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ENZYMATIC POST-COLUMN CLEAVAGE AND ELECTROCHEMICAL DETECTION OF GLYCOSIDES SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Crude extract of *Helix pomatia*, commercially available as β-glucuronidase, was immobilized on porous glass and packed in a column (50 × 3 mm I.D.). Similarly, β-glucuronidase from bovine liver, immobilized on agarose beads, was used as a post-column reactor in the high-performance liquid chromatography of phenolic glycosides. Electrochemical oxidation by glassy carbon electrodes was used for the detection of the phenolic products formed by the enzymatic reaction and proved to be useful for the identification and sensitive detection of phenolic glycosides. Enzymatic activities were in the range 0.1–1 I.U. The detection limits of various phenolic glycosides were 3–23 pmol. Peak broadening and the linearity of the system were evaluated. The reactor containing immobilized *Helix pomatia* crude extract was also shown to posses enzymatic activity towards cyanogenic glycosides.

INTRODUCTION

Glycosidases (E.C. 3.2.1) have been used extensively in studies of drug metabolites[1] and secondary plant products[2]. Polar glycosides can be transformed into less polar aglycones, thereby facilitating purification, *e.g.*, by extraction with organic solvents. For identification purposes, glycosidases are often used in purified form because of the high specificity encountered by such enzymes with regard to the nature of the sugar and the anomeric configuration of the glycosidic bond[2,3].

Immobilized enzymes have proved valuable in the chromatographic separation of various substrates by improving either the detection limits or the selectivity[4–6]. Recently, β-glucuronidase (E.C. 3.2.1.31) has been covalently attached to alkylamine on controlled-pore glass via a glutaraldehyde immobilization scheme. The reactor was used for on-line cleavage of urinary estriol conjugates before high-performance liquid chromatographic (HPLC) analysis[7,8].

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In this work, bovine liver β-glucuronidase was purchased as immobilized enzyme on agarose beads and evaluated for use in post-column reactors in HPLC. *Helix pomatia* crude extract was immobilized on controlled-pore glass according to known procedures[9–11] and similarly used in post-column reactors. The *Helix pomatia* extract has recently been shown to possess hydrolytic activity towards all known types of cyanogenic glycosides[12], and it has now been shown to retain the enzymatic activity after immobilization. Possibilities for the future use of post-column reactors for the determination of cyanogenic glycosides are considered, as only few, non-specific methods are available (for a review, see ref. 13).

In connection with HPLC, the two types of reactors have been used for the determination of phenolic glycosides by selective detection of the phenolics formed by the post-column reactions. For this purpose the electrochemical detector (ED) is ideal, owing to the sensitivity and selectivity previously demonstrated in the determination of phenolics and other easily oxidized compounds[14].

EXPERIMENTAL

Esculin (6,7-hydroxycoumarin 6-glucoside) was obtained from N.B. Co. (Cleveland, OH, U.S.A.). β-Glucuronidases, nitrophenyl glycosides, estrone glucuronide and phenolphthalein glucuronide were obtained from Sigma (St. Louis, MO, U.S.A.) and prunasin [β-D-glucopyranosyloxy-(*R*)-mandelonitrile] from Roth (Karlsruhe, G.F.R.). Gynocardin [1-(β-D-glucopyranosyloxy)-1-cyano-1,(4,5-*trans*)-trihydroxy-2-cyclopentene] was available in our laboratories from earlier work by Eyjolfsson[15]. Urine from a pig that had been given [^{14}C]trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyridmine, TMP) was kindly supplied by the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen. All other chemicals were of analytical-reagent grade.

Controlled-pore glass (CPG) with a pore size of 550 Å was purchased from Pierce (Rockford, IL, U.S.A.) and enzyme columns were either Omnifit glass or steel, both 50 × 3 mm I.D., with Omnifit or Swagelock end fittings, respectively. The columns were packed by sucking the slurry of agarose or by combined sedimentation and suction of CPG immobilized enzyme, using a peristaltic pump.

Aminopropyl-CPG was prepared by the procedure of Messing *et al.*[9], also described by Scouten[10]. Immobilization of *Helix pomatia* enzymes was carried out according to Robinson *et al.*[11].

Deactivation of the reactors was accomplished by flushing the reactors with 50% methanol.

