in [6] except that MU-Xyl (2.8 mM) was dissolved in the medium prior to sterilization by filtration.

Thin-layer chromatograms were done as in [5] but using the following solvent mixtures:
(A) *n*-Butanol:ethanol:water, 4:1:5 (by vol.);
(B) *n*-Butanol:acetic acid:water, 6:1:2 (by vol.);
(C) Ethyl acetate:pyridine:acetic acid:water, 5:5:1:3 (by vol.).
The separated zones were located by low temperature phosphorescence as in [7]. Chromatography was also performed on columns (1 × 100 cm) of Sephadex G-10 equilibrated and eluted at 15 ml/h and 5°C using sequentially de-ionized, ultrafiltered and boiled water. Elution profiles were determined by monitoring optical transmission at 280 nm, or by direct fluorescence measurements on the fractions (3 ml) using the spectrofluorimeter in [8], or by hydrolysis in 2.5 N HCl at 100°C before fluorimetric measurements following [9].

β-D-Xylosidase (EC 3.2.1.37) hydrolyses were performed at 25°C for 60 min in 60 mM sodium acetate buffer (pH 5.2) using 1.19 munits of a fungal preparation from Sigma (London) Ltd. Under these conditions the relative activities towards 25 nmol MU-Xyl, MU-Glc and MU-GlcU were 56, 82 and 0, respectively. β-D-Glucuronidase (EC 3.2.1.31) hydrolyses were performed at 37°C for 60 min in 0.2 M sodium acetate buffer (pH 3.6) using the partially purified (stage 3) preparation of rat preputial enzyme in [10]. Its specificity was checked by the effect on its activity of freshly-prepared 0.1 mM saccharinolactone (see table 1). Glucose was determined using the Multistix system by Ames Ltd and uronic acid estimations were according to [11].

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Table 1
Properties of fluorescence substances isolated from, or relevant to, the fibroblast culture medium

Substance of interest	Fluorescence maxima (nm)		Fluorimetric estimate (μM) of substance in medium			K_{av} on Sephadex G-10	R_F on thin-layer chromatography in solvent		
	Activation	Emission	Direct	After acid hydrolysis	After enzyme hydrolysis		A	B	C
Peak I	322	375	8.7	10.5		0.04	–		–
Peak II	325	373	35.4	38.6	36.2[a] 0.90[b]	0.54	0.43	0.54	0.55
Peak III	325	375	2640	2620	2670[c]	3.86	0.63	0.67	0.93
Peak IV	335[d] 370[e]	445[d] 445[d]	11.3	–	–	25.1	0.87	0.90	0.99
MU	335[d] 370[e]	445[d] 445 450[e]	–	–	–	24.4	0.86	0.89	0.99
MU-Xyl	326	374	–	(98 ± 3%)	(98 ± 3%)[c]	3.88	0.66	0.67	0.93
MU-Glc	325	375	–	–		2.00	0.60	0.59	0.85
MU-GlcU	325	372	–	(98 ± 3%)	(97 ± 3%)[a] (3%)[b]	0.52	0.47	0.52	0.53

[a] Hydrolysed for 60 min at 37°C and pH 3.6 with preputial enzyme; [b] Ditto but in presence of 0.1 mM saccharinolactone; [c] Hydrolysed for 50 min at 25°C and pH 5.2 with fungal enzyme; [d] Determined in 0.35 M sodium acetate buffer (pH 4.3); [e] Determined in 0.2 M sodium glycinate buffer (pH 10.4)

