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SEPARATION OF DIFFERENT UDP GLUCURONOSYLTRANSFERASE ACTIVITIES ACCORDING TO CHARGE HETEROGENEITY BY CHROMATOFOCUSING USING MOUSE LIVER MICROSOMES

THREE MAJOR TYPES OF AGLYCONES*

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Abstract—Hepatic UDP glucuronosyltransferase (EC 2.4.1.17) (GT) enzymes in control, phenobarbital- and 3-methylcholanthrene-induced microsomes from C57BL/6N mice have been fractionated according to charge heterogeneity on a chromatofocusing system using a pH 9.5 to 6 gradient. Transferase activities for eleven different substrates were determined on column fractions. Activities toward 3-hydroxybenzo[*a*]pyrene, phenolphthalein and estrone (type 1 substrates) were enhanced by both effector compounds and always eluted primarily at pH 8.5. In control and phenobarbital-induced microsomes, activities toward testosterone, 4-hydroxybiphenyl, morphine, naphthol and 9-hydroxybenzo[*a*]pyrene (type 2 substrates) eluted primarily at about pH 6.7. Activities toward *p*-nitrophenol, 4-methylumbelliferone and 2-hydroxybiphenyl (type 3 substrates) in control and phenobarbital-induced microsomes exhibited two peaks which eluted at pH 8.5 and 6.7. 3-Methylcholanthrene treatment increased almost exclusively activities which eluted at pH 8.5 for each of the three types of substrates. The pH value of elution corresponds to the approximate isoelectric point of the eluted protein. Immunoabsorption studies with an antibody preparation raised against a purified low pI form confirmed that a 51,000-dalton transferase form, GT_{M1}, eluted primarily at pH 6.7 and that a 54,000-dalton form, GT_{M2}, eluted at pH 8.5. A mathematical treatment of the ratios of activity after 3-methylcholanthrene treatment to that after phenobarbital treatment versus pH produced six patterns of activity. A minimum of two enzymes at the low pH region and one enzyme at the high pH region, all with broad-substrate specificity, could account for these patterns.

Most studies concerned with the heterogeneity of the drug detoxifying enzyme GT‖ have been conducted in the rat and rabbit. Groups of GT activities have been inferred from developmental studies [1] and preferential induction of activities for a particular group of substrates by prototypic inducers [2–5]. Previously, it was shown in rat [2–4] and, more recently, in mouse [5, 6] that PB treatment enhances activities for one group of substrates, typically represented by 2-OH-BiP, 4-OH-BiP, phenolphthalein and morphine, while 3-MC treatment enhances activities for a separate group of substrates typically represented by PNP, 4-MU, 3-OH-BP, and naphthol. It is not known in any of these studies whether a group of substrates is conjugated by one enzyme or whether more than one enzyme is induced and is responsible for conjugating a particular set of substrates. Several GT forms have been isolated from rat [6–11] and rabbit [12] and a single form from mouse [13]. The number of other forms of the enzyme existing in these species to accommodate the large number of substrates which undergo glucuronidation is not known.

Since the studies on differential induction suggest that a given effector might induce a single enzyme with multiple substrate activities in mice, it was of interest to determine whether multiple enzymes,

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‖ Abbreviations and short terms: GT, UDP glucuronosyltransferase; GT_{M1}, the 51,000-dalton UDP glucuronosyltransferase from mouse liver; GT_{M2}, the 54,000-dalton UDP glucuronosyltransferase from mouse liver; 3-OH-BP, 3-hydroxybenzo[*a*]pyrene; ϕ, phenolphthalein; E, estrone; T, testosterone; 4-OH-BiP, 4-hydroxybiphenyl; M, morphine; N, 1-naphthol; 9-OH-BP, 9-hydroxybenzo[*a*]pyrene; PNP, *p*-nitrophenol; 4-MU, 4-methylumbelliferone; 2-OH-BiP, 2-hydroxybiphenyl; PB, phenobarbital; 3-MC, 3-methylcholanthrene; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; and DTT, dithiothreitol.

separable by isoelectric point differences, are responsible for the multiple activities affected or whether one enzyme peak is always associated with the various activities affected by a given compound. Furthermore, one can compare and group the activities according to approximate pI values from untreated, 3-MC- and PB-treated microsomal preparations. Hence, this study attempts to determine the pattern of elution of GT activities for eleven different substrates using solubilized microsomes and the chromatofocusing system. An earlier application of the chromatofocusing technique to the UDP glucuronosyltransferase system in mice showed that a minimum of two enzymes were responsible for glucuronidating the twelve benzo[*a*]pyrene phenols [14].

MATERIALS AND METHODS

Materials. Compounds used were: 2-OH-BiP and 4-OH-BiP (Eastman Kodak Co); 3-MC (Biochemical Laboratories, Inc.); morphine sulfate and sodium phenobarbital (Merck & Co.); and phenolphthalein, testosterone, naphthol, estrone, PNP, UDP glucuronic acid, and 4-methylumbelliferyl-β-D-glucuronide (Sigma Chemical Co.). Detergents used were: sodium cholate, Zwittergent [1–12] (Calbiochem) and CHAPS, synthesized according to Ref. 15. Radiolabeled compounds used were: [2, 4, 6, 7-^{3}H(N)]estrone (95.6 Ci/mmole), [*N-methyl*-^{3}H] morphine HCl (72.0 Ci/mmole), [1,2,6,7-^{3}H(N)] testosterone (93.9 Ci/mmole) (New England Nuclear Corp.), and NaB[^{3}H]$_4$ (Amersham). 3-OH-BP and 9-OH-BP were from the National Cancer Institute Chemical Repository (Bethesda, MD). Chromatography resins used were: phenyl Sepharose, polybuffer exchanger 94 (PBE-94), polybuffer 96 (PB-96) and polybuffer 74 (Pharmacia). 2-OH-BiP and 4-OH-BiP were recrystallized from *n*-hexane and methanol respectively. C57BL/6N mice were supplied by the Veterinary Resources Branch of the National Institutes of Health (Bethesda, MA). Male, 4- to 6-week-old mice were used in all experiments.