Instrumentation

Spectrophotometric measurements were made on a Kontron LCD 725 instrument. The chromatographic system (Fig. 1) consisted of an LDC Constametric Model III pump, equipped with a Rheodyne 7125 valve, a 100 × 4.6 mm I.D. Nucleosil 5, C_8 (Machery, Nagel & Co., Duren, G.F.R.) analytical column, an LDC spectromonitor III UV detector and a Metrohm 656/641 electrochemical detector. The electrodes of the wall-jet cell consisted of a silver–silver chloride–3 *M* potassium chloride reference electrode and glassy carbon working and auxiliary electrodes. For measurements of enzyme activities in the enzyme reactors, a Bifok FIA-08 peristaltic pump was used.

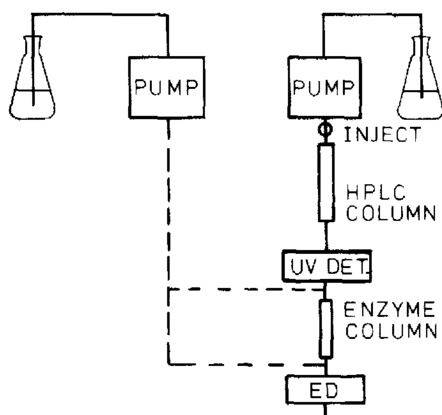

Fig. 1. HPLC system used for post-column cleavage using enzyme reactors. Additional mobile phase can be introduced for changing the pH before the enzymatic reaction or between the enzyme and the electrochemical detector, for optimum sensitivity (represented by broken lines).

Measurement of enzyme activity and V_{max}

Solutions (6–15 m*M*) of nitrophenylglycosides in 0.07 *M* phosphate buffer (pH 4.75) were passed through the enzyme reactors at a flow-rate of 2 ml/min. The eluates (0.50–2.0 ml) were collected in 1-cm cuvettes and 2.0 ml of 0.2 *M* glycine–sodium hydroxide buffer (pH 11) were added. The absorbance at 420 nm was measured and concentrations were determined from calibration graphs.

Prunasin was employed as a substrate in the following manner. A solution (2.2 m*M*) of prunasin in a 0.07 *M* phosphate buffer (pH 4.75) was passed through the enzyme reactor (*Helix pomatia*) at three different flow-rates (0.18, 0.40 and 0.92 ml/min). The eluates were collected in ice-cooled micro-test-tubes and aliquots of 10 μl were analysed for cyanogenic constituents according to Brimer *et al.*[12], after separation by thin-layer chromatography (TLC) using acetone–chloroform–water (85:10:5) and silica gel pre-coated plates (Merck, Darmstadt, G.F.R.).

Gynocardin was used as a substrate as follows. The enzyme reactor was flushed (1 ml/min) with a solution of the substrate (4.4 m*M*) in the buffer also used for prunasin for 2 min. The flow was stopped and the solution present in the enzyme reactor was eluted after various periods of time (residence time) with pure buffer, collecting a total of 2.0 ml in each instance. The mixture was analysed as described for prunasin, the amount applied in TLC being 50 μl.

Column void volume and dispersion

Void volumes (ml) and dispersion expressed as variances (σ^2, ml^2) of the enzyme reactors were determined with the chromatographic equipment by connecting the reactor between the injection valve and the detector, omitting the analytical column. The volume eluted before the first appearance of a peak due to injection of *p*-nitrophenyl galactoside was measured. A similar measurement was performed without the reactor connected for determination of other contributions to the void volume. The variance was measured from the peak width at half-height ($W_{1/2}$) ac-

cording to the equation

$$\sigma^2 = W_{1/2}^2/5.54 \quad (1)$$

Chromatography of trimethoprim metabolites

Urine from a pig was collected after i.v. administration of [^{14}C]TMP and fractionated by a previously described procedure[16], using Sephadex G-25 and G-10 gel chromatography. The fractions containing the radioactive glucuronides were pooled and further purification was carried out using preparative HPLC. Aliquots were then injected on to the analytical column (*cf.*, *Instrumentation*), using a mobile phase consisting of 15% methanol in 0.07 *M* phosphate buffer (pH 5.6) and the agarose enzyme reactor.