Figures in parenthesis refer to percentage of the theoretical value

3. Results

Four distinct peaks (designated I–IV) were detected when samples (0.5 ml) of the media from a 2 day culture of mouse 3T6 fibroblasts (containing ~2.8 mM MU-Xyl) were chromatographed on Sephadex G-10 and the eluate monitored for optical transmission and fluorescence (fig.1a). Essentially the same result was obtained by acid hydrolysis and fluorimetric estimation of the liberated MU (fig.1b). The fluorescence activation and emission spectra for peaks I, II and III (figs.2a- c; table 1), together with the absorption spectrum of peak III (317 nm maximum; 255 minimum and a shoulder at ~285 nm), were essentially the same as those of typical MU-glycoconjugates [12]. In contrast, the spectra for peak IV under acid and basic conditions were clearly not those of a MU-conjugate (fig.2d), but similar to those of MU itself [12]. The identity of peak IV with that of MU was confirmed by a comparison of their chromatographic properties in 3 different solvent systems (table 1) and on the Sephadex columns (fig.1a,c). The substances of peaks I, II and III also showed the 'increase in fluorescence' characteristic of MU-conju-

Fig.1. Elution profiles from Sephadex G-10 chromatography of substances in, or relevant to, MU-Xyl-containing fibroblast culture medium: (a) optical transmission at 280 nm (▪——▪) or fluorescence (– – –) activated and measured, respectively, at 317 and 380 nm (○) or 365 and 448 nm (●); (b) of fluorescence analysis in glycine buffer after acid hydrolysis (▪——▪) – relative uronic acid concentrations (▲——▲); (c) of authentic MU (○), MU-Xyl (●), MU-Glc (▪), MU-GlcU (□), glucose (▲) and blue dextran (▼); (c') enlarged section of early fractions shown in (c). Note especially the break in the abscissa between 280 and 1020 ml.

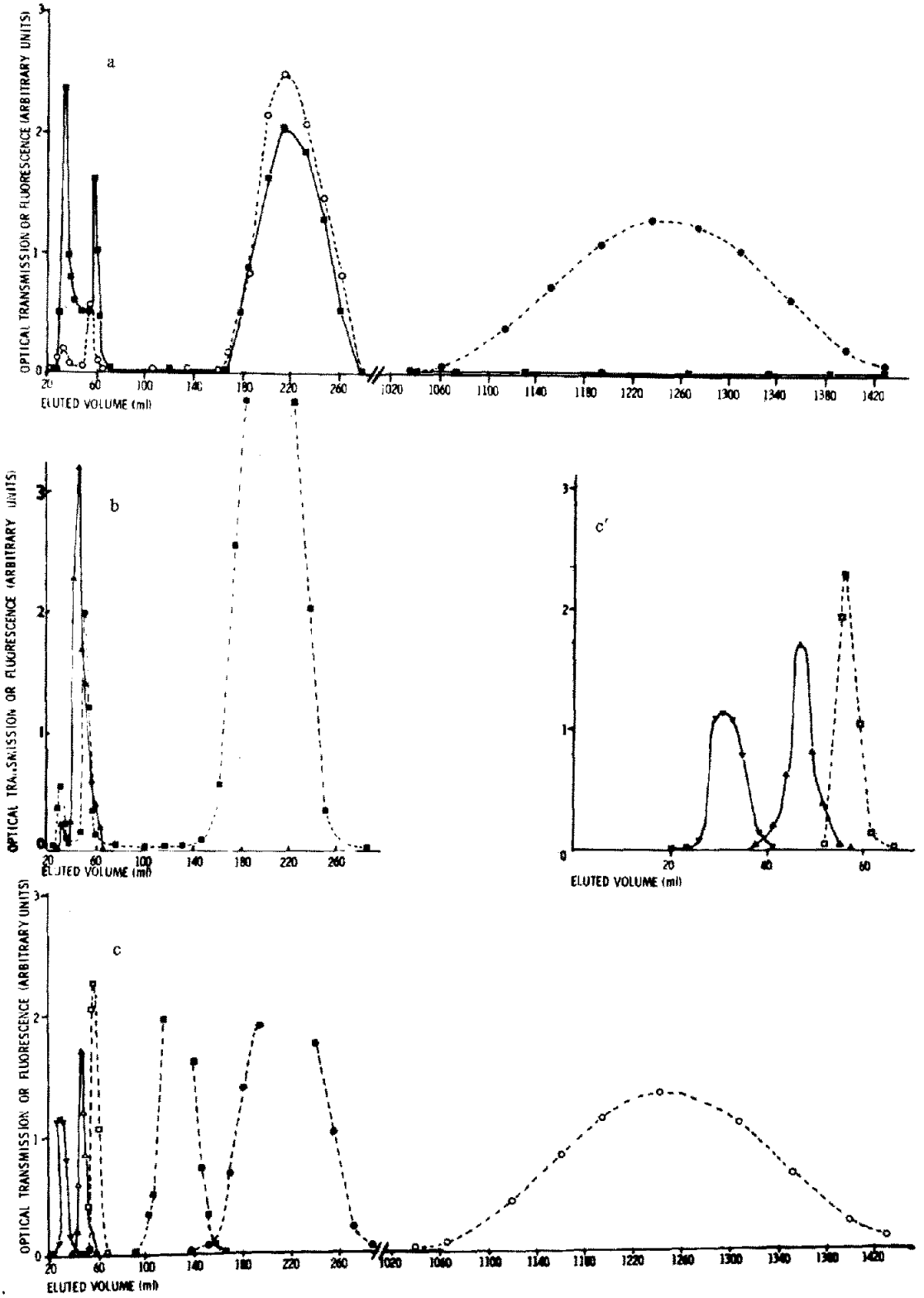
a
b
c
c'
OPTICAL TRANSMISSION OR FLUORESCENCE (ARBITRARY UNITS)
ELUTED VOLUME (ml)