Treatment of mice and preparation of microsomes. A single intraperitoneal dose of 3-MC (200 mg/kg) was administered to fifty C57BL/6N mice 6 days before they were killed. A second group of fifty mice received PB (0.75 g/l) in their drinking water for 2 weeks. These regimens gave maximum induction. After 24 hr of starvation and then exsanguination of the mice, pooled livers of each group (kept at 0–4° for all further manipulation) were homogenized in 0.25 M sucrose with an LKB polytron. After spinning the homogenate at 15,000 *g* for 15 min, the microsomes were pelleted at 100,000 *g* for 1 hr and then washed by resuspension in 0.15 M KCl containing 10 mM EDTA (pH 7.2) before a final 1-hr centrifugation at 100,000 *g*. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol such that the protein concentration was approximately 40 mg/ml and stored at −80° until used.

Chromatofocusing. The chromatofocusing system and recommended procedures of Pharmacia were used. All buffers were degassed before use. Microsomal protein was solubilized in 120 ml of 100 mM potassium phosphate buffer, pH 7.7, containing 10 mM CHAPS, 20% glycerol, 1 mM DTT and 1 mM EDTA. The detergent to protein ratio was 1.5. The suspension was stirred for 20 min at 4° and centrifuged at 100,000 *g* for 1 hr. The supernatant fraction was applied to a column of phenylSepharose (2.6 × 20 cm) to remove phospholipid and allow monodispersion of the proteins which is critical to isoelectric focusing. All subsequent buffers contained 20% glycerol and 10 mM CHAPS. The column was eluted with the application buffer at a flow rate of 15 ml/hr. A 25-ml aliquot (approximate 2 mg protein/ml) of the phenylSepharose fraction was extensively dialyzed against 25 mM ethanolamine–acetate buffer, pH 9.5, before application to a 16.5 × 1.25 cm column of PBE-94 resin previously equilibrated with 15 column volumes of the ethanolamine buffer. The pH gradient was initiated and developed by elution with polybuffer 96 previously adjusted to pH 6.0 with acetic acid. The flow rate was 30 ml/hr, and 4.5-ml fractions were collected. Determinations of pH and GT activities in the fractions were performed as quickly as possible to minimize enzyme denaturation. Since CHAPS had a high critical micelle concentration, it was possible to dilute the detergent out in the assay mixture such that no noticeable effect existed compared to preparations where the detergent was removed by dialysis. CHAPS proved throughout these studies to be an efficient solubilizing agent without causing significant loss of GT activity with the various manipulations.

UDP glucuronosyltransferase assays. Activities toward all substrates used except estrone and morphine were determined as already described [16]. Estrone GT activity was assayed according to Rao *et al.* [17] except that the steroid, present at 0.1 mM instead of 0.03 mM, was added to the reaction tube in ethanol, evaporated to dryness, and then preincubated with enzyme at 4° for 0.5 hr before adding the remaining reaction components. Tris–HCl buffer (0.1 M) at pH 7.8 instead of pH 8.8 was used in the incubation. GT activity toward morphine at 1.5 mM was determined according to Sanchez and Tephly [18]. Activity for each substrate was assayed under conditions of maximal velocity for control and treated microsomal preparations. Pooled enzyme activity at the peaks of high and low pH for the PB-treated microsomal preparation was reconstituted with phosphatidylcholine (20:1) before the assay [19].

Preparation of antibody. A low pI form of GT from mouse with a subunit molecular weight of 51,000 daltons was purified to homogeneity according to Mackenzie *et al.* [19], and anti-GT IgG was raised in goat as described by Vaitukaitis [20]. The IgG fraction was partly purified by ammonium sulfate fractionation.

Tritiation of protein eluted from chromatofocusing columns. The protein fractions containing the peaks of activity eluted from the chromatofocusing columns were pooled and concentrated by vacuum dialysis and tritiated with NaB[^{3}H]$_4$ by the method of Kreibich *et al.* [21]. The tritiated proteins were