RESULTS AND DISCUSSION

Chromatographic characteristics

Enzyme reactors packed with immobilized β-glucuronidase from bovine liver were used for preliminary work. Satisfactory results with regard to enzymatic activity and specificity of the enzyme were obtained. The agarose matrix was suitable for flow-rates of about 1 ml/min. The void volume at this flow-rate was 0.189 ml, but it was greatly increased by increasing the flow-rate to 2 ml/min, owing to compression of the matrix. The dispersion of solutes at a flow-rate of 1 ml/min was 0.036 ml^2.

Better results were obtained with crude extracts from *Helix pomatia* immobilized on CPG of 550 Å pore size. The void volume was similar to that of the agarose reactor (0.180 ml), being independent of flow-rate. The dispersion of the CPG reactor

TABLE I

GLYCOSIDASE POST-COLUMN REACTOR CHARACTERISTICS AND CALCULATED EFFECTS ON THE CHROMATOGRAPHIC RESOLUTION

V_v (ml) = post-column reactor void volume; V_r (ml) = retention volume of eluate from the chromatographic column; σ^2 (ml^2) = dispersion in the post-column reactor; N_P and N = number of theoretical plates of three chromatographic systems with and without the post-column reactor connected, respectively; N_P is calculated for theoretical values of V_r and N using the equation $N_P = V_r^2/(\sigma^2 + V_r^2/N)$.

Reactor type	V_v	V_r	σ^2	N	N_P	$\frac{N_P}{N} \cdot 100$
Helix pomatia enzyme/ controlled-pore glass	0.182	10	0.62×10^{-2}	10000	6173	62
	–	15	–	10000	7840	78
	–	10	–	5000	3817	76
	–	15	–	5000	4395	88
	–	10	–	2500	2165	87
	–	15	–	2500	2339	94
Bovine liver enzyme/ agarose beads	0.189	10	3.6×10^{-2}	10000	2174	22
	–	15	–	10000	3846	38
	–	10	–	5000	1234	24
	–	15	–	5000	2778	56
	–	10	–	2500	1316	53
	–	15	–	2500	1786	71

TABLE II

ENZYME ACTIVITIES AND V_{max} MEASURED AT 20°C AND pH 4.75 OF IMMOBILIZED GLYCOSIDASES FROM *HELIX POMATIA* AND BOVINE LIVER

V_{max} = rate of reaction for enzyme saturated with substrate, calculated from the enzyme activity (I.U.) and the void volume of the post-column reactor. Enzyme activity was measured on a new reactor and after 4 months (numbers in parentheses).

Reactor type	*Substrate*	V_{max} *(mmol/l · min)*	*Enzyme activity (I.U.) (µmol/min)*
Helix pomatia enzyme/ controlled-pore glass	*p*-Nitrophenyl β-D-glucuronide	2 (0.5)	0.4 (0.1)
	o-Nitrophenyl β-D-galactoside	1 (0.5)	0.2 (0.1)
	p-Nitrophenyl β-D-glucoside	– (5)	– (1.0)
Bovine liver enzyme/ agarose beads	Phenolphthalein β-D-glucuronide	1.0	0.2

was considerably smaller (0.006 ml^2) than that measured for the agarose reactor.

The effects of dispersion in the two types of reactors on the chromatographic resolution were calculated for various situations (Table I). It can be seen that 6173 and 7840 theoretical plates are retained when calculated for solutes with retention volumes of 10 and 15 ml, respectively, provided that an analytical column with an initial efficiency of 10,000 theoretical plates is connected upstream of the CPG reactor. The situation is greatly worsened using the agarose reactor, which nevertheless proved useful for the group identification of glucuronides in urine.

Enzymatic properties of the reactors

Enzymatic activities (I.U.) and rates of glycoside hydrolysis (V_{max}) measured under saturation conditions (6–14 m*M*) and at lower substrate concentrations are presented in Table II. Approximate Michaelis constants (K_M) of 0.001 *M* were found for *o*-nitrophenyl galactoside and *p*-nitrophenyl glucuronide using the *Helix pomatia* reactor. Inhibition of the enzyme was observed for *p*-nitrophenyl glycoside at concentrations higher than 0.0006 *M* and consequently calculation of V_{max} was based on the rate observed at a lower concentration (0.0002 *M*). The *Helix pomatia* reactor activity was measured when newly prepared and again after 4 months, showing a decline to approximately 25% of the initial activity for β-glucuronidase.

