Iodosylbenzene Derivatives as Oxygen Donors in Cytochrome P-450 Catalyzed Steroid Hydroxylations†

Jan-Åke Gustafsson,* Lars Rondahl, and Jan Bergman

ABSTRACT: The mechanism of cytochrome P-450 catalyzed steroid hydroxylations in rat liver microsomes has been investigated by employing derivatives of iodosylbenzene as oxygen donors. The model steroid substrate androstenedione which was hydroxylated in positions 7α, 6β, and 16α was used in reactions supported by NADPH, iodosylbenzene, and iodosylbenzene derivatives. Evidence for cytochrome P-450 involvement in iodosylbenzene-sustained androstenedione hydroxylation included inhibition by substrates and modifiers of cytochrome P-450. The most efficient oxygen donors were (diacetoxyiodo)-2-nitrobenzene > (diacetoxyiodo)-2-chlorobenzene > 2-nitroiodosylbenzene > (dinitratoiodo)-2-nitrobenzene > (diacetoxyiodo)benzene > (diacetoxyiodo)-2-methoxybenzene > 4-(diacetoxyiodo)toluene > iodosylbenzene. The capacity of the oxidation agents to serve as oxygen donors in cytochrome P-450 dependent steroid hydroxylation is probably dependent upon several factors such as the tendency of iodosyl compounds to associate, which decreases coordination with the heme iron, the presence of bulky substituents in the 2 position (decreases association), and the presence of electron-withdrawing substituents (tends to decrease coordination with the heme iron). The rates of 7α, 6β, and 16α hydroxylation of androstenedione catalyzed by (diacetoxyiodo)-2-nitrobenzene were 108-, 130-, and 167-fold higher, respectively, than the rates of the NADPH-supported reactions. These results strongly suggest that the rate-limiting step in NADPH-sustained cytochrome P-450 catalyzed reactions is the rate of reduction of cytochrome P-450.

Previous work in our laboratory has shown that $NaClO_2$ and $NaIO_4$ may support cytochrome P-450 catalyzed steroid hydroxylation in the absence of NADPH and molecular oxygen (Berg et al., 1975; Gustafsson et al., 1976; Hrycay et al., 1975a,b, 1976). This phenomenon occurred in liver microsomes (Hrycay et al., 1975a, 1976), in partially purified cytochrome P-450 preparations obtained from liver microsomes (Hrycay et al., 1975b) and from *Bacillus megaterium* (Berg et al., 1975), and in adrenocortical microsomal and mitochondria preparations (Gustafsson et al., 1976). Furthermore, Nordblom et al. (1976) have shown that highly purified cytochrome $P\text{-}450_{LM2}$ free of other known electron carriers catalyzes the hydroperoxide-dependent hydroxylation of a variety of substrates in the absence of NADPH, NADPH-cytochrome P-450 reductase, and molecular oxygen. In an extension of our work, we found that iodosylbenzene and (diacetoxyiodo)benzene were highly efficient oxygen donors in ω2 hydroxylation of fatty acids (Gustafsson & Bergman, 1976). These results indicated that the active oxygenated intermediate of cytochrome P-450 catalyzing ω2 hydroxylation only contained one oxygen atom and was possibly identical with the ferryl ion complex ($Fe^{4+}O^{-}$) possessing a formal oxidation state of +5 and equivalent to compound I of horseradish peroxidase (Gustafsson et al., 1976; Hrycay et al., 1975a,b, 1976). Simultaneously and independently, Lichtenberger et al. reported on iodosylbenzene-supported O-dealkylation of 7-ethoxycoumarin in rat liver microsomes (Lichtenberger et al., 1976). In view of these findings it was considered of interest to synthesize various derivatives of iodosylbenzene and (diacetoxyiodo)benzene in order to investigate their potency to support cytochrome P-450 dependent hydroxylation reactions. It was reasoned that information from this type of work should help in elucidating factors of importance in formation of the active oxygenated intermediate of cytochrome P-450.

The present investigation is a comparison between several iodosylbenzene derivatives as oxygen donors in cytochrome P-450 catalyzed steroid hydroxylation in rat liver microsomes.

Materials and Methods

Steroids. 4-[4-^{14}C]Androstene-3,17-dione (specific radioactivity, 60 mCi per mmol) was purchased from New England Nuclear (Boston, MA) and purified by thin-layer chromatography prior to use (Einarsson et al., 1973). Unlabeled 4-androstene-3,17-dione was kindly donated by Dr. J. Babcock (Upjohn Co., Kalamazoo, MI).

Oxidation Agents. Iodo compounds were obtained commercially or prepared by standard methods. The trivalent and pentavalent aromatic iodine compounds were synthesized as follows.

Most of the aryl diacetoxyiodo compounds were prepared by oxidation of aryl iodides with peracetic acid (method A). The reagent was either prepared from hydrogen peroxide (30%, v/v) and acetic anhydride (Pausacker, 1953) or purchased as 40% (v/v) acid (Leffler & Story, 1967). There seemed to be no advantage to use the 40% acid.