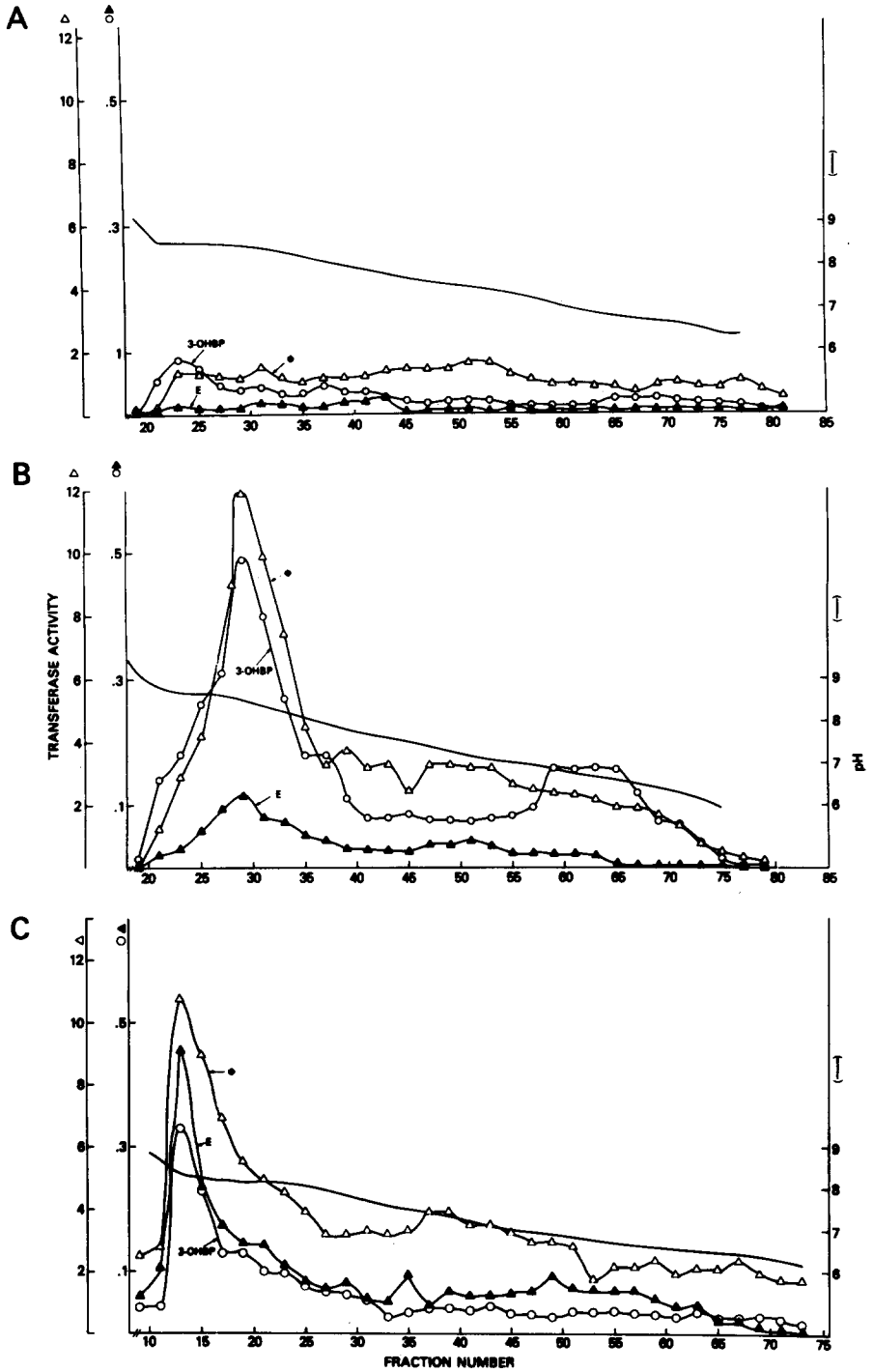

Fig. 1. Chromatofocusing of UDP glucuronosyltransferases (type 1 substrates) using solubilized hepatic microsomes. A, B, and C represent control (54 mg protein), PB- (69 mg protein) and 3-MC-induced (26 mg protein) microsomes, respectively, with fractions assayed for 3-hydroxybenzo[*a*]pyrene (3-OH-BP, O); phenolphthalein (ϕ, △); and estrone (E, ▲) GT activities. Activities toward estrone and phenolphthalein are expressed as nmoles/hr × ml, and that toward 3-OH-BP is expressed as nmoles/min × ml. Microsomes were chromatofocused according to Materials and Methods. Activities toward estrone and phenophthalein which showed a 2-fold greater recovery from 3-MC-treated microsomes have been corrected in Fig. 1C.

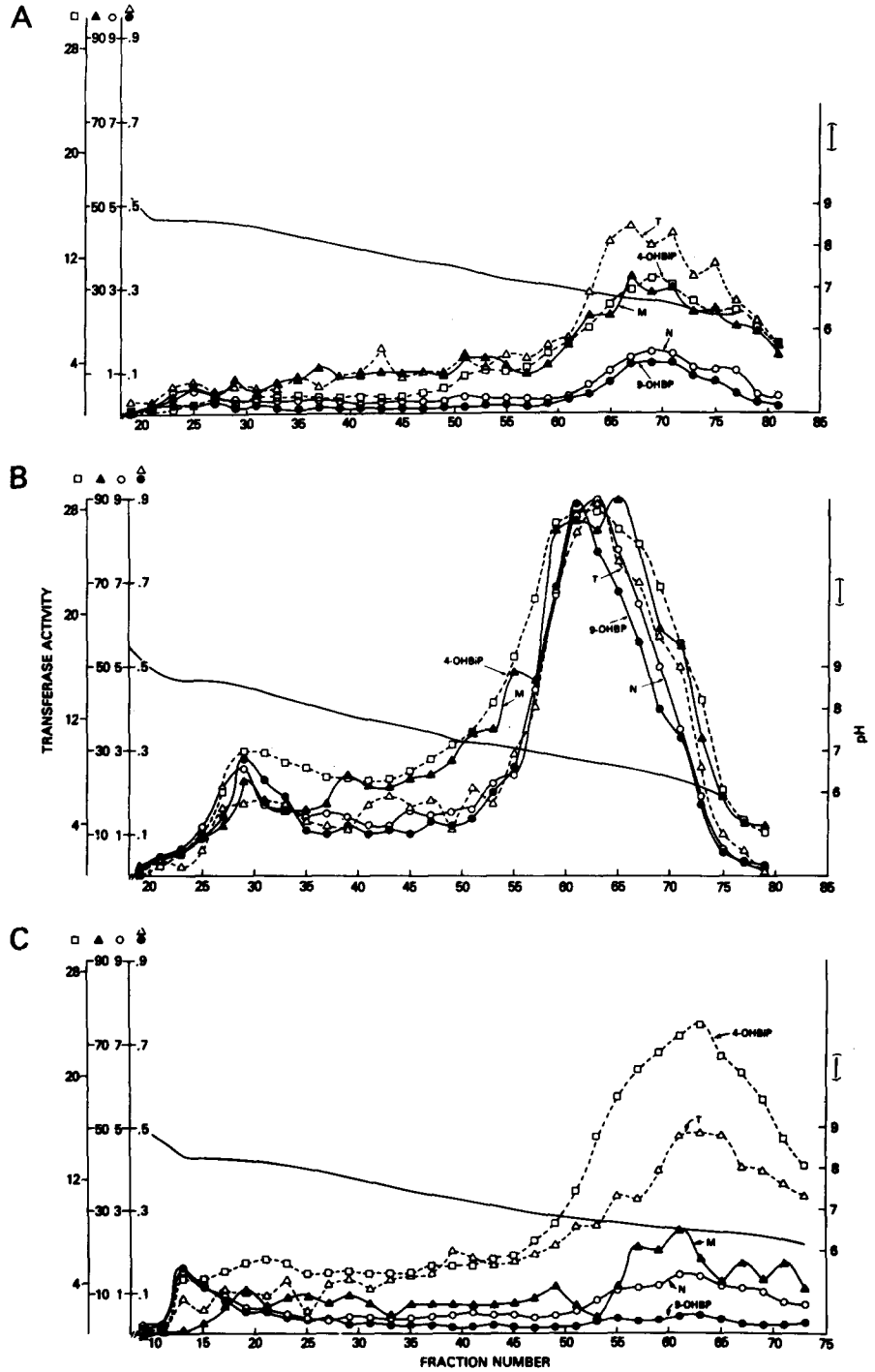

Fig. 2. Chromatofocusing of UDP glucuronosyltransferases (type 2 substrates) in solubilized hepatic microsomes. A, B, and C represent control, PB- and 3-MC-induced microsomes with fractions assayed for testosterone (T, △); 4-hydroxybiphenyl (4-OH-BiP, □); morphine (M, ▲); naphthol (N, O); and 9-hydroxybenzo[*a*]pyrene (9-OH-BP, ●) GT activities. Glucuronidation of 4-OH-BiP, 9-OH-BP, and 1-naphthol is expressed as nmoles/min × ml, and glucuronidation of morphine and testosterone is expressed as nmoles/hr × ml.