Fig.1.

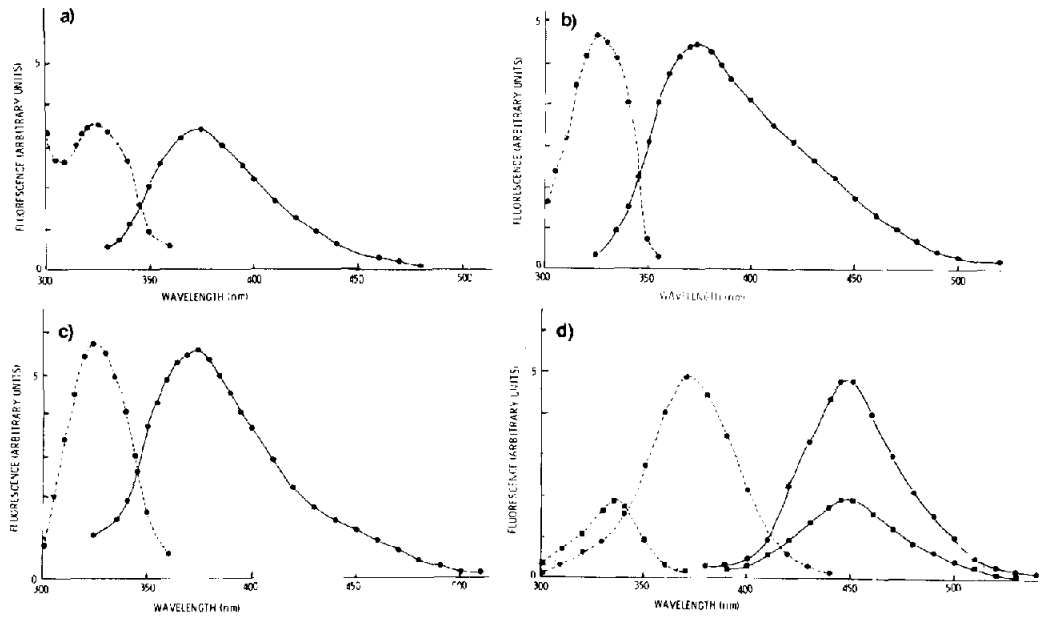

Fig.2. Uncorrected fluorescence activation (– – –) and emission (——) spectra determined on the spectrofluorimeter in [7] for the substances of: (a) peak I; (b) peak II; (c) peak III; and (d) peak IV in 0.2 M glycine buffer (pH 10.4) (•) and 0.35 M acetate buffer (pH 4.3) (▪).

gates after acid hydrolysis (~40-fold) [13] and the liberated material exhibited, under both acid and basic conditions, fluorescence activation and emission spectra typical of free MU (i.e., similar to those in fig.2d).