Some derivatives of (diacetoxyiodo)benzene were prepared by converting the corresponding substituted iodobenzene into the dichloroiodo compound which was hydrolyzed to the iodosyl compound. Treatment of this compound with glacial acetic acid yielded the diacetoxyiodo compound (Willgerodt, 1899) (method B).

Some of the substituted iodosylbenzenes were prepared by alkaline hydrolysis of the corresponding diacetoxyiodo compound (method C).

Dinitratoiodo compounds were prepared by treating the corresponding diacetoxyiodo compounds with fuming nitric acid and pouring the mixture into ice (Yamada et al., 1976) (method D).

Iodyl compounds were synthesized by oxidation of the corresponding dichloroiodo compound with sodium hypochlorite (Formo & Johnson, 1955) (method E).

(Dibenzoyloxyiodo)-2-nitrobenzene was prepared by the action of ethereal benzoic acid on the corresponding diacetoxyiodo compound (Fox & Pausacker, 1957) (method F).

Treatment of *o*-iodobenzoic acid with chlorine in chloroform and alkaline hydrolysis of the solid obtained yielded 1-

† From the Department of Medical Nutrition and the Department of Chemistry, Karolinska Institutet (J.-Å.G.), and the Department of Organic Chemistry, Royal Institute of Technology (L.R. and J.B.), Stockholm, Sweden. *Received April 24, 1978; revised manuscript received October 27, 1978.* This work was supported by grants from the Swedish Medical Research Council (No. 13X-2819).

0006-2960/79/0418-0865$01.00/0

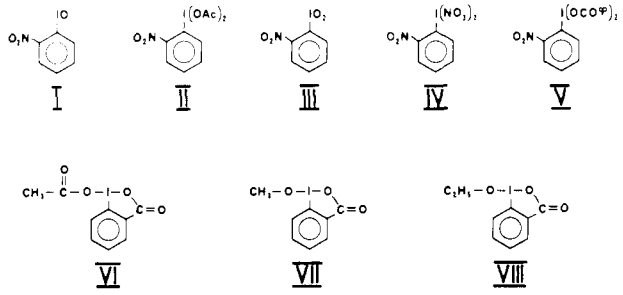


FIGURE 1: Structural formulas of some iodosylbenzene derivatives investigated in the present study. (I) 2-Nitroiodosylbenzene; (II) (diacetoxyiodo)-2-nitrobenzene; (III) 2-nitroiodylbenzene; (IV) (dinitratoiodo)-2-nitrobenzene; (V) (dibenzoyloxyiodo)-2-nitrobenzene; (VI) 1-acetoxy-4,5-benziodoxolin-3-one; (VII) 1-methoxy-4,5-benziodoxolin-3-one; (VIII) 1-ethoxy-4,5-benziodoxolin-3-one.

hydroxy-4,5-benziodoxolin-3-one which failed to give an acetoxy compound on treatment with acetic acid. Gentle boiling for a few minutes in acetic anhydride, methanol, or ethanol yielded the 1-acetoxy, 1-methoxy, or 1-ethoxy derivative, respectively (Baker et al., 1965) (method G).

(Diacetoxyiodosyl)benzene was prepared by treating iodylbenzene with acetic acid (Yagupolskii et al., 1977) (method H).

The compounds and their melting points (uncorrected) are given in Table I. A few structures are shown in Figure 1. Microanalyses were carried out by Centrala Analyslaboratoriet, Uppsala, Sweden. The structures of all new diacetoxyiodo compounds were confirmed by elemental analyses and infrared spectroscopy and mass spectrometry. Mass spectra were recorded at 20 eV with an LKB 9000 instrument.

Preparation of Microsomes. Microsomal fraction was prepared from livers of male Sprague-Dawley rats (220–250 g). The animals were killed by decapitation and the livers were taken out immediately and chilled on ice. Liver homogenates, 50% (w/v), were prepared in a modified Bucher medium (Bergström & Gloor, 1955), pH 7.4, with a Potter-Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. The homogenate was centrifuged at 20000*g* for 15 min. The microsomal fraction was obtained by centrifuging the 20000*g* supernatant fluid at 105000*g* for 70 min. The microsomal fraction was suspended in 12 mL of the homogenizing medium and was homogenized with a loosely fitting pestle. The resulting suspension was recentrifuged at 105000*g* for 70 min and the obtained sediment was suspended and homogenized in 36 mL of Bucher medium and used for incubations.