Table 1. Approximate isoelectric point of UDP glucuronosyltransferases determined by chromatofocusing using pH 9.5 to 6 gradient

Type	Substrates	Approximate isoelectric points		
		Control	PB	3-MC
1	Estrone		8.5	8.5
	Phenolphthalein		8.5	8.5
	3-OH-BP	8.5	8.5*, 6.7	8.5
2	4-OH-BiP	6.7	8.5, 6.7*	8.5, 6.7*
	Testosterone	6.7	8.5, 6.7*	6.7
	Morphine	6.7	8.5, 6.7*	6.7
	Naphthol	6.7	8.5, 6.7*	8.5, 6.7*
	9-OH-BP	6.7	8.5, 6.7*	8.5
3	PNP	8.5, 6.7	8.5, 6.7	8.5*, 6.7
	4-MU	8.5, 6.7	8.5, 6.7	8.5*, 6.7
	2-OH-BiP	8.5, 6.7*	8.5, 6.7*	8.5, 6.7

* Indicates that the large majority of the activity was eluted at that pH. Various GT activities were chromatofocused as described in Figs. 1–3. The pH of peak activity eluting from a column was designated the approximate isoelectric point.

extensively dialyzed against 100 mM potassium phosphate, pH 7.4, containing 0.1% sodium cholate and 0.01% Zwittergent and used for immunochemical studies.

Immunoprecipitation of GT from pooled chromatofocusing fractions. An aliquot (200 μl) of the tritiated proteins from each chromatofocusing column reacted with excess anti-GT IgG for 1 hr and then excess protein A-Sepharose for 2 hr while the samples rotated at 4°. The protein A-Sepharose-bound immune complexes were washed extensively according to Negishi *et al.* [22], subjected to SDS-polyacrylamide gel electrophoresis [23], and visualized by treating the gel with Autofluor and subsequent autoradiography.

Mathematical analyses of possible number of enzyme forms. The data for the eleven substrates in Figs. 1–3 were corrected for differences in protein amount applied to the chromatofocusing column and divided into twenty-two different pH levels. Control activity for each substrate was subtracted from both PB and 3-MC activities. The resulting activities were then plotted against each other for each of the twenty-two pH levels. This is schematically represented in Fig. 5 for one pH level. The direction of the arrow (measured from the horizontal) as determined from Fig. 5 was plotted versus pH for each of the eleven substrates in Fig. 6A and 6B. These eleven curves are grouped into six distinct patterns for the substrates as shown in Fig. 6C.

Protein assays. Protein content was determined by either the method of Lowry *et al.* [24] or Bearden [25] using crystalline bovine albumin as standard.

RESULTS

Microsomes were chromatofocused on a PBE-96 resin, eluted with a 9.5 to 6 pH gradient, and solubilized microsomes and column fractions were assayed for glucuronidation of eleven different substrates. The microsomal preparations were differentially induced by PB and 3-MC for the various substrates. Typically, activity toward morphine was induced 2-fold and that toward 2-OH-BiP and 4-OH-BiP was induced 3-fold by PB. On the other hand, activities toward 4-MU and 3-OH-BP were induced 1.5- to 3-fold by 3-MC. Since proteins in this focusing system elute from the column near their isoelectric point, the activities are referred to by apparent pI values. The substrates were grouped into three types on the basis of elution profiles for control activity as indicated in Table 1.

Type 1 substrates. Figure 1, panels A–C, shows the elution profiles for activities toward phenolphthalein, estrone, and 3-OH-BP from control, PB- and 3-MC-treated mice respectively. Activity toward esterone was barely detectable in any fraction from control microsomes, whereas a low level of activity for phenolphthalein eluted between pH 7.25 and 8.5 and for 3-OH-BP at pH 8.5. Chromatofocused microsomes from PB- and 3-MC-treated mice (Fig. 1B and 1C) showed enhanced activities and revealed that the majority of the activities for estrone, phenolphthalein, and 3-OH-BP eluted at pH 8.5. 3-OH-BP as a substrate showed a new and smaller peak of GT activity at approximately pH 6.7 only after PB treatment. Activity in the microsomes toward phenolphthalein was induced 6- and 8-fold, that toward estrone was induced 5- and 1.5-fold, and that toward 3-OH-BP was induced 1.5- and 3-fold by 3-MC and PB treatment respectively. Glucuronidating activities toward estrone, phenolphthalein, and most of the 3-OH-BP under the conditions used in this study was always eluted at pH 8.5 as indicated in Table 1. The enzyme(s) responsible for these activities was designated a high pI form(s), and these substrates were considered type 1.

Type 2 substrates. Glucuronidation studies with the substrates morphine, naphthol, testosterone, 4-OH-BiP, and 9-OH-BP and with chromatofocused fractions from control microsomes indicated that

