Microsomal Hydroxylation of Specifically Deuterated Monosubstituted Benzenes. Evidence for Direct Aromatic Hydroxylation†

Robert P. Hanzlik,* Kerstin Hogberg, and Charles M. Judson

ABSTRACT: The aromatic hydroxylation of six pairs of selectively deuterated monosubstituted benzenes was investigated with rat liver microsomes of various induction states. The substrates studied included 3,5-$D_2C_6H_3X$ (**1a–6a**) and 2,4,6-$D_3C_6H_2X$ (**1b–6b**), where X = Br, CN, NO_2, OCH_3, CH_3, or Ph, respectively. The deuterium content of the ortho, meta, and para hydroxylated metabolites, as well as side chain oxidation products from **4** and **5**, was determined by capillary gas chromatography–mass spectroscopy. These data were analyzed according to a hypothetical model in which a molecule of substrate can undergo either *direct* aromatic hydroxylation (defined as obligatory and complete loss of deuterium from the site of hydroxylation) or *indirect* aromatic hydroxylation (defined as the obligatory and complete shift of deuterium to an adjacent position, followed by its partial loss as governed by a kinetic deuterium isotope effect). From this and other analyses of the data the following conclusions were reached. (1) The relative extent of meta hydroxylation increased and the total yield of metabolites decreased as the substituents X became more electron withdrawing. (2) The induction state of the microsomes altered the regioselectivity of hydroxylation (2, 3, 4, or side chain) noticeably and predictably but had little or no effect on the retention or loss of deuterium during each hydroxylation. (3) With each substrate and at each ring position hydroxylation was found to occur by a *combination* of direct and indirect mechanisms. (4) The relative importance of direct vs. indirect mechanisms did not vary in a simple manner with either the position of hydroxylation or the nature of the substituent X. (5) For 20 different hydroxylations the value of the isotope effect (k_H/k_D) governing retention or loss of deuterium was 4.05 ± 0.2. These observations provide strong independent support for the existence of "direct" pathways for aromatic hydroxylation and for the obligatory intermediacy of a cyclohexadienone in "indirect" aromatic hydroxylations.

Enzymic aromatic hydroxylation is generally thought to proceed by way of arene oxide intermediates that rearrange nonenzymically to phenols (Low & Castagnoli, 1980; Jerina & Daly, 1974). In a few cases epoxide intermediates have been isolated or trapped as such, but in most cases evidence for their discrete existence is indirect, e.g., isolation of dihydrodiols and/or premercapturic acids. Numerous cases of NIH shift processes during aromatic hydroxylations have also been documented, but this in itself is not unambiguous evidence for an epoxide intermediate (Trager, 1980). The general absence of large (i.e., primary) kinetic deuterium (or tritium) isotope effects on the rate of hydroxylation of aromatic compounds is consistent with epoxidation as the rate-limiting enzyme-catalyzed step, since no C–H bonds are broken. However, several apparent exceptions to this generalization have been reported (Tomaszewski et al., 1975; Billings & McMahon, 1978) and, together with other evidence (Selander et al., 1975; Preston & Allen, 1980; Forgue & Allen, 1982; Preston et al., 1983), have been taken to suggest the existence of an alternate or "direct" pathway for aromatic hydroxylation.

Potential chemical mechanisms for cytochrome P-450 catalyzed aromatic hydroxylations have been thoroughly analyzed (Tomaszewski et al., 1975; Trager, 1980), as summarized in Figure 1. As seen in this figure, direct pathways to phenol formation may exist with (d, f) and without (c), an epoxide intermediate. Similarly, indirect pathways involving 1,2 shifts may also exist with (d, g) and without (e) epoxide intermediates.

Experimental differentiation between all of these possibilities would likely to be extremely difficult. In the present work we have therefore adopted an operationally more useful classification for mechanisms of aromatic hydroxylation. We define direct aromatic hydroxylation processes as those leading only to complete loss of deuterium from the position of hydroxylation. Similarly, indirect aromatic hydroxylations lead only to complete migration of deuterium to an adjacent ring position, from which its net retention or loss is governed by a kinetic isotope effect (Schemes I and II below).

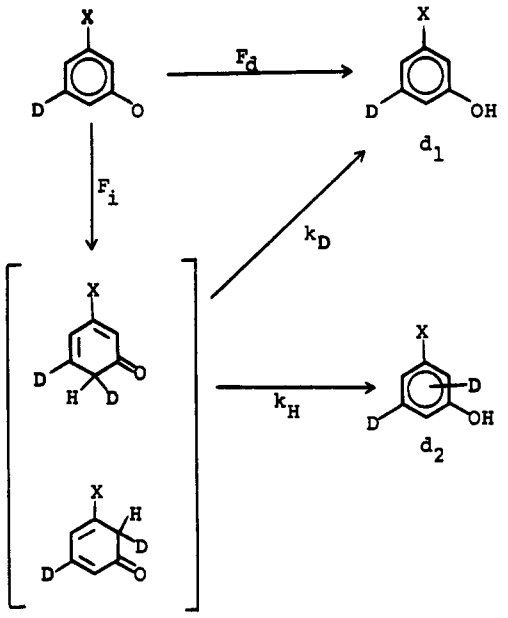
We have now tested the model with data from the microsomal ortho, meta, and para monohydroxylation of 15 isotopic variants of six monosubstituted benzenes. With minor exceptions, this model qualitatively and quantitatively accommodates our data and indicates that both types of processes contribute to the net hydroxylation of each substrate. However, their relative contribution is not a simple function of either the position of hydroxylation or the nature of the substituent. These findings are discussed in relation to other studies indicating the existence of a direct aromatic hydroxylation pathway.