Standard Incubation Conditions. The radioactive steroid substrate was diluted with unlabeled steroid in acetone prior to use. A 50-μL solution of the steroid (100 μg; 400000 counts/min) was added to a 2-mL final volume containing microsomes (15 mg of protein/mL) and Bucher medium. Oxidation agent-supported reactions were started by adding the following final concentration of reagent: $NaClO_2$ (10 mM); $NaIO_4$ (20 mM); iodosylbenzene, (diacetoxyiodo)-benzene, and derivatives of these compounds (5 mM). NADPH-supported reactions were started by adding 1 mM (final concentration) NADPH. The incubations were performed in air at 37 °C for 2 s up to 15 min and reactions were terminated with chloroform/methanol (2/1, v/v). The conversions of the steroid substrate in the NADPH-supported reactions were linear with respect to incubation time and protein concentration. With the oxidizing agents, velocities were linear with respect to microsomal protein concentration and time for only the first few seconds after which velocities began to level off. It was necessary in some instances to use a 1-min incubation time in order to obtain a suitable yield of hydroxylated products for quantitation. However, for comparison purposes, all reaction rates were calculated from the linear portion of the time curve and expressed as nmol of hydroxylated product formed per mg of protein per min of incubation time.

The influence of CO on the hydroxylation reactions was studied by bubbling a gas mixture of 40% CO, 56% N_2 and 4% O_2 through the incubation mixture during the incubation. The controls were bubbled with 96% N_2 and 4% O_2.

Quantitation of Metabolites. Incubations were terminated by addition of 20 vol of chloroform/methanol (2/1, v/v), the precipitate was filtered off, and 0.2 vol of 0.9% (w/v) NaCl solution was added. The chloroform phase was collected, the solvent evaporated, and the residue dissolved in 0.5 mL of methanol and applied onto precoated silica gel plates (250 μm, Merck, Darmstadt, West Germany). The extracts were developed once in chloroform/ethyl acetate (4/1, v/v). The silica gel plates were autoradiographed with an exposing time of 5 days; the radioactive zones were determined exactly from the X-ray film, scraped off carefully, eluted with methanol, and measured for radioactivity in a liquid scintillation counter using Instagel as scintillation liquid. The identities of the hydroxylated metabolites of androstenedione were checked with a gas chromatograph–mass spectrometer (Einarsson & Gustafsson, 1973; Einarsson et al., 1972, 1973).

Results

Hydroxylated Metabolites of Androstenedione. Following incubation of ^{14}C-labeled androstenedione with liver microsomes fortified with NADPH or various oxidizing agents, three major hydroxylated products were formed, 7α-, 6β-, and 16α-hydroxyandrostenedione.

Rate Estimates of Androstenedione Hydroxylation. Reaction velocities for NADPH-fortified hydroxylations of [^{14}C]androstenedione were linear up to 10 min at 37 °C and were found to be: 7α hydroxylation, 0.13 nmol (mg of protein)$^{-1}$ min^{-1}; 6β hydroxylation, 0.25 nmol (mg of protein)$^{-1}$ min^{-1}; and 16α hydroxylation, 0.07 nmol (mg of protein)$^{-1}$ min^{-1}.

$NaClO_2$- and $NaIO_4$-catalyzed hydroxylations of androstenedione were complete after 2 min of incubation (Figures 2A and 2B), whereas iodosylbenzene-catalyzed hydroxylations were complete 30 s after initiation of incubation (Figure 2C). With (diacetoxyiodo)benzene as a catalyst, formation of hydroxylated products ceased already 10 s following the start of the incubation. No hydroxylated metabolites of androstenedione were formed when incubations were carried out in the absence of hydroxylating agent or with boiled microsomal fractions, indicating the enzymic nature of the reactions.

Figures 3A–C show the effects of increasing concentrations of oxidizing agent on androstenedione hydroxylation in liver microsomes. The saturating concentrations for $NaClO_2$, $NaIO_4$, iodosylbenzene, and (diacetoxyiodo)benzene were 2.5, 20, 2.5, and 2.5 mM, respectively. The most active hydroxylase in all cases was the 6β-hydroxylase. The highest total conversions were obtained with iodosobenzene.

Inhibition of Androstenedione Hydroxylation by Modifiers of Cytochrome P-450. Aniline, a compound that produces type II spectral changes when interreacting with cytochrome P-450, was a potent inhibitor of iodosylbenzene- and (diacetoxyiodo)benzene-supported 7α, 6β, and 16α hydroxylation of androstenedione (Table II). Imidazole, SKF-525 A, *p*-aminoglutethimide, and metyrapone that are all well-known inhibitors of cytochrome P-450 decreased iodosobenzene diacetate-catalyzed androstenedione hydroxylation by up to 50% (Table II). These findings indicate an involvement of