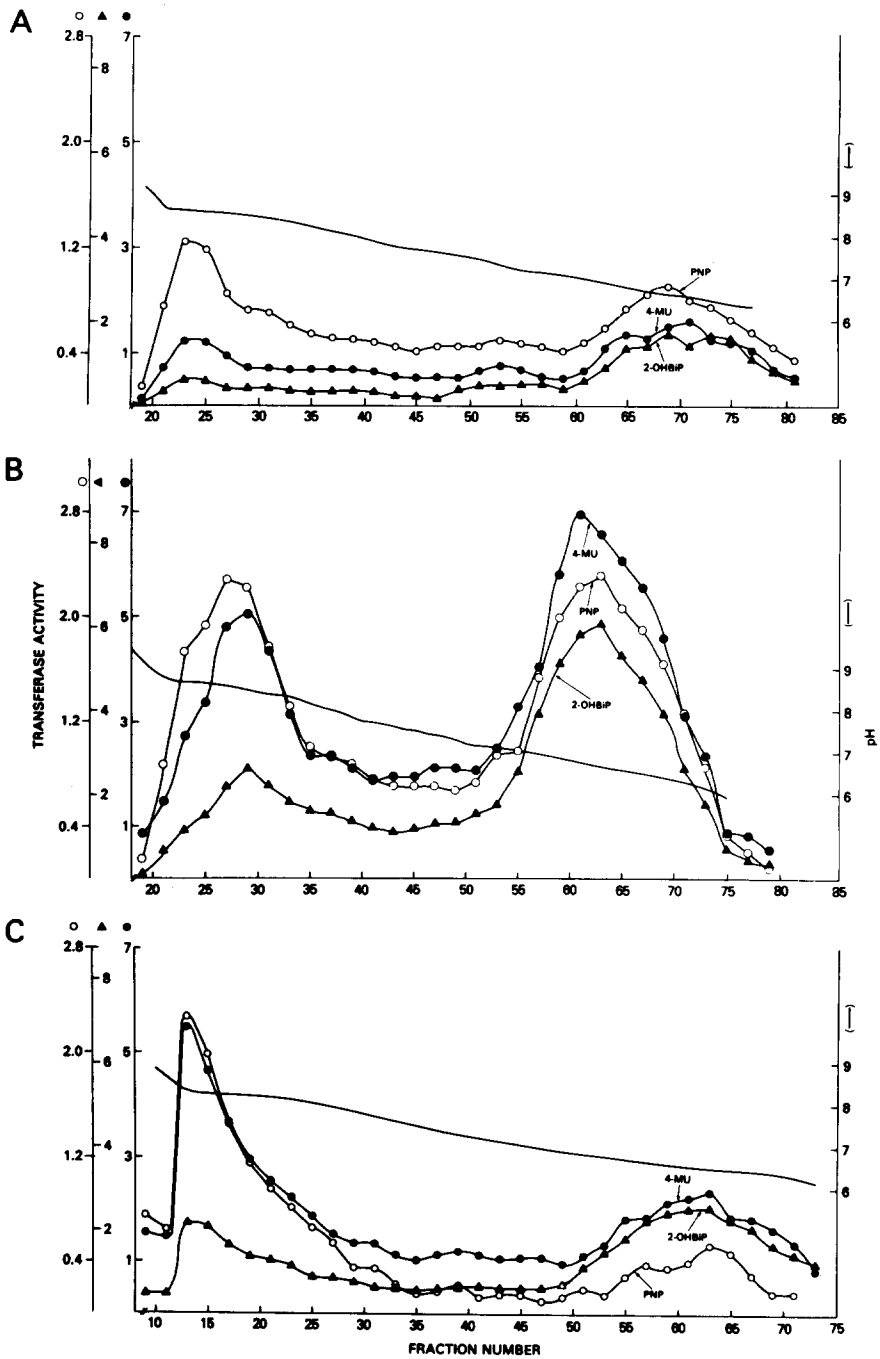

Fig. 3. Chromatofocusing of UDP glucuronosyltransferases (type 3 substrates) in solubilized microsomes. A, B, and C represent control, PB- and 3-MC-induced microsomes with fractions assayed for *p*-nitrophenol (PNP, O); 4-methylumbelliferone (4-MU, ●); and 2-hydroxybiphenyl (2-OH-BiP, ▲) GT activities. All activities are expressed as nmoles/min × ml.

most of these activities eluted below pH 7.0 (Fig. 2A). These substrates were designated type 2. If one compares PB-induced (Fig. 2B) microsomes for these five substrates, the majority of the activities was again eluted below pH 7.25 and, in addition, new and smaller peaks of activities were eluted at about pH 8.5.

A further comparison of GT activities for morphine, naphthol, testosterone, 4-OH-BiP, and 9-OH-BP with fractions from chromatofocused 3-MC-induced microsomes (Fig. 2C) showed that again most of the activities for morphine, testosterone, and 4-OH-BiP were eluted around pH 6.7. Activity for naphthol was eluted about equally between the low pH fractions and a peak near pH 8.5. The major GT activity for 9-OH-BP from the 3-MC-treated microsomes appeared in the pH 8.5 peak, while activity was lost from the low pH region compared

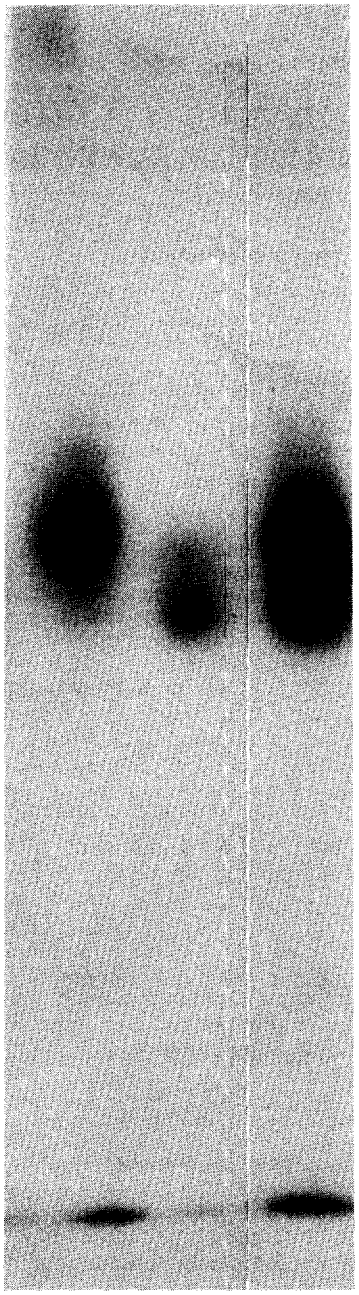

Fig. 4. Immunoprecipitation of ^{3}H protein fractions eluted from chromatofocusing column with anti-GT IgG and protein A-Sepharose. Fractions containing peak activities (12–25 and 55–69) eluted from the chromatofocusing column using the 3-MC-induced microsomes (Figs. 1C, 2C and 3C) were pooled and concentrated and the protein was tritiated and reacted with anti-GT IgG for 1 hr at 4° and with protein A-Sepharose for 2 hr. The sample was prepared for autoradiography as described under Materials and Methods. The *left lane* represents immunoprecipitated GT in the pooled fractions between 12 and 25 (Figs. 1C, 2C and 3C), and the *middle lane* represents immunoprecipitated GT in the pooled fractions between 55 and 69 from the same chromatofocusing run. The *right lane* shows the results of combining the immunoprecipitated material similar to that used in the *left* and *middle lanes* before applying to the gel.