Hydroxylation Model. Consider the meta hydroxylation of a 3,5-dideuterio substrate (Scheme I) and the related 2,4,6-trideuterio substrate (Scheme II). Of the total amount of metabolite formed in each case a certain fraction of it, F_d, may arise by direct aromatic hydroxylation, while the remaining fraction, F_i, will arise by indirect or shift-type mechanisms (i.e., $F_i + F_d = 1$). In the latter instance the kinetic isotope effect on enolization of the dienone intermediates is k_H/k_D, where $k_H + k_D = 1$. Note that F_i, F_d, k_H, and k_D are *fractional* rate constants for the steps indicated in the schemes. If the mole fraction of substrate or product containing n atoms of deuterium is represented by D_n and d_n, respectively, eq 1 and 2 can readily be derived. If it is assumed (see Results and Discussion) that k_H/k_D does not vary significantly among the regioisomeric and/or chiral forms of a given dienone intermediate, or between the various deuterated forms of a given substrate, one can solve eq 1 and 2 for explicit

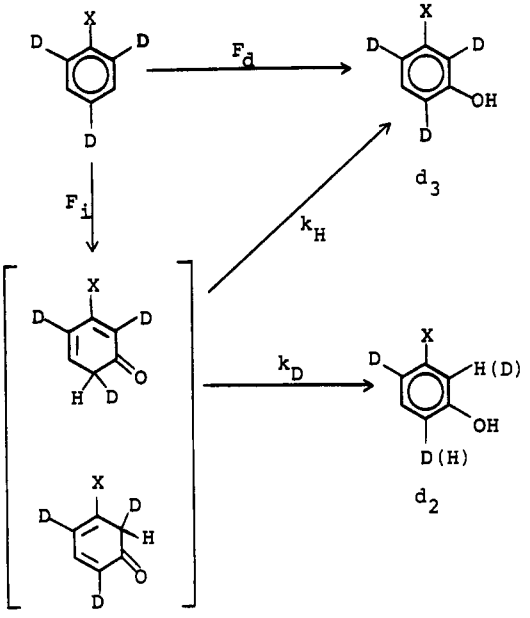
† From the Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045-2500. *Received September 27, 1983.* Financial support for this work was provided in part by the National Institutes of Health (GM-21784) and the University of Kansas General Research Fund. Funds for the purchase of the mass spectrometer used in these studies were provided by NIH Grant GM-27430.

0006-2960/84/0423-3048$01.50/0

Scheme I



Scheme II



values of F_i and k_H for the meta hydroxylation of each substrate Ph–X (eq 3 and 4). For para hydroxylation, similar models may be derived (not shown), resulting in eq 5 and 6.

$$d_2 = D_2 k_H F_i \quad (1)$$

$$d_3 = D_3(F_d + F_i k_H) \quad (2)$$

$$F_i = 1 - d_3/D_3 + d_2/D_2 \quad (3)$$

$$k_H = d_2/(D_2 F_i) \quad (4)$$

$$F_i = 1 - d_2/D_2 + d_3/D_3 \quad (5)$$

$$k_H = d_3/(D_3 F_i) \quad (6)$$

For ortho hydroxylation, the situation is potentially more complex (Schemes III and IV); *in principle*, it can proceed via (i) direct hydroxylation (F_d), (ii) indirect hydroxylation with shift of H or D from C-2 to C-3 (bF_i), or (iii) indirect hydroxylation with shift of H or D from C-2 to C-1 (aF_i), where $a + b = 1$ and F_i and F_d are as defined above. A fourth mechanism would involve ipso attack of enzyme at C-1 with

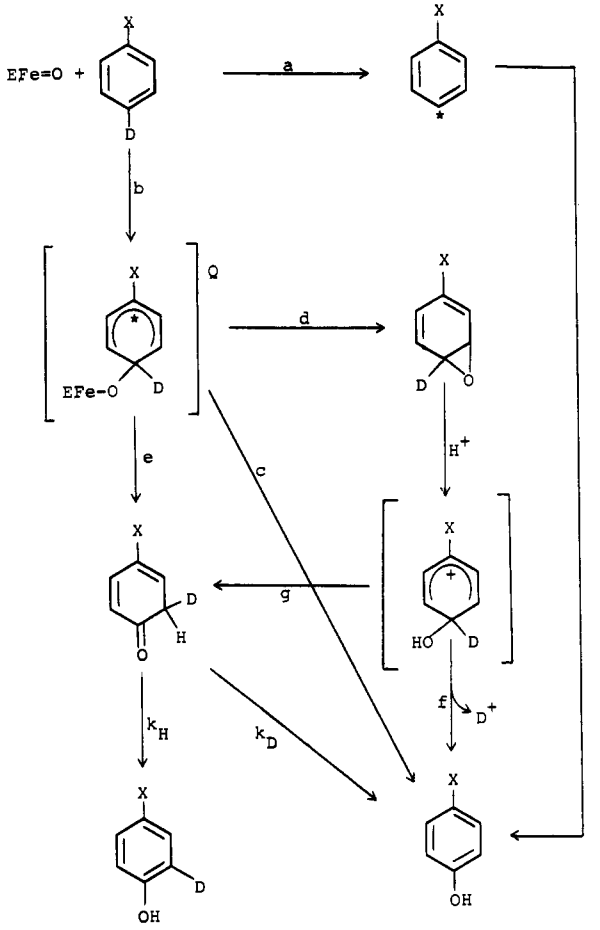
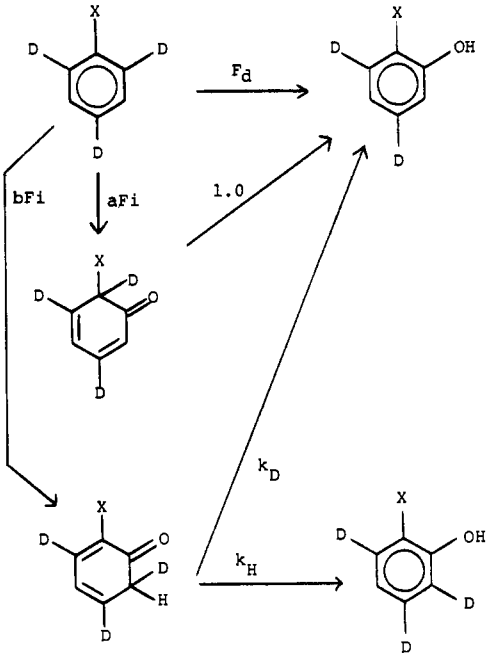


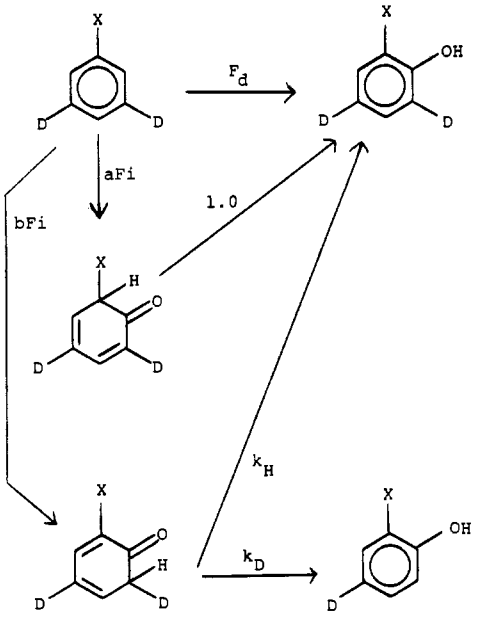
FIGURE 1: Potential mechanisms for aromatic hydroxylation. EFe represents cytochrome P-450, and an asterisk represents a reactive intermediate. For details see text.