The fluorescent material in the (large) peak III also showed the low temperature phosphorescence of MU-conjugates [7] and was further identified as residual MU-Xyl (~95% recovery) by comparison of its behaviour with the authentic compound on 3 thin-layer chromatographic solvent systems (table 1), and on Sephadex G-10 chromatography (fig.1a,c). This conjugate was also hydrolysed rapidly by fungal β-D-xyloside to yield close to the theoretical quantity of MU (calculated from its spectrofluorimetrically-determined concentration, table 1).

The conjugate of peak II was found to be susceptible to rapid hydrolysis by the (very specific) preputial β-D-glucuronidase test system used here, and its identity with MU-GlcU was further confirmed by chromatography on the 3 thin-layer systems and on the Sephadex G-10 column (table 1; fig.1a–c). Analysis of peak II for bound MU and total uronic acid gave a ratio of 1:13.4, but a comparison of fig.1b,c shows that peak II overlaps a large (free uronic acid?) peak with an elution profile closely similar to that of free glucose.

These results strongly indicate that MU-GlcU and free MU were present in the cell culture medium to ~36 and 11 μM, respectively, and since neither was found in control cultures (without added MU-Xyl), these materials were probably formed metabolically from the β-D-xyloside precursor. Although the material of peak I was not studied intensively here, the results in fig.1 and table 1 indicate that it is probably a high M_r, uronic acid containing conjugate of MU. By analogy with [2,3] it is presumably a glycosaminoglycan conjugate of MU formed metabolically by the fibroblasts from MU-Xyl and found in the medium at ~10 μM.

4. Discussion

Although MU-Xyl has been added to the media in the course of numerous studies of connective-tissue cells in culture, little or no attention had been paid to the study of any low-M_r products of the cellular metabolism of the xyloside.

This work indicates that in addition to high-M_r

conjugates of MU (presumably analogous to those in [4]), the fibroblast cultures also convert the MU-xyloside into free MU and its β-D-glucuronide. While the cellular mechanisms of such metabolic conversions have not been investigated here, preliminary experiments (K. F. Z., D. H. L., unpublished) indicate that these transformations occur largely (if not wholly) within the cells, by metabolic hydrolysis to MU followed by glucuronidation of the liberated MU. Although glucuronidation of MU is known to take place in intact rabbits [14], this appears to be the first report of 'de-toxicative' glucuronidation taking place in connective-tissue cells [15]. Such processes have been investigated extensively in the livers of various species [15] but it is interesting that similar mechanisms may also be important in the highly-differentiated cells of connective-tissues. This could have importance for substances (e.g., drugs) targetted at connective-tissues.

The chromatography of MU-conjugates by aqueous-elution from Sephadex G-10 follows the observations [16] of the technique's convenience and valuable resolving power. The extremely high affinities of MU-Xyl and (especially) of MU for the stationary phase are noteworthy compared with those of MU-Glc, MU-GlcU, glucose and the high-M_r MU-conjugates. The latter (glycosaminoglycan) conjugates are presumably excluded from the Sephadex (at least in part) by their molecular size, whereas the MU-Glc and MU-Glc.U are presumably less strongly absorbed due to their more polar monosaccharide residues. The high affinity of MU-Xyl and MU for the gel suggests strong non-polar interactions [16] and this, in turn, suggests that these substances may traverse passively the membranes of living cells into the lysosomes (for hydrolysis of the xyloside) or into the endoplasmic reticulum (for saccharide prolongation of the xyloside and for glucuronidation of liberated MU). Such fluorogenic substances could perhaps provide useful markers of such processes taking place within living cells in culture.

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