to control microsomes. Activity in the solubilized microsomes toward morphine, naphthol, 4-OH-BiP and 9-OH-BP were induced 2- to 3-fold by PB and that toward testosterone and naphthol was induced 1.5-fold by 3-MC. Since most of the GT activities for morphine, 1-napthol, testosterone, and 4-OH-BiP eluted with peak activities near pH 6.7 (as summarized in Table 1) the enzyme(s) with these glucuronidating activities was designated a low pI form(s). 9-OH-BP GT activities are of both the low and high pI forms under the appropriate conditions, as indicated above.

Type 3 substrates. GT activities in fractions from control and PB-induced microsomes for PNP, 4-MU, and 2-OH-BiP eluted at both pH 8.5 and pH 6.7 (Fig. 3A and 3B). These substrates are designated type 3. The ratios of activities at pH 8.5 to pH 6.7 for PNP, 4-MU, and 2-OH-BiP were 0.7, 0.5, and 0.25, respectively, in both control and PB-induced microsomes. After induction by 3-MC (Fig. 3C), the ratios were 2.6, 1.2, and 0.5. These ratios reveal that 3-MC had a dominant effect on enhancing activities eluting at pH 8.5 for all three substrates. Activities were enhanced by PB treatment but to the same extent in both peaks (Fig. 3B). Activities in the solubilized microsomes toward 4-MU and 2-OH-BiP were induced 3-fold by 3-MC and PB, and activity toward PNP was induced about 1.5-fold by either inducer. Since major activity for PNP, 4-MU, and 2-OH-BiP in either microsomal preparation tested eluted in both high and low pH fractions, these substrates were considered to be glucuronidated by both high and low pI forms.

Table 1 shows a summary of the distribution of the GT activities eluted from the chromatofocusing system for the three types of substrates. Activity toward 9-OH-BP was unique; activity for this phenol eluted in the low pH region from control and PB-induced microsomes, but this activity was lost and activity appeared at pH 8.5 after 3-MC treatment.

In a separate experiment, the peak activities from the PB-treated microsomes when reconstituted with phosphotidylcholine before the assay showed no enhancement or a 1.4- to 2-fold enhancement of activity for the eleven substrates used.

The relative recovery of each activity applied to the chromatofocusing column was determined for each substrate. The recovery ranged from 50 to 70% for the various substrates although the difference in recovery did not vary more than 15% for a particular substrate for the three different microsomal preparations except for phenolphthalein and estrone from the 3-MC-treated microsomes. The 2-fold greater recovery of activity for these substrates from the chromatofocusing column was corrected in Fig. 1C.

Initially, the chromatofocusing system was run with PBE-74 from pH 7.5 to 4.0 with polybuffer 74, and activities were determined as already described. Under these conditions, the activities eluted with the same pattern of high and/or low pI forms except the high pI forms eluted above pH 7.0 and the low pI forms eluted at about pH 6.25. Significant amounts (15–35%) of the high pI activities, however, did not bind to this column system but emerged in the flow-through, whereas no significant activity appeared in

the flow-through in the pH 9.5 to 6 gradient. In comparing the pH 7.5 to 4.0 and 9.5 to 6 gradients, the activities remained true to the order presented here with no stable activities eluting below pH 6.0.

The reliability of the chromatofocusing system to separate discretely different membrane proteins according to isoelectric points without cross-contamination due to mixed micelles is indicated by the single location of activities at pH 8.5 toward 3-OH-BP, phenolphthalein and estrone and the primary location of activities for testosterone, naphthol, 4-OH-BiP, and 9-OH-BP at pH 6.7. The quality of the separation was also assessed by the immunogenicity of GT in the two peaks of activities eluted from the chromatofocusing column using anti-GT IgG raised against a low pI form that was purified by affinity chromatography to 95% homogeneity according to SDS-polyacrylamide and two-dimensional gel electrophoreses [19]. The subunit molecular weight of the low pI form is 51,000 daltons.* The immunoadsorption of an aliquot of the protein fractions which were tritiated after elution from the chromatofocusing column with anti-GT IgG shows in Fig. 4 (left lane) that a GT form with a subunit molecular weight of 54,000 daltons is eluted at pH 8.5 and, that primarily, a GT form with a subunit molecular weight of 51,000 daltons (middle lane) is eluted at pH 6.7. The latter form is consistent with the molecular weight of the antigen. When the immunoadsorbed protein fractions eluted at pH 8.5 and 6.7 are combined (Fig. 4, right lane) the 54,000- and 51,000-dalton protein bands migrate to the expected positions. The 51,000- and 54,000-dalton forms have been described recently in a purification and immunochemical study.

Minimum number of different GT enzymes. To determine the minimum number of enzyme forms necessary to explain the data in Figs. 1–3, the ratios of activity after 3-MC treatment to that after PB treatment versus pH values were plotted as described under Materials and Methods. Such plots should show similarities in profiles for substrates which share a common enzyme form in every region of the pH gradient or unique profiles for substrates catalyzed by unique forms. A novel form is most likely to elute

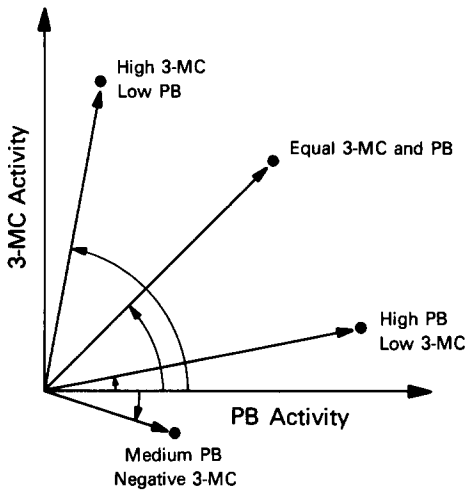

Fig. 5. Schematic representation of the activities after 3-MC or PB treatment by the direction of the *arrow* as an angle in radians from the horizontal. Angles described in this manner at each pH level were used to construct the curves in Fig. 6, panels A and B.

in the region of the curve which shows the greatest amount of variability. Based on the angles described in Fig. 5, two different types of profiles are evident in Fig. 6A. The upper profile encompasses activities for estrone and phenolphthalein to form pattern 1 (Fig. 6C), while the lower profile encompasses activities for 2-OH-BiP, 4-OH-BiP, 4-MU, 3-OH-BP, and naphthol to form pattern 2 (Fig. 6C).