Scheme III



shift of the X group to C-2, followed by the total loss of H or D originally at C-2. While examples of NIH shifts of Cl and CH_3 are known, they are not common, and it is not known what other substituents, if any, can shift in this way. Therefore, we have chosen to ignore this potential pathway in our model and to represent total ortho hydroxylation by $aF_i + bF_i + F_d = 1$. From Schemes III and IV one can derive

Scheme IV



eq 7–10 by analogy to the derivation of eq 1–6; note that eq 9 and 10 simplify to eq 5 and 6 when $a = 0$.

$$d_3 = D_3 b F_i k_H \quad (7)$$

$$d_2 = D_2(F_d + aF_i + bF_i k_H) \quad (8)$$

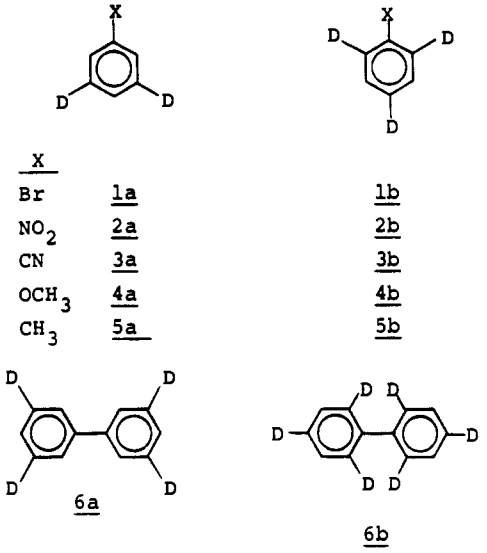
$$bF_i = 1 - d_2/D_2 + d_3/D_3 \quad (9)$$

$$k_H = d_3/(D_3 bF_i) \quad (10)$$

The usual type of NIH shift experiment (viz., Scheme I) measures the extent to which deuterium present in the substrate is shifted *and retained* in the product. By representation of this fraction as δ, it can be seen that for the meta hydroxylation of 3,5-$D_2C_6H_3X$, $\delta = d_2/D_2$, while for the ortho or para hydroxylation of 2,4,6-$D_3C_6H_2X$, $\delta = d_3/D_3$ (cf. eq 4 and 6). One factor that differentiates our study from the usual study of NIH shift processes is the concurrent measurement of hydrogen shift and deuterium loss (viz., Scheme II) as a complement to the measurement of δ values. This enables the explicit evaluation of F_i and k_H through eq 3–6.

Experimental Procedures

Synthesis of Deuterated Materials. Hydrochloride salts of the meta- and para-monosubstituted anilines corresponding to compounds **1–5** were prepared and heated in D_2O (4–6 mL/g) to effect deuterium exchange at ring positions ortho and para to the amine substituent (Swain et al., 1975). After 48 h of refluxing, the mixture was lyophilized, more D_2O added, and refluxing continued for a further 48 h. After a third such treatment, NMR and mass spectral analysis showed that the desired deuterium exchanges had occurred cleanly and almost quantitatively. The amine group was then removed by a sequence of diazotization (Roe, 1949) and reduction (Korzeniowski et al., 1977) to give compounds **1a–5a** from the para-substituted anilines and **1b–5b** from the meta-substituted to anilines. Bromobenzene-*4-d* (**1c**) was prepared by addition of D_2O to *p*-bromophenylmagnesium bromide. Each final compound was isolated by extraction (CH_2Cl_2 or ether), removal of solvent by fractional distillation, and bulb-to-bulb distillation (<0.1 torr); overall yields ranged from 50 to 80%. For the synthesis of **6a** and **6b**, bromobenzenes **1a** and **1b**, respectively, were converted to their Grignard derivatives by refluxing with excess Mg in ether under nitrogen. Addition of Grignard reagent to a rapidly stirred suspension of finely



powdered anhydrous $FeCl_3$ (2 mol/mol of Grignard) in ether under nitrogen effected oxidative coupling and afforded biphenyls **6a** and **6b** in ca. 80% yield after extraction and column chromatography over active silica gel. The chemical purity of each deuterated compound was >98% as determined by NMR and GC/MS analysis (see below).

Metabolic Studies. Male Sprague-Dawley rats (200–250 g) were used throughout this study. In most cases, they were pretreated with a single ip injection of Arochlor 1254 (25 mg in 0.25 mL of corn oil per rat) 48 h prior to killing. In other cases, phenobarbital (60 mg/kg) was administered in 0.9% NaCl (1 mL/kg) by ip injection 72, 48, and 24 h prior to killing. For microsome preparation the livers were removed, chopped, and homogenized at 0–3 °C in phosphate buffer (pH 7.4, 0.1 M) containing 1 mM ethylenediaminetetraacetic acid (EDTA) (4 mL/g of liver). The homogenate was centrifuged at 12000*g* (30 min) and again at 105000*g* (90 min); the microsome pellet was then rehomogenized in phosphate buffer and recentrifuged at 105000*g* (90 min). The final microsomal pellet was resuspended in phosphate buffer (10 mL/g of microsomes) and used immediately for incubations.

Incubations were conducted in glass-stoppered 25-mL Erlenmeyer flasks, which were shaken (90–120 Hz) under air at 35 °C for 1 h. Each incubation contained 6.5 mL of microsome suspension, 0.65 mL of NADPH-generating system, and sufficient substrate dissolved in 50–75 μL of MeCN to give a final concentration of 4 mM. The NADPH-generating system contained 7 mg of NADP, 26 mg of glucose 6-phosphate, 10 IU of glucose-6-phosphate dehydrogenase, and 100 μmol of $MgCl_2$ per milliliter of phosphate buffer.