The effect of pH on the agarose reactor–ED system was studied (Fig. 2). The relative response of the electrochemical detector, measured in the pH range 3.5–6.5, showed an optimum between pH 4.0 and 4.5, while irreversible inhibition of the enzyme was observed at pH 3.5. Usually the reactor was employed at pH 4.75 or 5.60 at room temperature (20°C), the mobile phase containing 30% or less of methanol. These conditions were efficient for the elution of the most lipophilic glucuronides tested, estrone and phenolphthalein glucuronide.

Detection limits and detector selectivity

Detection limits were measured for standard solutions of substrates (Table

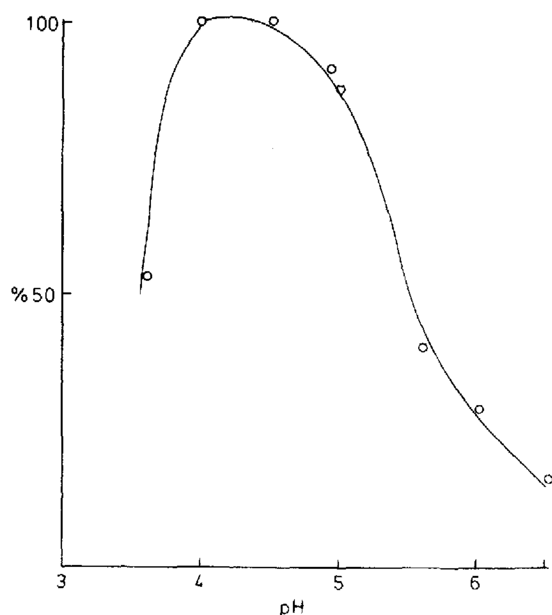

Fig. 2. Relative electrochemical detector response to *p*-nitrophenyl glucuronide after cleavage on agarose-bound bovine liver glucuronidase at various pH values.

III), with the exception that the TMP metabolite was quantitated on the basis of ^{14}C radioactivity. The UV and ED detection limits were similar, the advantage of the ED being the selectivity encountered. By choosing appropriate potentials, only phenolics were oxidized, leaving the corresponding glycosides unaffected by the ED. Nitrophenyl glycosides were not oxidized as such at 1200 mV, while other glycosides were unaffected only at lower potentials. Thus, esculin was oxidized above 500 mV, while the

TABLE III

ENZYMATIC POST-COLUMN CLEAVAGE OF GLYCOSIDES

Glycoside	*Electrochemical detection*		*UV detection*	
	Potential (mV)	*Detection limit* (pmol)*	*Wavelength (nm)*	*Detection limit* (pmol)*
o-Nitrophenyl β-galactoside	1200	6	254	7
m-Nitrophenyl α-galactoside	1200	21	254	7
p-Nitrophenyl β-glucuronide	1200	3	254	14
Estrone glucuronide	1200	6	254	11
Esculin (glucoside)	500	23	330	13
TMP M-4-glucuronide	700	18	280	1

* Signal-to-noise ratio = 3.

