Biodehalogenation and Metabolism of [^{125}I]-4-Iodobiphenyl

J. E. SINSHEIMER [x] **and Y. Y. SHUM** [*]

Received January 31, 1980, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.* Accepted for publication October 14, 1980. *Present address: Warner-Lambert/Parke-Davis Pharmaceutical Resarch Division, Ann Arbor, MI 48106.

Abstract □ [^{125}I]-4-Iodobiphenyl has been used as a model compound to investigate the *in vivo* metabolism of iodinated aromatic compounds in the rat. Material balance studies showed that 57.6% of the injected dose was excreted *via* feces and 41.2% *via* urine. Distribution studies indicated uptake of inorganic iodide in the thyroid. In the feces, 2.2% of the dose was unmetabolized iodobiphenyl, 2.6% was 2-hydroxy-4′-iodobiphenyl, 21.9% was 4-hydroxy-4′-iodobiphenyl, and 20.9% was a polar fraction of which only 10.3% could be silylated. In urine, 1.6% of the dose was 2-hydroxy-4′-iodobiphenyl, 4.9% was 4-hydroxy-4′-iodobiphenyl, 1.9% was 2-hydroxy-4′-iodobiphenyl, 17.7% was 4-hydroxy-4′-iodobiphenyl glucuronide and sulfate conjugates, and 8.2% was inorganic iodide. About 3.0% of the dose in the urine polar fraction could be methylated.

Keyphrases □ 4-Iodobiphenyl—metabolism in rats, distribution studies □ Metabolism—4-iodobiphenyl and metabolites, distribution studies in rats □ Distribution—4-iodobiphenyl and metabolites, metabolism and distribution studies in rats

The increasing medicinal use of iodinated organic compounds as positive X-ray contrast media (1) as well as diagnostic, scanning, and radiopharmaceutical agents (2) prompted this study of the biodehalogenation and metabolism of iodocompounds. *In vitro* mechanisms for the biodehalogenation of some model compounds were reported previously (3). This paper describes the *in vivo* biodehalogenation and metabolism of 4-iodobiphenyl (I) as a model compound used in rats.

EXPERIMENTAL[1]

Chromatographic Solvent Systems—Benzene–methanol–formic acid (90:9:1) (Solvent System 1) and benzene–methanol (85:15) (Solvent System 2) were used for TLC[2], *n*-butyl alcohol–0.5 *M* NH_4OH–ethanol–water (20:20:2:1) (Solvent System 3) was used for paper chromatography, and *n*-butyl alcohol–acetone–concentrated ammonium hydroxide–water (65:20:10:5) (Solvent System 4) was used for resin paper chromatography.

[^{125}I]-4-Iodobiphenyl—This compound (specific activity, 9.0 μCi/mg; radiochemical purity by reverse isotopic dilution, 99.7%) was synthesized by an exchange reaction described previously (3).

4-Hydroxy-4′-iodobiphenyl (II)—Compound II was prepared from 4-hydroxybiphenyl and benzoyl chloride according to a literature method (4). The product was recrystallized in chloroform, mp 202° [lit. (4) mp 199°]; IR (KBr): 3300 (OH), 1580, 1520 (aromatic H), 1470–1420 (CH), 1380, 1250 (aromatic OH), 1120, 1000, 810, and 720 (aromatic H) cm^{-1}; NMR (acetone-d_6): δ 8.57 (broad s, 1H, aromatic OH), 7.92 (d of d, 2H, J_{ab} = 9.0 Hz, J_{ac} = 2.6 Hz, aromatic H), and 7.07 (d of d, 2H, J_{ab} = 9.0 Hz, J_{ac} = 2.0 Hz, aromatic H); mass spectrum (75 ev): *m/z* (relative intensity) 298 (1.0%) $[M + 2]^+$, 297 (14.1) $[M + 1]^+$, 296 (100) $[M]^+$, 267 (1.3), 169 (7.5) $[M - I]^+$, 168 (10.6) $[M - HI]^+$, 152 (3.8) $[M - HOI]^+$, 141 (19.7), 139 (8.0), 118 (metastable), 115 (14.3), and 96.5 (metastable).