For extraction each incubation was poured into a 16 × 150 mm culture tube (Teflon-lined screw cap), and two 100-μL portions of 15% $ZnSO_4$ solution were added with mixing after each addition. Except as noted below, metabolites were isolated as follows. Each tube was extracted with 4 mL of ether/hexane (20:80) and centrifuged as needed to break emulsions; this procedure was repeated three additional times with 3, 2, and 2 mL of solvent, and the combined organic phases were then extracted with 2 mL of 0.1 M NaOH. The aqueous phase was acidified with 0.2 mL of 10% HCl, and the metabolites were extracted into ether (1 mL). The ether phase was concentrated in vacuo and applied to a silica gel TLC plate (20 × 20 × 0.025 cm). After elution with ethyl acetate/hexane (20:80), the metabolite bands were identified under UV light with the aid of authentic standards run at the edges of the plate. Metabolite-containing bands were scraped

Table I: Deuterium Analyses of Bromobenzene Substrates and Bromophenol Metabolites

bromobenzene,[a] species (mol %)	expt[b]	bromophenols ortho	meta	para
3,5-$D_2C_6H_3Br$[c]				
d_1 (7.5)	A	20.6	50.0	20.4
d_2 (91.9)		77.0	38.8	71.2
d_1 (1.1)	B	11.0	52.3	18.3
d_2 (98.4)		88.9	43.4	81.8
2,4,6-$D_3C_6H_2Br$				
d_2 (1.7)	C	36.5	13.8	23.6
d_3 (98.3)		63.9	84.4	76.5
4-DC_6H_4Br[d]				
d_1 (93.0)	D	92.4	83.3	62.0
	E	83.4	51.3	65.2
	F	88.3	76.2	68.3

[a] The term d_n gives the percentage of material containing n deuterium atoms. [b] Each letter corresponds to a different preparation of microsomes. Unless otherwise state Arochlor-induced microsomes were used. [c] Two separate preparations of this compound were made and independently submitted to microsomal oxidation. [d] This material was submitted to microsomal oxidation on three separate occasions over an 8-month period.

Table II: Deuterium Analyses of Nitrobenzene Substrates and Nitrophenol Metabolites

nitrobenzene,[a] species (mol %)	expt[a]	nitrophenols ortho	meta	para
3,5-$D_2C_6H_3NO_2$				
d_1 (13.2)	A	15.9	82.6	18.1
d_2 (86.2)		84.1	17.4	81.9
	B	16.0	94.9	18.7
		84.0	5.1	81.3
	C	15.4	75.0	16.6
		84.6	25.0	83.4
2,4,6-$D_3C_6H_2NO_2$				
d_2 (3.3)	B	79.2	5.9	88.5
d_3 (96.7)		20.8	93.4	4.0
	C	60.6	5.8	89.9
		29.1	94.4	6.0
	D	46.4	8.9	75.6
		56.3	91.9	24.4
	E	*b*	7.3	72.0
			93.7	28.0
	F	30.9	9.7	76.4
		69.1	88.7	12.9

[a] See Table I for explanation. [b] Yield of metabolite too low to obtain deuterium analysis.

Table III: Deuterium Analyses of Benzonitrile Substrates and Cyanophenol Metabolites

benzonitrile,[a] species (mol %)	expt[a]	cyanophenols ortho	meta	para
3,5-$D_2C_6H_3CN$[b]				
d_1 (15.0)	A	18.8	86.6	16.4
d_2 (84.2)		81.2	13.4	83.6
d_1 (2.1)	B	17.5	69.1	7.1
d_2 (97.4)		82.5	30.9	92.9
	B	17.2	75.9	8.0
		82.8	24.1	92.0
2,4,6-$D_3C_6H_2CN$				
d_2 (3.8)	B	39.3	8.7	78.6
d_3 (96.3)		59.3	89.4	19.5
	C	48.7	3.5	81.0
		49.5	96.0	17.0

[a] See Table I for explanation. [b] Two separate preparations of this compound were made and independently submitted to microsomal oxidation. The second preparation was incubated in duplicate and both sets of results are presented.

into small glass columns (Pasteur pipets), and the silica was eluted with 10 mL of ether/hexane (20:80). After another cycle of extraction into 0.1 M NaOH, acidification, and reextraction into ether (1 mL), the material was evaporated to dryness in vacuo in a 1-mL conical tube. The residue was treated with 25 μL of BSTFA silylating reagent (60 °C for 1 h) and subjected to GC/MS analysis. The only exceptions to this procedure involved the use of ether/pentane (20:80) to extract the toluene metabolites and the omission of acid–base extractions in the case of the biphenyl metabolites.

GC/MS Analysis. Both the starting substrates and their metabolites were submitted to GC/MS analysis using a Nermag R10-10 quadrupole gas chromatograph/mass spectrometer and data system. Samples were introduced with a moving needle injection device and were separated on a 30-m fused silica capillary column coated with Carbowax 20M stationary phase. Substrates were chromatographed isothermally at 80–100 °C. For metabolite analysis, injections were made with an initial column temperature of 80–100 °C, followed immediately by a programmed rise of 3 °C min^{-1} for 10 min and 5 min at the peak temperature prior to recycling for the next injection. For metabolite Me_3Si ethers the peak widths at half-height were in the order of 2–5 s, and the chromatographic resolution of ortho, meta, and para isomers (in that order of elution) was >90–95% with cresols and complete in all other cases.

Full spectra (50–350 amu, 2-ms integration time at each mass peak) were collected throughout the chromatographic separation to allow verification of metabolite identity. For deuterium analysis selected ion monitoring was used across a 5–8 amu mass range encompassing the molecular ions (for substrate analyses) or the $ArOSiMe_2^+$ fragment ions for metabolite analyses (50–100 scans per GLC peak). Intensity data for each relevant mass peak were collected for each metabolite peak and averaged, and similarly averaged background data were subtracted to obtain corrected relative intensities at each mass. Similar data were collected for nondeuterated standards of both substrates and metabolites. The matrix–algebraic procedure of Brauman (1965) was used to determine the true mole fraction of each deuterated species in the mixture.

Because of major interference from M − 1 fragment ions, the deuterium content of toluene and biphenyl could not be determined accurately by direct mass spectral analyses. The data reported in Table V for toluenes **5a** and **5b** are, in fact, those of the respective deuterated toluidine precursors; their validity is confirmed by the deuterium analysis of the benzyl alcohol metabolite from these substrates (see Results and Discussion). The deuterium analyses reported for biphenyls **6a** and **6b** were calculated from the square of the observed distribution of deuterated species in their respective precursors, **1a** and **1b**.

Results and Discussion

The deuterium analyses of substrates **1–6** and of their derived monohydroxy metabolites are presented in Tables I–VI, respectively. The relative proportions of ortho, meta, and para isomers of the metabolites formed are given in Table VII. Before attempting to interpret these data, it is worthwhile to consider several aspects of the quality of the data themselves.

For deuterium analysis of the substrate, ample material was available and the results were always reproducible within ±1%, and often much less. Repetitive analyses of most metabolites were reproducible within ±3%, except for very minor metabolites for which the signal-to-noise ratio was relatively low

Table IV: Deuterium Analyses of Anisole Substrates and Methoxyphenol Metabolites

anisole,[a] species (mol %)	expt[a]	methoxyphenols ortho	meta	para
3,5-$D_2C_6H_3OCH_3$				
d_1 (2.8)	A	11.3	84.6	17.5
d_2 (88.6)		82.4	15.1	79.3
d_3 (8.6)		6.2	0.3	3.2
	B	9.8	86.8	16.7
		84.2	12.9	80.5
		6.0	0.3	2.8
2,4,6-$D_3C_6H_2OCH_3$				
d_2 (18.3)	A	58.2	*b*	38.5
d_3 (80.3)		31.1		57.0
	B	61.7	*b*	40.7
		28.2		54.3

[a] See Table I for explanation. [b] Yield of metabolite too low to obtain deuterium analysis.