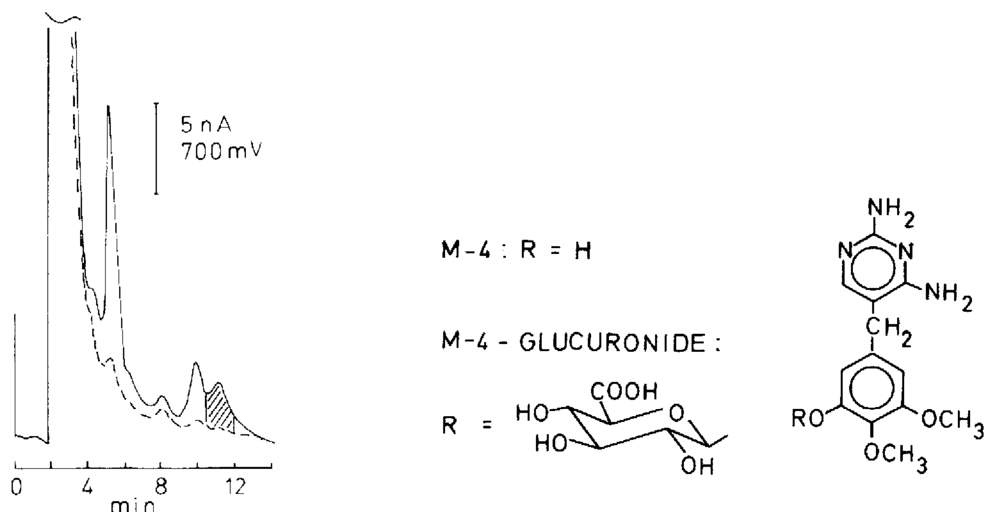

Fig. 3. Group-selective detection of glucuronides provided by consecutive chromatograms with (solid line) and without (broken line) an enzyme reactor connected after the HPLC column (100 × 4.6 mm I.D. Nucleosil 5, C_8). Shaded area corresponds to a trimethoprim metabolite glucuronide, identified by the radioactive label present in urine collected from a pig after administration of [^{14}C]trimethoprim. The mobile phase consisted of 15 % methanol in 0.07 *M* phosphate buffer (pH 5.6). The ED was maintained at a potential 700 mV.

TMP metabolite M-4-glucuronide (and TMP[17]) was oxidized above 900 mV. This behaviour is ascribed to the presence of a free phenolic group in esculin and other electroactive groups in the M-4-glucuronide. For the selective detection of various glycosides, the lowest feasible potential for oxidation of the corresponding phenolics was applied. In the case of M-4, obtained by hydrolysis of the glucuronide, this potential was 700 mV, *i.e.*, well below the limit for oxidation of the native glucuronide. This allowed the selective determination, by differential measurements of the glucuronide, as shown in the following application.

Chromatography of a partly purified fraction of urine obtained from a pig after administration of ^{14}C-labelled TMP showed the presence of at least three glucuronides (Fig. 3), one of which contained the radioactive label. Two chromatograms of the same urine fraction were obtained, one with an active enzyme reactor connected and the other with a deactivated column connected after the analytical column. The only differences between the two chromatograms were due to the phenolics produced by the enzymatic reaction, and the chromatograms therefore provided a picture of the glucuronides present in the urine sample.

Detector linearity

An exponential form (eqn. 2) of the integrated rate expression of the Michaelis–Menten model shows that the exponential term becomes independent of product concentration when $(P) \ll V_{max}\, t$.

$$(S_0) = (P)\,\{1 - \exp\,[(P)/K_M - V_{max}\, t/K_M]\}^{-1} \qquad (2)$$

where (S_0) = substrate concentration, (P) = product concentration, K_M = Michaelis

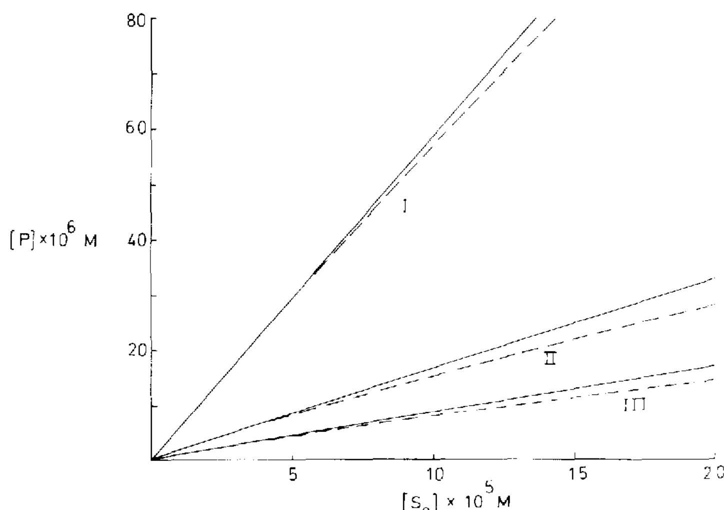

Fig. 4. Relationship between the calculated product concentration (P) of the eluate from the enzyme reactors and the substrate concentration, $(S_0) = (P)\{1 - \exp[(P)/K_M - V_{max}\, t/K_M]\}^{-1}$, at the inlet of the reactors. When $(P) \ll V_{max}\, t$ a linear relationship between (P) and (S_0) exists (shown as solid lines). If (P) approximates $V_{max}\, t$, a deviation from this relationship can be predicted (broken lines). Experimental values are as follows (*cf.*, Table II): t = 10.8 sec, K_M = 0.001 *M*, V_{max} = 0.005 *M*/min (I), V_{max} = 0.001 *M*/min (II), V_{max} = 0.0005 *M*/min (III).

constant, V_{max} = maximum rate of substrate hydrolysis and t = residence time in the enzyme reactor.