Table I: Data on Synthesized Substituted Iodosyl- and Iodylphenyl Derivatives

compounds	synthetic method	melting point[a] (°C) found	melting point[a] (°C) lit.	%C found	%C calcd	%H found	%H calcd	MS data, *m/e* (rel intensity)
(diacetoxyiodo)benzene	A	157–158	158[e]					
(diacetoxyiodo)-2-nitrobenzene	B	146–148	145[f]					308 (16), 249 (100), 203 (30), 92 (12), 76 (16), 43 (19)
(diacetoxyiodo)-3-nitrobenzene	B	145–150	155[e]					249 (100)
(diacetoxyiodo)-2-chlorobenzene	A	137–139	140[g]					299 (17), 297 (51), 240 (39), 238 (100), 113 (8), 111 (25), 43 (7)
(diacetoxyiodo)-3-chlorobenzene	A	153–155	154–155[h]					299 (10), 297 (32), 240 (32), 238 (100), 113 (12), 111 (34), 43 (7)
(diacetoxyiodo)-4-chlorobenzene	A	188–191	185–190[h]					299 (9), 297 (28), 240 (33), 238 (100), 113 (16), 111 (30), 43 (7)
(diacetoxyiodo)-2-trifluoromethylbenzene	A	155		34.0	34.0	2.2	2.2	272 (100)
(diacetoxyiodo)-3-trifluoromethylbenzene	A	139–140		33.9	34.0	2.7	2.3	331 (42), 272 (100), 145 (34), 43 (7)
(diacetoxyiodo)-4-trifluoromethylbenzene	A	167–169		32.0	34.0	2.2	2.3	272 (100), 145 (33)
(diacetoxyiodo)-2-methylbenzene	A	140–141	140–142[i]	39.4	39.3	3.9	3.9	
(diacetoxyiodo)-4-methylbenzene	A	101–104	109[e]					
(diacetoxyiodo)-2-methoxybenzene	A	147–149		37.6	37.5	3.7	3.7	293 (14), 235 (7), 234 (100), 219 (13), 114 (9), 92 (8), 77 (7), 43 (5)
(diacetoxyiodo)-3-methoxybenzene	A	131–132		37.5	37.5	3.7	3.7	293 (10), 235 (7), 234 (100), 166 (5), 107 (17), 92 (7), 43 (8)
(diacetoxyiodo)-2,6-dimethoxybenzene	A	194–195		37.6	37.7	4.0	3.9	323 (3), 264 (100), 249 (4), 221 (7), 154 (5), 107 (4)
(diacetoxyiodo)-2-cyanobenzene	A	146–148		38.1	38.1	2.9	2.9	288 (26), 229 (100), 102 (38), 43 (10)
(diacetoxyiodo)-4-bromobenzene	B	115–117		29.9	29.9	2.5	2.5	343 (12), 341 (10), 284 (100), 282 (100), 157 (29), 155 (30), 43 (91)
(diacetoxyiodo)-4-phenylbenzene[b]	B	138–142	141–144[j]					339 (8), 281 (10), 280 (100), 170 (23), 153 (16), 152 (23), 43 (5)
1-(diacetoxyiodo)naphthalene	A	176–178	170–175[k]	45.2	45.2	3.6	3.5	254 (100), 144 (7), 187 (35), 43 (6)
(dibenzoyloxyiodo)-2-nitrobenzene	F	90–91						
(dinitratoiodo)benzene	D	175–178	105–106[l]					
(dinitratoiodo)-2-nitrobenzene	D	217–218						
p-bis(diacetoxyiodo)benzene	A	223–226[c]	220–223[m]					389,[d] 330 (100), 220 (13), 203 (46), 76 (17)
1-hydroxy-4,5-benziodoxolin-3-one	G	225–230 (dec)	231–233 (dec)					249 (9), 248 (100), 231 (32), 65 (11)
1-acetoxy-4,5-benziodoxolin-3-one	G	165–168	167–169					306 (M^+ 5), 248 (13), 247 (100), 220 (12), 203 (44), 60 (12), 43 (24)
1-methoxy-4,5-benziodoxolin-3-one	G	160–165	166–168					278 (M^+ 19), 253 (27), 248 (17), 247 (62), 234 (48), 219 (89), 203 (100), 92 (24), 76 (39), 77 (39)
1-ethoxy-4,5-benziodoxolin-3-one	G	120–125	126–128					
iodylbenzene	E	238–240 (dec)						220,[d] 204 (100), 77 (40)
2-nitroiodylbenzene	E	217–218 (dec)	210 (dec)					
(diacetoxyiodosyl)benzene	H	189–191	192[n]					220,[d] 204 (100), 77 (21)

[a] Recrystallized from acetic acid or acetic acid and water. [b] Recrystallized from acetic acid and ether. [c] Washed with water. [d] Weak diagnostic peak. [e] Pausscker, 1953. [f] Willgerodt, 1893b. [g] Willgerodt, 1893c. [h] Willgerodt, 1893d. [i] Karek & Neilands, 1970. [j] Leffler & Story, 1967. [k] Ortolevas, 1900. [l] Dasent & Waddington, 1960. [m] Yamada et al., 1976. [n] Yagupolskii, 1977.