Table V: Deuterium Analyses of Toluene Substrates and Cresol Metabolites

toluene,[a] species (mol %)	expt[a]	cresols ortho	meta	para
3,5-$D_2C_6H_3CH_3$				
d_1 (2.9)	A	13.7	*b*	21.5
d_2 (95.8)		86.3		78.5
	B	10.4	84.0	12.9
		84.6	16.0	87.1
	C	12.5	74.7	8.7
		87.5	25.3	91.3
2,4,6-$D_3C_6H_2CH_3$				
d_2 (9.0)	B	58.3	10.2	36.4
d_3 89.7		35.8	87.8	60.9
	C	57.9	12.7	46.1
		34.3	84.0	48.5
	D	54.6	*b*	25.9
		38.6		71.0

[a] See Table I for explanation. [b] Too little metabolite for deuterium analysis.

and/or the background corrections were relatively high. The reproducibility of results from one incubation to the next is best gauged by inspection of the data presented in Tables I–VI. In many cases, reproducibility was quite good, but in others, the variability was greater than could be accounted for in terms of analytical problems with minor metabolites. With some substrates (e.g., nitrobenzene, Table II) interincubation reproducibility was good, even with minor metabolites when metabolic attack occurred adjacent to deuterium, but relatively poor when it occurred at a position bearing deuterium. No explanation for this is readily apparent, nor was there any obvious analytical problem that would justify discarding particular pieces of data. Therefore, all replicate data reported in Tables I–VI were averaged and used in the calculations described below.

Several control experiments were performed to verify that no spurious losses of deuterium from the phenolic metabolites occurred during their isolation. For example, C_6D_5Br and $C_6D_5NO_2$ were incubated with liver microsomes from Arochlor-induced rats and their phenolic metabolites isolated and analyzed in the usual way. In both cases all three isomeric phenols were found to contain exactly 4.0 atoms of deuterium. A second check against spurious loss of deuterium was provided by analysis of the phenol formed by O-demethylation of **4a** and **4b** and the benzyl alcohol formed by benzylic hydroxylation of toluenes **5a** and **5b**. These metabolites were formed concurrently with ring-hydroxylated products (Table VII). In both cases, the deuterium content of these metabolites

Table VI: Deuterium Analyses of Biphenyl Substrates and Phenylphenol Metabolites

biphenyl,[a] species (mol %)	expt[a]	phenylphenols ortho	meta	para
3,5,5′,5′-d_4				
d_3 (13.8)	A	22.2	25.7	27.5
d_4 (84.5)		75.4	70.9	69.3
	B	23.0	27.0	27.8
		73.8	70.0	68.5
	C	22.3	30.0	27.3
		74.3	66.0	69.0
	D[b]	23.3	38.6	28.4
		73.6	56.6	70.3
	E[c]	22.8	36.3	28.2
		73.9	59.5	68.1
2,4,6,2′,4′,6′-d_6				
d_5 (3.4)	C	47.6	31.8	24.6
d_6 (96.6)		49.1	65.9	73.6
	F	45.7	29.2	25.5
		52.1	65.5	71.3
	D[b]	47.8	19.5	28.4
		50.0	79.4	70.3
	E[c]	45.9	19.9	25.5
		49.2	79.2	73.1

[a] See Table I for explanation. [b] This experiment utilized microsomes from phenobarbital-induced rats. [c] This experiment utilized microsomes from noninduced rats.

Table VII: Relative Proportions of Metabolites Formed from Monosubstituted Benzenes in Vitro

substrate	microsome type[a]	hydroxylation products ortho	meta	para	other
1, PhBr	A	19–46	10–20	44–69	
2, $PhNO_2$	A	2–16	33–44	49–54	
3, PhCN	A	45–46	21–29	26–33	
4, $PhOCH_3$	A	15–24	1–3	62–75	11–15[b]
5, $PhCH_3$	A	6–10	1–2	5–7	82–88[c]
	P	9–19	1–2	19–30	50–72[c]
	N	16–18	1–2	18–20	60–64[c]
6, PhPh	A	34–40	10–11	50–55	
	P	24–35	6–15	50–70	
	N	8–14	3–4	83–89	

[a] A, Arochlor 1254 induced; P, phenobarbital induced; N, noninduced. [b] Phenol. [c] Benzyl alcohol.

was within experimental error of that of their respective precursors (data not shown).

The total amounts of metabolites formed in each experiment were not specifically quantitated in these studies, but it was quite obvious that, as reported by Daly et al. (1968), compounds **2** and **3** were hydroxylated much less efficiently than the others. With each substrate the relative proportions of ortho, meta, and para hydroxylation product (Table VII), as well as the moderate degree of variability in these ratios from one incubation to the next, are in general agreement with other published studies involving simple aromatic compounds. Both the decrease in overall yield and the shift of product ratios toward meta hydroxylation as the substituents become more electronegative are consistent with electrophilic character on the part of the attacking enzymic species. Differences in substrate deuterium labeling pattern (i.e., 3,5-d_2 vs. 2,4,6-d_3) had no apparent effect on the regioselectivity of the hydroxylations (data not shown), although a small effect (±5%) might have been obscured by the run-to-run variability in isomer ratios mentioned above. On the other hand, changes in induction status of the microsomes used had a very noticeable effect on the regioselectivity of hydroxylation of toluene and biphenyl (Table VII).

Table VIII: Fractional Retention of Deuterium during Microsomal Aromatic Hydroxylation of Monosubstituted Benzenes[a]

substrate	δ value[b] ortho	meta	para
1, PhBr	0.65	0.43	0.78
			0.70[d]
2, $PhNO_2$	0.45	0.18	0.16
3, PhCN	0.56	0.24	0.18
4, $PhOCH_3$	0.37	*c*	0.69
5, $PhCH_3$	0.40	0.22	0.67
6, PhPh	0.52	0.82	0.75
	0.51[e]	0.73[e]	0.74[e]

[a] Except as noted all incubations used liver microsomes from rats pretreated with Arochlor 1254. [b] See text for definition; 100δ = % NIH shift. [c] Too little metabolite formed to obtain accurate measurement of deuterium content. [d] Computed for *p*-DC_6H_4Br. [e] From incubations using liver microsomes from phenobarbital-pretrated rats; microsomes from noninduced rats gave identical δ values (see Table VI).