Each activity for morphine, testosterone, 9-OH-BP, and PNP has a unique profile (as schematically smoothed in Fig. 6C) forming patterns 3 through 6. This analysis, therefore, classifies the substrate activities into six patterns as schematically represented in Fig. 6C. Morphine appears to form a unique pattern but may be combined with pattern 2.

DISCUSSION

Solubilized liver microsomes from control, PB-, and 3-MC-treated C57BL/6N mice were focused on a polybuffer exchange resin by developing a pH 9.5 to 6 gradient. Theoretically, the proteins elute from the column near their isoelectric point. Therefore, the pH of the buffer containing an eluted protein is close to the isoelectric point for that protein. Further evidence that the GT forms eluted from the chromatofocusing column according to their isoelectric point is indicated by the distribution of various activities on DEAE anion exchange chromatography [19]. When the same purification fraction derived from PB-induced microsomes was applied to a DEAE anion exchange column adjusted to pH 7.7 instead of the chromatofocusing column, 60–90% of the activity for naphthol, testerone, and morphine bound to the column, whereas 80–95% of the activity for estrone, 3-OH-BP and phenolphthalein emerged in the flow-through without binding. Approximately 50% of the activity for 4-MU and PNP bound to the column at pH 7.7 and 50% emerged in the flow-through. The binding of the activities for testerone and morphine to the

* In an earlier study [19], the anti-GT IgG preparation was shown to immunoprecipitate *directly* 88, 35 and 15% of the activity towards naphthol, PNP, and 3-OH-BP, respectively, from PB-treated ^{3}H microsomes at the same time that only the 51,000-dalton protein band was recovered on SDS-polyacrylamide gel electrophoresis. On the other hand, the adsorption of all immune complexes with protein A-Sepharose without prior direct immunoprecipitation showed the presence of two bands with molecular weights of 51,000 and 54,000 daltons. Under these conditions, 70–85% of the activities for naphthol, PNP, and 3-OH-BP was removed. The appearance of the 54,000-dalton band coincided with the increased removal of activities toward 3-OH-BP and PNP. In separate experiments, the GT immune complexes bound to protein A-Sepharose, when washed extensively with high detergent concentrations and re-equilibrated with detergent-free buffer, were found to contain GT activities for substrates representative of the high and low pI forms. This result indicated that the GT forms were not trapped by the immune complexes but were immunogenically removed without inhibition of catalytic activity.

anion column at pH 7.7 is consistent with a protein(s) with an isoelectric point at about 6.7 (Fig. 2), and the lack of binding of those activities for 3-OH-BP, estrone and phenolphthalein is consistent with a protein(s) with an isoelectric point close to 8.5 (Fig. 1). The distribution of activities for 4-MU and PNP on the DE-52 column is consistent with 50% of a low pI form(s) and 50% of a high pI form(s) (Fig. 3). Further evidence that the high and low pI forms of GT do not cross-contaminate by mixed micelles is provided by the immunoprecipitates of the chromatofocused proteins after tritiation. The antibody preparation raised against the purified low pI form developed precipitin lines in the Ouchterlony double diffusion assay only against the fractions which were eluted at pH 6.7 (P. I. Mackenzie and I. S. Owens, unpublished results), suggesting that the antigen was present only in those fractions. Also, the anti-GT IgG immunoprecipitates primarily the 51,000-dalton form (GT_{M1}) from the protein fractions which eluted at pH 6.7 (Fig. 4, second lane) and not from the fractions which eluted at pH 8.5. On the other hand, the GT_{M2} form can only be immunoadsorbed with anti-GT IgG from the fractions eluting at pH 8.5 (Fig. 4, first lane). The small amount of GT with a molecular weight greater than 51,000 eluting at pH 6.7 (Fig. 4, second lane) is most likely different from the 54,000-GT band (Fig. 4, first lane) associated with 3-OH-BP and phenolphthalein glucuronidation since these activities were not detected in the pH 6.7 fractions after 3-MC treatment (Fig. 1C).

Microsomal proteins from untreated C57BL/6N mice, separated according to this procedure, showed primarily three types of elution profiles for GT activities with the eleven different aglycones used in this study.* Activities when detectable for one set of substrates were always eluted primarily in the high pH region (8.5); activities for a second set were eluted primarily in the low pH region (6.7); and activities for a third set were eluted in both the high and low pH regions. Table 1 summarizes the region of the gradient which contains most of the activity for each substrate used in this study.

These results provide new information concerning the charge heterogeneity of GTs which contribute to the glucuronidation of certain aglycones when using intact microsomes from untreated, PB- and 3-MC-treated mice. From the GT purification study in mice [13], for example, there is no indication that more than one enzyme form metabolizes a particular substrate. The evidence in Figs. 2 and 3 and Table 1 indicates that at least eight substrates used in this study are metabolized to a significant extent by more than one form of GT. Activities toward 4-MU and PNP, preferentially induced by 3-MC in microsomal studies [2–5], showed approximately equal responsiveness in the low and high pH fractions to PB treatment whereas only activities in the high pH fractions were affected by 3-MC. Activity towards naphthol, also typically induced by 3-MC, was the most responsive in the low pH fractions by PB, but at the high pH fraction by 3-MC treatment. On the other hand, activity toward 3-OH-BP, an activity