Table II: Inhibition of C_6H_5IO- and $C_6H_5I(OAc)_2$-Supported Hydroxylation of Androstenedione by Modifiers of Cytochrome P-450

	hydroxylation (in % of control)								
	C_6H_5IO			$C_6H_5I(OAc)_2$			NADPH		
modifier	7α	6β	16α	7α	6β	16α	7α	6β	16α
control	100	100	100	100	100	100	100	100	100
aniline (5 mM)	37	9	0	80	18	0	57	30	49
aniline (10 mM)	44	9	0	80	18	0			
metyrapone (0.5 mM)	44	61	93	50	50	91	54	20	58
progesterone (0.2 mM)	71	62	59	107	99	124	61	53	50
testosterone (0.2 mM)	100	81	93	129	53	86	71	57	59
imidazole (2 mM)	54	68	71	63	59	62			
imidazole (4 mM)	39	46	43	66	55	76			
SKF-525 A (0.5 mM)	100	100	95	75	48	69			
SKF-525 A (2 mM)	72	74	57	92	53	60			
p-aminoglutethimide (50 μg/mL)	97	106	117	52	44	67			

Table III: Catalysis of Androstenedione Hydroxylation in Rat Liver Microsomes by (Diacetoxyiodo)benzene and Iodosylbenzene Derivatives

	hydroxylations (nmol (mg of protein)$^{-1}$ min^{-1})		
substituents	7α	6β	16α
(Diacetoxyiodo)benzene Derivatives			
unsubstituted	14.0	32.6	11.7
2-nitro	29.0	91.4	43.7
3-nitro	3.8	15.8	5.8
2-chloro	32.3	68.6	23.2
3-chloro	1.0	9.2	3.2
4-chloro	4.6	7	0
2-trifluoromethyl	8.8	23.8	18.4
3-trifluoromethyl	6.8	11.2	4.8
4-trifluoromethyl	5.6	11.6	8.6
2-methyl	6.6	16.8	7.0
4-methyl	2.4	3.2	0
2-methoxy	11.0	31.2	10.8
3-methoxy	4.8	10.8	3.8
2,6-dimethoxy	0.5	2.1	0
2-cyano	0	0	0
4-bromo	0.6	1.8	0
4-phenyl	0	0	0
2,3-benzo(1-(diacetoxyiodo)naphthalene)	3.8	6.4	3.0
Iodosylbenzene Derivatives			
unsubstituted	10.5	28.9	9.1
2-nitro	11.7	48.2	19.3
3-nitro	2.4	6.5	2.3
2-chloro	2.0	7.2	2.4
3-chloro	0	0	0
4-chloro	0.9	1.5	0
3-trifluoromethyl	2.1	6.0	1.9
4-trifluoromethyl	0	0	0
4-methyl	6.8	29.2	4.3
2-methoxy	8.6	18.2	5.0
3-methoxy	1.6	2.0	0
2,6-dimethoxy	1.3	2.2	0
2,3-benzo	0	0	0
4-bromo	0	0	0

cytochrome P-450 in iodosylbenzene- and (diacetoxyiodo)-benzene-supported liver microsomal hydroxylation of androstenedione.

NADPH-catalyzed androstenedione hydroxylations were inhibited by carbon monoxide in varying degrees: 7α hydroxylation by 88%, 6β hydroxylation by 51%, and 16α hydroxylation by 60%. In contrast, iodosylbenzene- or (diacetoxyiodo)benzene-supported androstenedione hydroxylations were not inhibited by carbon monoxide.

Efficiency of Substituted Derivatives of Iodosylbenzene and (Diacetoxyiodo)benzene in Supporting Steroid Hydroxylation. Tables III and IV summarize results obtained from liver

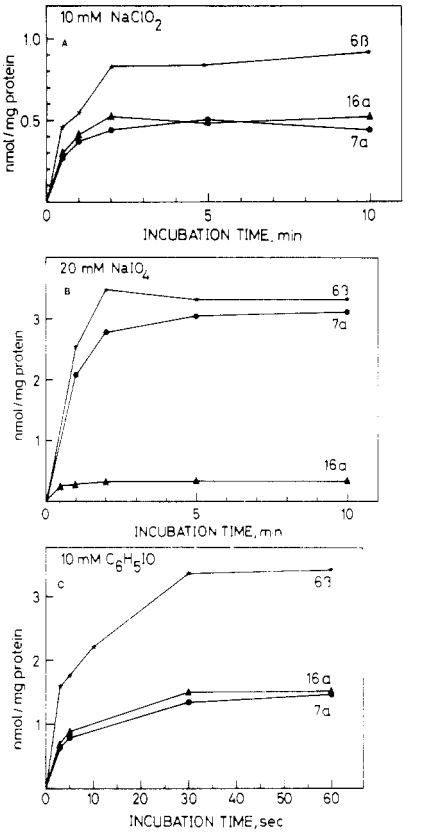


FIGURE 2: $NaClO_2$ (A), $NaIO_4$ (B), and iodosylbenzene (C) supported hydroxylations of androstenedione as a function of incubation time. Reaction rate is expressed as nmol of 6β-, 7α-, or 16α-hydroxyandrostenedione formed per mg of protein per unit of incubation time. For incubation conditions, see Materials and Methods.