Changes in regioselectivity with induction status and/or animal species are commonly observed with substrates that give rise to multiple metabolites. This phenomenon is usually attributed to changes in the relative proportions of various cytochrome P-450 isozymes, each of which may have a distinct pattern of substrate selectivity and/or regioselectivity. In contrast, induction status had no discernible effect on deuterium retention (i.e., NIH shift) in the ortho and para hydroxylation of biphenyl, although a small effect was seen in the case of meta hydroxylation (Talbe VI). This relative lack of change in deuterium retention with induction status (or even species differences) has also been noted with 4-$DC_6H_4OCH_3$, 4-DC_6H_4Cl, and 4-DC_6H_4F (Daly et al., 1969), warfarin-*7-d* (Bush & Trager, 1982), and phenytoin-*4-d* (Claeson et al., 1982).

The fact that induction status significantly alters overall regioselectivity while having little or no effect on deuterium retention suggests that these two issues can and should be considered separately. In particular, it is appealing to propose that the differences in regioselectivity seen with a given substrate are associated with differences in amino acid composition in the regions comprising the active sites of the various isozymes and that fundamental mechanistic details such as deuterium migration during aromatic hydroxylation derive mainly from the properties of heme–oxygen–substrate transition state complexes with relatively little influence from the protein (Bush & Trager, 1982; Hanzlik & Shearer, 1978). This proposal is highly germane here because most of the data presented in Tables I–VI came from incubations that used liver microsomes from rats pretreated with Arochlor 1254, an agent that induces more than one isozyme of cytochrome P-450.

Table VIII lists the δ values associated with the hydroxylation of substrates **1–6**. The δ values we obtained for para hydroxylation of anisole, toluene, and biphenyl are comparable to those reported by Daly et al. (1968) (0.60, 0.54, and 0.64, respectively) and are similar to those reported for the para hydroxylation of phenytoin (0.68–0.72) (Claeson et al., 1982) and 4-chlorobiphenyl (0.79) (Safe et al., 1975). For ortho hydroxylation of anisole and toluene, Daly & Jerina (1969) report δ values of 0.48 and 0.60, whereas our estimates are somewhat lower (0.40 and 0.37, respectively). Our δ values for para hydroxylation of benzonitrile (0.19) and nitrobenzene (0.16) are significantly lower than those reported by Daly et al. (1968) (0.41 and 0.40, respectively). However, the latter values were determined from in vivo experiments, only trace amounts of metabolites were available for analysis, and meta hydroxylation products were not detected in the urine, whereas they were major metabolites in our in vitro system. Most other δ values in the literature are for polysubstituted or fused-ring aromatic systems and cannot be directly compared to our data. In any case, δ values are only lower estimates of the amount of deuterium that actually shifts (F_i), since not all of the shifted deuterium is retained (i.e., $k_D > 0$). The estimates of F_i and k_H given in Table IX provide greater insight to the nature of aromatic hydroxylation processes than can be obtained from δ values alone.

The data in Table IX represent the most detailed investigation of the enzymic hydroxylation of monosubstituted benzene derivatives to date. Only two of these numbers require special comment before considering the data at large. First, the value of k_H/k_D for ortho hydroxylation of nitrobenzene is conspicuously larger than the others. Because of the hyperbolic nature of eq 6, the calculation of k_H becomes extremely sensitive to the value of the term d_2/D_2 as this value approaches unity. Thus, although the particular data appear quite reproducible (Table II), any error in d_2 and/or D_2 sufficient to reduce their *ratio* by only 4% would reduce the calculated value of k_H/k_D from 19.53 to 7.16, which is much less out of line with the others. Second, the calculated value of F_i for meta hydroxylation of biphenyl with microsomes from Arochlor-induced rats is greater than 1.0, which by definition is not possible. As the data from which this calculation is made also appear to be highly reproducible (Table VI), no explanation for this anomaly is readily apparent. It may represent a case in which one or more of the assumptions made in deriving the model are not valid. On the other hand, this situation did not occur when the hydroxylation of the same substrates (**6a** and **6b**) was carried out with microsomes from either phenobarbital-induced or noninduced animals.

With the two exceptions mentioned above, the values presented in Table IX are not unreasonable. The mere fact that eq 3–6 accommodate all of the data in Tables I–VI and

Table IX: Characteristics of Microsomal Aromatic Hydroxylation of Monosubstituted Benzenes[a]

substrate	ortho F_i	ortho k_H	ortho k_H/k_D	meta F_i	meta k_H	meta k_H/k_D	para F_i	para k_H	para k_H/k_D
1, PhBr	0.779	0.834	5.03	0.573	0.753	3.05	0.975	0.798	3.95
							0.898[b]	0.781[b]	3.55[b]
2, $PhNO_2$	0.476	0.951	19.53[c]	0.229	0.801	4.03	0.203	0.771	3.37
3, PhCN	0.674	0.832	4.96	0.316	0.762	3.22	0.226	0.839	5.20
4, $PhOCH_3$	0.458	0.808	4.20				0.855	0.811	4.30
5, $PhCH_3$	0.504	0.800	4.00	0.259	0.836	5.10	0.776	0.863	6.29
6, PhPh	0.642	0.816	4.43	1.136[c]	0.718	2.55	0.935	0.803	4.07
6, PhPh[d]	0.637	0.810	4.26	0.901	0.812	4.03	0.917	0.809	4.24

[a] The terms F_i, k_H, and k_H/k_D are as defined in Schemes I and II and eq 1–6. [b] Computed for *p*-DC_6H_4Br (see Tables I and VIII). [c] See text for discussion. [d] From incubations using liver microsomes from phenobarbital-pretreated rats; microsomes from noninduced rats gave similar δ values (see Table VI).

lead to values of the sort expected would appear to provide some justification for the model depicted in Schemes I and II as well as for the associated assumptions. In examining these results, one is struck by the observation that *in each case hydroxylation appears to occur by a combination of "direct" and "indirect" types of mechanisms*. Although the balance between the two varies considerably, in no case does F_i approach zero and in only two instances does it approach unity. The value of F_i varies less among the ortho hydroxylations than among the meta or para hydroxylations, but in none of these series does F_i vary in a simple way with the nature of the substituent. Finally, there appears to be no consistent relationship between F_i values for ortho, meta, and para hydroxylation among the various substrates. The values of F_i and k_H for ortho hydroxylation given in Table IX were computed from eq 5 and 6. Their computation by use of eq 9 and 10 has no special advantage since the value of b must be estimated and there is no rational basis for making such an estimate at present. Since the aF_i pathway is *operationally* equivalent to the F_d pathway, it seems preferable to use eq 5 and 6 rather than eq 9 and 10 to analyze data for ortho hydroxylation.