At low concentrations the measured product concentration should therefore be proportional to the substrate concentration (S) shown as solid lines in Fig. 4. When (P) approaches $V_{max}t$, the exponential term causes a deviation from this proportionality and the curves shown as broken lines in Fig. 4 are obtained for given values of V_{max}, t and K_M. So far this deviation from linearity has not been observed in practice. A linear calibration graph (r = 0.9986) was obtained on using *p*-nitrophenyl glucuronide as the substrate (0.01–2 m*M*) and measuring peak heights (H):

$$H = 6.5 \cdot 10^4\, (S_0) + 0.105 \qquad (3)$$

Detection of cyanogenic glycosides

In order to examine wether the *Helix pomatia* reactor contained sufficient enzymatic activity towards cyanogenic glycosides, prunasin and gynocardin were applied to the reactor. Prunasin is a substrate for commercial β-glucosidase (almond), whereas gynocardin cannot be cleaved by this enzyme[18]. Hydrolysis of prunasin under flow conditions (0.2–1.0 ml/min) could readily be observed, whereas stop-flow experiments were necessary using gynocardin.

The TLC analysis of the product–substrate mixture eluted from the column allowed the determination of both the unstable cyanohydrin (aglycone) formed and of the authentic glycoside (substrate) left in the case of prunasin, whereas only the substrate could be detected and quantified in the case of gynocardin. The results for prunasin (Table IV) permitted calculations of enzyme activity in the reactor (0.3–0.4 I.U.), and were found to be of the same magnitude as those found for *p*-nitrophenyl

TABLE IV

CLEAVAGE OF THE CYANOGENIC GLUCOSIDE PRUNASIN BY *HELIX POMATIA* ENZYME REACTOR, UNDER FLOW CONDITIONS

Flow-rate (ml/min)	*Substrate concn. before reactor (mM) (a)*	*Substrate concn. after reactor (mM) (b)*	*Aglycone (cyanohydrin) concn. after reactor (mM) (c)*	*Recovery (%)* $\left(\frac{b+c}{a} \cdot 100\right)$	*Degree of cleavage (%)*	*Substrate cleaved (μmol/min) (activity, I.U.)*
0.18	2.2	0.68	1.68	109	74	0.29
		0.57	1.82			
0.40	2.2	1.62	0.88	113	38	0.33
		1.48	0.98			
0.92	2.2	1.82	0.44	105	19	0.39
		1.91	0.44			

glucoside. On the other hand, results from stop-flow experiments with gynocardin indicated a much lower activity with this substrate, as 7.5, 38, 75 and 83% of gynocardin were hydrolysed after 1, 5, 10 and 20 min, respectively. Future aspects for the determination of cyanogenic glycosides by use of post-column reactors are considered.

Cyanohydrins (aglycones) formed by enzymatic cleavage of cyanogenic glycosides are easily converted into aldehydes and cyanide in basic solution. Cyanide has previously been determined by electrochemical oxidation at a silver electrode[19] or by colorimetry[20]. Thus, a feasible procedure for determination of cyanogenic glycosides may be HPLC combined with post-column enzymatic cleavage and electrochemical or colorimetric detection.

CONCLUSION

The potential of enzyme reactors seems to be broad, and these reactors may be useful for the determination of other groups of compounds than those so far described. The advantages of post-column compared with pre-column cleavage lie in the possibilities for selective detection, *e.g.*, using a chromophore or the electroactive properties of one of the enzymatic products. Using enzymes from various sources, a broad pH interval can be covered, *e.g.*, *E. coli* glucuronidase has optimum activity at about pH 7, whereas many other sources contain glucuronidases of optimum pH in the range 4–6. Adjustment of the pH between the analytical column and the enzyme reactor and control of temperature are other possibilities for enhancement of enzyme activities (*cf.*, Fig. 1). Disadvantages such as decreased resolution, caused by the reactor, or lack of sensitivity due to incomplete reaction can be overcome by employing purified enzymes, allowing smaller reactor volumes. Purification of the crude enzyme mixture of *Helix pomatia* is required in order to obtain increased substrate specificity and activity. The possibilities for the use of CPG reactors for the detection of other conjugates such as sulphate esters and acetates are currently being investigated.

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