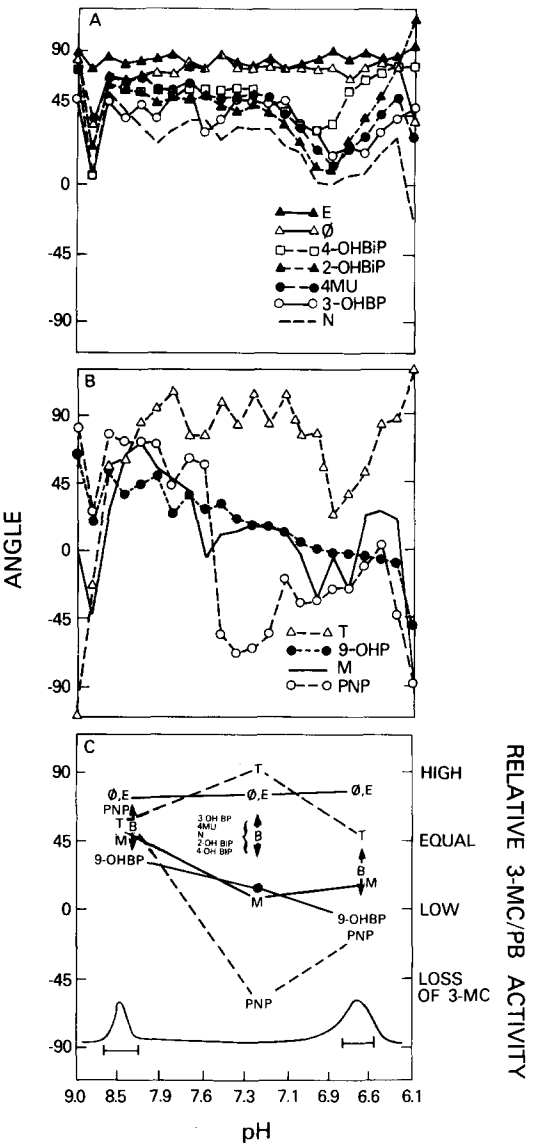

Fig. 6. (A and B) Direction of the angle for a substrate activity versus the pH of elution of that activity. The angles were determined from twenty-two plots of the ratio of 3-MC activity minus control activity to PB activity minus control activity for the eleven substrates at twenty-two pH levels as illustrated in Fig. 5. The curve for each substrate is labeled. Before the angles were determined, the activities were corrected for differences in protein applied to the chromatofocusing column and differences in recovery of the various activities from the column. (C) Averaged representation of the curves in Fig. 6A and 6B. The curves in Fig. 6A were averaged according to similarities in profiles such that estrone and phenolphthalein (E, ϕ) formed pattern 1 and 3-OH-BP, 4-MU, naphthol, 2-OH-BiP, and 4-OH-BiP formed pattern 2. Each curve in Fig. 6B was considered unique and not part of any other pattern. They are schematically represented in Fig. 6C. The symbols are defined in the abbreviation footnote at the beginning of the paper. The schematic curve at the *bottom* of the graph defines the position of peak 1 and peak 2 in Figs. 1–3.

* The discussion of types 1, 2, or 3 substrates in this paper considers the region of the pH curve where the majority of *control* activities elute, whereas the six patterns discussed for Fig. 6 considers the possibility of overlapping substrate specificity throughout the gradient.

inducible by 3-MC [2–5], was responsive only at the high pH peak to either inducer. Activities toward 4-MU, PNP, naphthol and 3-OH-BP at the microsomal level were induced 2.0-fold by either PB or 3-MC in this study. A consideration of activities responsive preferentially to PB treatment showed that 2-OH-BiP and 4-OH-BiP were overwhelmingly localized to the low pH region, and all the activity toward phenolphthalein was in the high pH region from either microsomal preparation. Hence, studies concerned with preferential induction of transferase activities by PB for a particular subset of substrates [5, 6] most likely work by increasing the level of multiple enzymes and not one particular GT form. It appears that at least two forms in mice separable by chromatofocusing are induced by PB but that only one form defined by this system is responsive to 3-MC. Furthermore, the homogeneity of angle at the high pH fractions suggests that PB and 3-MC may, in fact, induce the same form.

The specificity of a particular glucuronidating enzyme also complicates interpretations regarding heterogeneity of forms. It is known from studies [9, 26] with purified GT that a particular form can exhibit broad substrate specificity. Overlapping substrate specificity is demonstrated in this study by the observation that no substrate activity is ever zero at any region in any gradient. Furthermore, the low pI form which has been purified [19] exhibited rather broad substrate specificity in that it preferentially glucuronidated 1-naphthol, testosterone and morphine and had intermediate activity for 4-nitrophenol and 4-methylumbelliferone.

The complex induction profiles in Figs. 1–3 were analyzed for the minimum number of different forms necessary to explain the data. The schematic representations (Fig. 6C) of the patterns indicate that at peak 2 (low pH region) a wide range in the ratios (angles) of activity for 3-MC induction to PB induction exists. The existence of a minimum of two enzymes, E_1 and E_2, which are differentially induced by 3-MC and PB and each with varying K_m values for the eleven substrates could account for the substrate activities observed. It is possible, however, that more than two enzyme forms exist in this region of the gradient. In peak I (high pH) region, the ratio of substrate activities between inductions showed greater homogeneity than at pH 6.7. It is possible, therefore, that one enzyme form, E_3, might be sufficient to explain the observed uniformity in the level of activities in this region.

The variability of the patterns in the midrange (Fig. 6C) was greater than in any other region, suggesting the possibility of two different enzyme forms, which failed to focus properly into a peak. During two to three replications of each of the chromatofocusing runs shown in Figs. 1–3 for control, PB- and 3-MC-treated microsomes, there was never an indication of significant activity in the midrange in spite of the variability of the patterns in this area.

3-MC was equal to or more effective than PB as an inducer in the high pH region for the three types of substrates (Figs. 1–3, 6A and 6B). Activities toward estrone and phenolphthalein clearly showed greater inducibility by 3-MC when values were corrected for the amount of protein applied to the columns and recovered. Activity for 4-MU, previously shown [27] to be genetically associated with the *Ah* locus, was increased more than twice as much in the high pH region compared to that in the low pH region. This result suggests that the GT(s) which is genetically regulated by the *Ah* locus has a high pI.

The successful separation of membrane-bound GT forms by chromatofocusing enables one to categorize these activities according to approximate pI values and, thus, design ion exchange chromatography to select for substrate-specific forms.

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