In contrast to the variability in F_i, the calculated value of k_H is remarkably constant across all of the individual hydroxylations, i.e., 0.802 ± 0.033 (mean ± SD, n = 20). The corresponding mean kinetic isotope effect responsible for retention of deuterium once it shifts is thus 0.802/0.198 = 4.05, which is not unreasonable for an isotope effect of this sort (Boyd et al., 1972). The relatively constant value of k_H across Table IX, despite significant variations in F_i, is consistent with the cyclohexadienone structure depicted in Figure 1 being an intermediate common to *all* indirect or shift-type hydroxylations. In addition, it provides post facto justification for the assumptions made in deriving eq 1–6, although direct experimental support for them unfortunately remains lacking. If a cyclohexadienone is common to all indirect hydroxylations, then the variability in F_i must arise from events that occur prior to the formation of this intermediate. The same must also be true of the extent to which an arene oxide is or is not formed along the way (path d vs. paths c + e in Figure 1), although NIH shift data cannot illuminate this aspect of the mechanism.

The data presented in Table IX clearly support the existence of mechanisms for direct aromatic hydroxylation not involving arene oxides or NIH shifts. Other evidence for the existence of direct aromatic hydroxylation has come from two basically different types of observations. One of these involves the isolation of major amounts of a particular hydroxylation product that cannot be accounted for by the rearrangement of an epoxide precursor. Examples include the meta hydroxylation of chlorobenzene (Selander et al., 1975) and 2,5,2′,5′-tetrachlorobiphenyl (Preston & Allen, 1980; Forgue & Allen, 1982; Preston et al., 1983). A related example in a nonaromatic system involves the formation of chloral as a major metabolite of trichloroethylene (Miller & Guengerich, 1982). Such observations effectively rule out the intermediacy of epoxide metabolites (which may be of considerable toxicological interest), but they do not preclude an NIH shift through a mechanism such as path e in Figure 1. Although information on NIH shifts during the metabolism of the tetrachlorobiphenyl mentioned above is lacking, a δ value of 0.24 has been reported for the meta hydroxylation of chlorobenzene (Daly & Jerina, 1968), and the formation of chloral from trichloroethylene can only occur via chlorine migration, an event not unprecedented among aromatic hydroxylations (Daly et al., 1972).

The second line of evidence previously cited in support of direct mechanisms for aromatic hydroxylation involves observations of normal kinetic deuterium isotope effects on the rates of hydroxylation of deuterated vs. nondeuterated substrates (Tomaszewski et al., 1975; Billings & McMahon, 1975; Bush & Trager, 1982). However, "significant" isotope effects were observed in only a few cases, and they were rather small compared to the isotope effects that could have been expected if events such as steps a or c in Figure 1 were rate limiting in the turnover of cytochrome P-450 during these hydroxylations. The observation of a large kinetic isotope effect on an enzyme-catalyzed reaction can signify that a particular chemical step is rate limiting in turnover, but when the isotope effect observed is negligible or only a fraction of its expected value, it becomes ambiguous. It could be that the isotope effect in question is intrinsically small or that the isotopically sensitive step is not rate limiting in turnover or both. Furthermore, if more than one reaction pathway contributes to product formation (viz., Figure 1), only an average isotope effect will be observed. Thus, it is difficult to draw much support for the existence of direct aromatic hydroxylation pathways from the intermolecular isotope effect data currently available. Perhaps further measurements of intramolecular isotope effects on aromatic hydroxylation would help to resolve this question.

Conclusion

The results of this study provide clear support for the existence of "direct" pathways for aromatic hydroxylation involving neither arene oxides nor shift of deuterium. As is evident from Figure 1, the observation of an NIH shift is not in itself sufficient grounds to implicate an arene oxide intermediate. However, the observation of nearly identical values for the isotope effect governing retention or loss of deuterium once it has shifted (i.e., k_H/k_D = 4.05) provides strong support for the intermediacy of a cyclohexadienone in all indirect or NIH shift type hydroxylations. In addition, results of recent calculations[1] modeling the competing pathways c, d, and e in Figure 1 indicate, assuming that the Fe–O bond in the tetrahedral intermediate Q breaks first, that epoxide formation (d) is favored over direct phenol formation (c) and ketone formation (e). These calculations further indicate that once an epoxide is formed, protonation and ring opening can lead to both direct (f) and indirect (g) phenol formation. These latter pathways were found to be competitive and sensitive to substituent effects.

The extent to which direct hydroxylation occurs with a given substrate varies with the position of hydroxylation and/or the nature of the substituent group, but there appears to be no simple recognizable pattern to this variation. In contrast, the extent to which direct hydroxylation occurs is relatively insensitive to the animal source or induction status of the microsomes used despite the fact that these factors, as well as the nature of the substituent on the aromatic ring, have marked effects on the overall regioselectivity of the hydroxylation process. These findings are consistent with the proposal (Hanzlik & Shearer, 1978; Bush & Trager, 1982) that (i) the basic mechanistic details of substrate oxygenations effected by cytochrome P-450 are determined by the intrinsic chemical reactivity at various sites in the substrate molecule and the properties of the derived heme–oxygen–substrate transition state complexes but that (ii) differences in *apoprotein* structure among various P-450 isozymes may override the above factors

[1] K. R. Korzekawa, D. S. Spangler, W. F. Trager, and G. H. Loew, private communication.

and influence the regioselectivity of attack on a given substrate.

Acknowledgments

We are grateful to Drs. Donald Jerina, Gilda Loew, William Trager, and Gerald Miwa for providing helpful and constructive criticism of the manuscript. We also thank Lisa Theissen and Deborah Blanchard for their help in the synthesis of some of the deuterated materials used in this study.