Table IV: Catalysis of Androstenedione Hydroxylation in Rat Liver Microsomes by Iodosophenyl Derivatives Other Than Diacetoxyiodo Compounds and Iodosylbenzenes

	hydroxylations (nmol (mg of protein)$^{-1}$ min^{-1})		
compound	7α	6β	16α
iodylbenzene	1.2	1.8	0
2-nitroiodylbenzene	0.8	12.6	4.8
(dibenzoyloxyiodo)-2-nitrobenzene	4.2	13.2	6.2
(dinitratoiodo)-2-nitrobenzene	12.8	45.7	26.6
p-bis(diacetoxyiodo)benzene	2.4	3.0	3.2
1-hydroxy-4,5-benziodoxolin-3-one	0	0	0
1-acetoxy-4,5-benziodoxolin-3-one	0	0	0
1-methoxy-4,5-benziodoxolin-3-one	0	0	0
1-ethoxy-4,5-benziodoxolin-3-one	0	0	0
(diacetoxyiodosyl)benzene	3.4	3.6	0

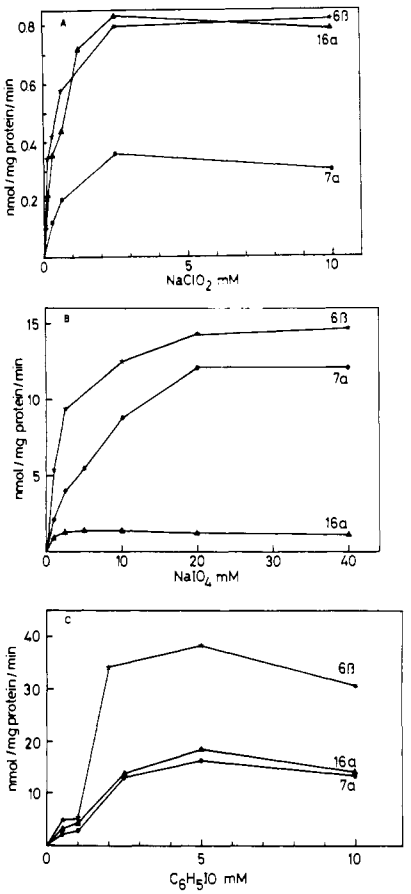
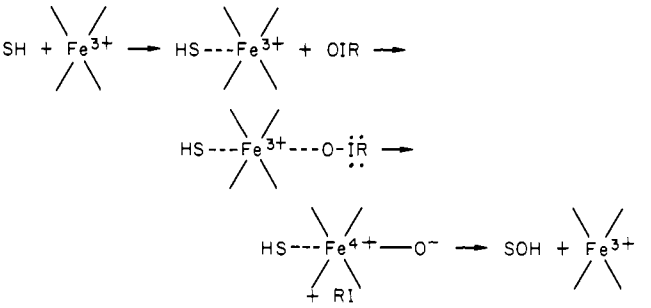


FIGURE 3: 6β, 7α, or 16α hydroxylation of androstenedione as a function of concentration of $NaClO_2$ (A), $NaIO_4$ (B), and iodosylbenzene (C). Reaction rate is expressed as nmol of 6β-, 7α-, or 16α-hydroxyandrostenedione formed per mg of protein per min of incubation time. For incubation conditions, see Materials and Methods.

microsomal incubations with androstenedione fortified with various derivatives of iodosylbenzene. The most efficient fortifier of androstenedione hydroxylation was (diacetoxyiodo)-2-nitrobenzene that was about three times as active as unsubstituted (diacetoxyiodo)benzene and several hundred times as active as NADPH (Table III). (Diacetoxyiodo)-2-chlorobenzene was about twice as active as unsubstituted (diacetoxyiodo)benzene, whereas (diacetoxyiodo)-2-trifluoromethyl- and (diacetoxyiodo)-2-methoxybenzene were about as active as the unsubstituted agent. When the substituent was transferred from the ortho position of the benzene ring to the para and the meta positions, as in 3-nitro-, 3-chloro-, 4-chloro-, 3-trifluoromethyl-, 4-trifluoromethyl-, and 3-methoxy(diacetoxyiodo)benzene compounds, the catalytic activity of the oxidizing agent decreased. Insertion of a methyl or a cyano group in position 2 or a bromine atom or a phenyl group in position 4 of (diacetoxyiodo)benzene also decreased its catalytic activity. Other substitutions that resulted in a less efficient catalytic agent were 2,6 insertion of methoxy groups (diacetoxyiodo-2,6-dimethoxybenzene) or condensation with another benzene ring (1-(diacetoxyiodo)naphthalene) (Table III).