Registry No. **1**, 108-86-1; **1**, *o*-OH, 95-56-7; **1**, *m*-OH, 591-20-8; **1**, *p*-OH, 106-41-2; **2**, 98-95-3; **2**, *o*-OH, 88-75-5; **2**, *m*-OH, 554-84-7; **2**, *p*-OH, 100-02-7; **3**, 100-47-0; **3**, *o*-OH, 611-20-1; **3**, *m*-OH, 873-62-1; **3**, *p*-OH, 767-00-0; **4**, 100-66-3; **4**, *o*-OH, 90-05-1; **4**, *m*-OH, 150-19-6; **4**, *p*-OH, 150-76-5; **5**, 108-88-3; **5**, *o*-OH, 95-48-7; **5**, *m*-OH, 108-39-4; **5**, *p*-OH, 106-44-5; **6**, 92-52-4; **6**, *o*-OH, 90-43-7; **6**, *m*-OH, 580-51-8; **6**, *p*-OH, 92-69-3; phenol, 108-95-2; benzyl alcohol, 100-51-6; 3,5-$D_2C_6H_3Br$, 7291-07-8; 2,4,6-$D_3C_6H_2Br$, 13122-41-3; 4-DC_6H_4Br, 13122-33-3; 3,5-$D_2C_6H_3NO_2$, 89908-09-8; 2,4,6-$D_3C_6H_2NO_2$, 28147-77-5; 3,5-$D_2C_6H_3CN$, 37960-86-4; 2,4,6-$D_3C_6H_2CN$, 13137-55-8; 3,5-$D_2C_6H_3OCH_3$, 55415-90-2; 2,4,6-$D_3C_6H_2OCH_3$, 2567-25-1; 3,5-$D_2C_6H_3CH_3$, 16954-38-4; 2,4,6-$D_3C_6H_2CH_3$, 13122-37-7; 3,5-$D_2C_6H_3C_6H_3D_2$-3,5, 6120-98-5; 2,4,6-$D_3C_6H_2C_6H_2D_3$-2,4,6, 6081-66-9.

References

Billings, R. E., & McMahon, R. E. (1978) *Mol. Pharmacol. 14*, 145–154.

Boyd, D. R., Daly, J. W., & Jerina, D. M. (1972) *Biochemistry 11*, 1961–1966.

Brauman, J. (1966) *Anal. Chem. 38*, 607–609.

Busch, E. D., & Traiger, W. F. (1982) *Biochem. Biophys. Res. Commun. 104*, 626–632.

Claeson, M., Moustafa, M. A. A., Aldine, J., Vandervorst, D., & Poupaert, J. H. (1982) *Drug Metab. Dispos. 10*, 667–671.

Daly, J., & Jerina, D. (1969) *Arch. Biochem. Biophys. 134*, 266–268.

Daly, J., Jerina, D., & Witkop, B. (1968) *Arch. Biochem. Biophys. 128*, 517–527.

Daly, J., Jerina, D., Farnsworth, J., & Guroff, G. (1969) *Arch. Biochem. Biophys. 131*, 238–244.

Daly, J. W., Jerina, D. M., & Witkop, B. (1972) *Experientia 15*, 1129–1164.

Forgue, S. T., & Allen, J. R. (1982) *Chem.-Biol. Interact. 40*, 233–245.

Jerina, D. M., & Daly, J. (1974) *Science (Washington, D.C.) 185*, 573–582.

Korzeniowski, S. H., Blum, L., & Gokel, G. W. (1977) *J. Org. Chem. 42*, 1469–1470.

Low, L. K., & Castagnoli, N., Jr. (1980) *Burger's Medicinal Chemistry* (Wolff, M. E., Ed.) 4th ed., Part 1, pp 171–226, Wiley, New York.

Miller, R. E., & Guengerich, F. P. (1982) *Biochemistry 21*, 1090–1097.

Preston, B. D., & Allen, J. R. (1980) *Drug Metab. Dispos. 8*, 197–204.

Preston, B. D., Miller, J. A., & Miller, E. C. (1983) *J. Biol. Chem. 258*, 8304.

Roe, A. (1949) *Org. React. (N.Y.) 5*, 193–228.

Safe, S., Hutzinger, O., & Jones, D. (1975) *J. Agric. Food Chem. 23*, 851–853.

Selander, H. G., Jerina, D. M., & Daly, J. W. (1975) *Arch. Biochem. Biophys. 168*, 309–321.

Swain, G. C., Sheats, J. E., Gorenstein, D. G., & Harbison, K. G. (1975) *J. Am. Chem. Soc. 97*, 791–795.

Tomaszewski, J. E., Jerina, D. M., & Daly, J. (1975) *Biochemistry 14*, 2024–2031.

Trager, W. F. (1980) *Concepts in Drug Metabolism* (Jenner, P., & Testa, B., Eds.) Part A, pp 178–209, Marcel Dekker, New York.

Monoclonal Antibody Specific for Yeast Elongation Factor 3†

James S. Hutchison, Barry Feinberg, Teva C. Rothwell, and Kivie Moldave*

ABSTRACT: Hybridomas have been prepared by fusing mouse myeloma (P3 × 63 Ag8) cells with spleen cells of mice immunized with a yeast fraction enriched with respect to nonribosomal translational components. Cloned hybridoma lines were grown in the form of ascites tumors, and the monoclonal antibodies produced were purified from the ascites fluid by chromatography on DEAE-Affi-Gel Blue. One of the antibodies, from a hybridoma cell line designated as PSH-1, inhibited the translation of natural mRNA and poly(U) and polysomal chain elongation in a cell-free protein-synthesizing system from yeast. Resolution and partial purification of the elongation factors indicated that the monoclonal antibody from PSH-1 did not interact with EF-1 or EF-2 but reacted with and inactivated EF-3, the 125 000 molecular weight additional elongation factor specifically required with yeast ribosomes. The EF-3 purified from the cytosol by immunoaffinity chromatography was comparable to that prepared by ion-exchange chromatography. Evidence was obtained which indicated that EF-3 was essential for the translation of natural mRNA as well as poly(U), was associated with polysomes but not ribosomal subunits, and was required for every cycle in the elongation phase of protein synthesis.

Immunological techniques have provided important tools for the biological characterization of components involved in protein synthesis and the reactions that they carry out. Specific polyclonal antisera to prokaryotic and eukaryotic protein synthesis components, such as ribosomes and translational factors, have been prepared (Howe et al., 1978; Petryshyn et al., 1979; Khanh et al., 1979; Van Duin et al., 1979; Lelong et al., 1979; Zinker, 1980; Politz & Glitz, 1980; Ghosh-Dastidar et al., 1980; Tanaka et al., 1980; Stoffler et al., 1980; Kahan et al., 1981; Fahnestock et al., 1981; Van Duin & Wijnands, 1981; Kastner et al., 1981; Brown-Luedi et al., 1982; Meyer et al., 1982; Howe & Hershey, 1982; Olson et al., 1982). One of the problems inherent in the use of polyclonal antibodies has been the requirement for pure specific proteins for immunization, which in many cases are available only in

† From the Department of Biological Chemistry, College of Medicine, University of California, Irvine, Irvine, California 92717. *Received November 10, 1983.* This work was supported in part by Research Grants AM-15156 and GM-27999 from the National Institutes of Health.

0006-2960/84/0423-3055$01.50/0