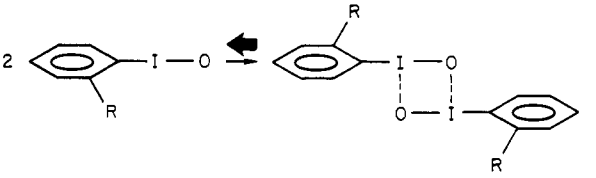
In general, iodosylbenzenes were less efficient catalysts of liver microsomal androstenedione hydroxylation than the equivalent (diacetoxyiodo)benzene (Table III). Also in the iodosylbenzene series ortho-substituted derivatives were more efficient catalysts than meta- or para-substituted derivatives (2-nitro > 3-nitro, 2-chloro > 3-chloro, 2-chloro > 4-chloro, 2-methoxy > 2-methoxy).

Scheme I



(SH = substrate; RIO = oxidation agent; Fe = heme iron in cytochrome P-450)

Scheme II



As can be seen from Table IV, (dinitratoiodo)-2-nitrobenzene was a potent fortifier of liver microsomal androstenedione hydroxylation. Other examples of good catalysts were 2-nitroiodylbenzene and (dibenzoyloxyiodo)-2-nitrobenzene. Interestingly, ortho substitution of iodylbenzene with a nitro group increased the catalytic activity of the oxidizing agent quite significantly.

Irrespective of catalytic agent, the ratio between 7α-, 6β-, and 16α-hydroxyandrostenedione formed during liver microsomal hydroxylation of androstenedione was generally in the range of 1:2:1 when the reactions were fortified with iodosylbenzene derivatives. The stable ratio indicates that these catalytic agents act through a common mechanism.

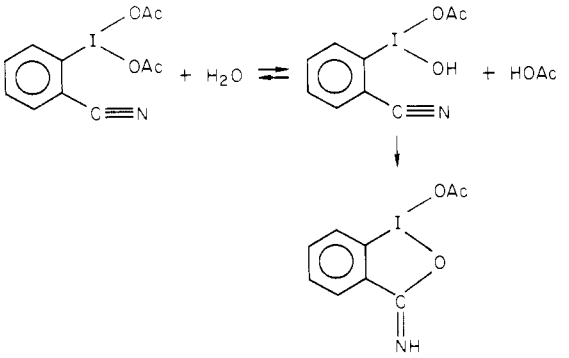
Discussion

The results obtained in this investigation have shown that, in addition to NADPH, several derivatives of iodosylbenzene and (diacetoxyiodo)benzene can fortify steroid hydroxylation in rat liver microsomes. These hydroxylations appear to be cytochrome P-450 dependent since various modifiers and substrates of the enzyme were efficient inhibitors. The similar ratio between the hydroxylated products in the reaction mixture obtained with all oxygen donors indicates that these interact with the hemoprotein in a similar fashion. Furthermore, the sites of hydroxylation of the employed substrate, androstenedione, were the same as in NADPH-supported reactions (6β, 7α, and 16α) suggesting a similar nature of the "activated oxygen" species generated in oxidation agent- and molecular oxygen-fortified hydroxylations. The present results point toward a transfer step involving a monooxygen species rather than a dioxygen species. A possible mechanism may be outlined as shown in Scheme I.

The insensitivity of the oxidation agent catalyzed hydroxylations to carbon monoxide is natural in view of the proposed mechanism, since only the ferrous form of cytochrome P-450 binds carbon monoxide.

The second step in Scheme I, involving the coordination of RIO to Fe^{3+}, should be hampered (or even inhibited) by association of RIO molecules to oligomers as well as by introduction of electron-withdrawing substituents in 2 and/or 4 position. On the other hand, for steric reasons, the formation of associates (e.g., dimers) should be less pronounced with substituents in the 2 position or in the 2,6 positions; i.e., the anticipated reactive oxene transfer species, the monomer,

Scheme III

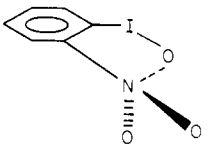


should exist in a higher concentration (Scheme II).

Introduction of very bulky substituents, e.g., 2,6-dimethoxy, should, however, disfavor the coordination with Fe^{3+} due to steric interaction with the enzyme, even if the tendency to oligomerization should be expected to be very low in this case.

The efficiency of 2-nitroiodosylbenzene as an oxygen donor in cytochrome P-450 catalyzed steroid hydroxylation might thus be explained in the following way. The relatively bulky nitro group prevents oligomerization, perhaps by inducing the iodosyl group into the following conformation with the nitro group blocked from coplanarity, preventing any electron withdrawal from the iodosyl group by resonance. The only operating electron withdrawal will therefore be through inductive deactivation (Chart I).

Chart I



In 2-cyanoiodosylbenzene, which does not act as an oxygen donor in cytochrome P-450 catalyzed hydroxylation, the cyano group gives strong resonance and inductive deactivation, apparently rendering the iodosyl group too poor for coordination properties. Another possibility would be that 2-cyanoiodosylbenzene exists as some kind of a cyclic structure. However, infrared spectroscopy data indicated that neither 2-cyanoiodosylbenzene nor its diacetoxy compound was cyclized (data not shown). On the other hand, e.g., the diacetoxy compound might be converted to a cyclized (and therefore inactive) form via the reactions shown in Scheme III.

Iodosylbenzoic acid derivatives exist in cyclized form (cf. Figure 1) and are consequently inactive as oxygen donors in cytochrome P-450 catalyzed hydroxylation.

The (diacetoxyiodo)benzene derivatives probably operate in the same manner as the iodosylbenzenes. In this connection it should be noted that the acetoxy groups are readily displaced by water or methanol (Seveno et al., 1977). (Dinitratoiodo)benzenes undergo rapid hydrolysis in water and (dinitratoiodo)-2-nitrobenzene may therefore be assumed to coordinate as 2-nitroiodosylbenzene.

In conclusion, the results of the present investigation indicate that the active oxygenated intermediate of cytochrome P-450 catalyzing steroid hydroxylation only contains one oxygen atom. The bypass of the reduction steps involved in NADPH-supported hydroxylation reactions provided by the iodosylbenzene derivatives resulted in a dramatic increase in the velocity of the hydroxylation reactions: the rates of 7α, 6β, and 16α hydroxylation of androstenedione catalyzed by (diacetoxyiodo)-2-nitrobenzene were 108-, 130-, and 167-fold higher, respectively, than the rates of the NADPH-sustained reactions. These data strongly suggest that the rate-limiting step in NADPH-supported cytochrome P-450 catalyzed reactions is the rate of reduction of cytochrome P-450.

Acknowledgments

The skillful technical assistance of Mrs. Gunilla Elfström is gratefully acknowledged.

References

Baker, G. P., Mann, G. F., Sheppard, N., & Tetlow, A. J. (1965) *J. Chem. Soc.*, 3721–3728.

Berg, A., Carlström, K., Gustafsson, J.-Å., & Ingelman-Sundberg, M. (1975) *Biochem. Biophys. Res. Commun. 66*, 1414–1423.

Bergström, S., & Gloor, U. (1955) *Acta Chem. Scand. 9*, 34–38.

Dasent, W. E., & Waddington, T. C. (1960) *J. Chem. Soc.*, 3350–3356.

Einarsson, K., & Gustafsson, J.-Å. (1973) *Eur. J. Biochem. 32*, 197–206.

Einarsson, K., Gustafsson, J.-Å., & Goldman, A. S. (1972) *Eur. J. Biochem. 31*, 345–353.

Einarsson, K., Gustafsson, J.-Å., & Stenberg, Å. (1973) *J. Biol. Chem. 248*, 4987–4997.

Formo, M. W., & Johnson, J. R. (1955) *Organic Syntheses Collect., Vol. III*, 486.

Fox, A. R., & Pausacker, K. H. (1957) *J. Chem. Soc.*, 295–301.

Gustafsson, J.-Å., & Bergman, J. (1976) *FEBS Lett. 70*, 276–280.

Gustafsson, J.-Å., Hrycay, E. G., & Ernster, L. (1976) *Arch. Biochem. Biophys. 174*, 440–453.

Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M., & Ernster, L. (1975a) *FEBS Lett. 56*, 161–165.

Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M., & Ernster, L. (1975b) *Biochem. Biophys. Res. Commun. 66*, 209–216.

Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M., & Ernster, L. (1976) *Eur. J. Biochem. 61*, 43–52.

Karek, B., Neilands, O. (1970) *Latv. PSR Zinat. Akad., Vestis, Khim. Sec.*, 587–590.

Leffler, J. E., & Story, L. J. (1967) *J. Am. Chem. Soc. 89*, 2333–2338.

Lichtenberger, F., Nastainczyk, W., & Ullrich, V. (1976) *Biochem. Biophys. Res. Commun. 70*, 939–946.

Nordblom, G. D., White, R. E., & Coon, M. J. (1976) *Arch. Biochem. Biophys. 175*, 524–533.

Ortoleva, G. (1900) *Gazz. Chim. Ital. 30*, 1.

Pausacker, K. H. (1953) *J. Chem. Soc.*, 107–109.

Seveno, A., Morel, G., Foucaud, A., & Marchand, E. (1977) *Tetrahedron Lett.*, 3349–3352.

Willgerodt, C. (1893a) *Ber. 26*, 357–362.

Willgerodt, C. (1893b) *Ber. 26*, 1802–1810.

Willgerodt, C. (1893c) *Ber. 26*, 1532–1534.

Willgerodt, C. (1893d) *Ber. 26*, 1947–1950.

Willgerodt, C. (1899) *J. Protein Chem. 59*, 198.

Yagupolskii, L. M., Maletina, I. I., Kondratenko, N. V., & Orda, V. V. (1977) *Synthesis*, 574–575.

Yamada, Y., Kashima, K., & Okawara, M. (1976) *J. Polym. Sci., Polym. Lett. Ed. 14*, 65–71.