2-Hydroxy-4′-iodobiphenyl (III)—Compound III was synthesized from 2-hydroxybiphenyl and benzoyl chloride using the same procedure as used for the preparation of II. The product was recrystallized in pentane, mp 75° [lit. (5) mp 74°]; IR (KBr): 3470–3020 (aromatic OH), 1470, 1390 (aromatic H), 1268 (aromatic OH), 1188, 1130, 1120, 1042, 1010, 834, 814, 756, 700, 612, and 540 (aromatic H) cm^{-1}; NMR ($CDCl_3$): δ 5.40 (s, 1H, aromatic OH), 6.90 (d of t, 1H, J_{ab} = 9.0 Hz, J_{ac} = 2.0 Hz, aromatic H), 7.20 (d of d, 2H, J_{ab} = 9.0 Hz, J_{ac} = 2.0 Hz, aromatic H), 7.40 (t, 1H, J_{ab} = 2.0 Hz, aromatic H), 7.68 (d of d, 2H, J_{ab} = 9.0 Hz, J_{ac} = 2.0 Hz, aromatic H), and 7.94 (d, 2H, J_{ab} = 9.0 Hz, aromatic H); mass spectrum (75 ev): *m/z* (relative intensity) 297 (14%) $[M + 1]^+$, 296 (100) $[M]^+$, 170 (20) $[M - I]^+$, 169 (10), 168 (2.0), 141 (9.0), and 115 (4.0).

Reverse Isotopic Dilution—In a manner analogous to previously reported analyses (6), ~1 g of II or III, accurately weighed, was transferred to a 300-ml round-bottom flask and dissolved completely in a small volume of acetone. Fecal extracts or urine samples were pipetted (1.0 ml) into the flask, and sufficient methanol was added to give a homogeneous solution. Solvents were removed *in vacuo*, and the residue was recrystallized from chloroform. Three samples of ~10 mg from each recrystallization were weighed accurately and counted for radioactivity in a γ-counting system. Recrystallization was repeated until constant specific activity was obtained.

***In Vivo* Studies**—Sprague–Dawley rats, 200–250 g, were housed in separate stainless steel metabolism cages and quarantined for at least 1 day prior to use. Rat food[3] and water were supplied *ad libitum*.

[^{125}I]-4-Iodobiphenyl was diluted with carrier 4-iodobiphenyl (I) and dissolved in sterilized peanut oil to give a final concentration of 110 μCi/100 mg/ml for intraperitoneal injection.

Tissue distribution studies were conducted after a single intraperitoneal injection of 0.5 ml of the peanut oil solution was given to 18 female rats. Sets of three rats were killed in an ether chamber at 3, 6, 24, 48, 72, and 96 hr after injection, and major tissues were excised and weighed. Representative samples of these tissues were weighed, and their radioactivities were determined (Table I).

Pretreatment with Lugol's solution was done with two rats, which were administered orally 0.3 ml of Lugol's solution (50 mg of elemental iodine and 100 mg of potassium iodide in 100 ml of water) once per day for 2 days. Control rats were dosed with distilled water under the same schedule. On the 3rd day, 3.9 μCi of [^{125}I]-4-iodobiphenyl in 0.5 ml of ethanol was administered to each rat by intraperitoneal injection. Rats were kept in separate metabolic cages for 24 hr before they were killed. Thyroid glands from each rat were excised, trimmed, weighed, and counted for activity in a γ-counting system.

Balance studies were conducted on three male rats, each receiving a single intraperitoneal injection of 1.0 ml of the dosage solution. Urine and fecal samples were collected for 18 days to observe excretion patterns of the metabolites.

Three rats received a single intraperitoneal injection of 1.0 ml of the dosage solution every 4 days for a total of 3.0 ml of solution to accumulate higher quantities of metabolites. Control rats were treated in a manner corresponding to each group but were injected only with sterilized peanut oil.

Rat urine and feces were collected separately after [^{125}I]-4-iodobiphenyl injections. Urine samples were quantitated for radioactivity, quick frozen in an acetone–dry ice bath to prevent mold growth, and stored at −25° until analyzed for metabolites. Feces were air dried and powdered, and samples (5–10 mg) were measured daily for radioactivity by γ-counting. They also were stored at −25° until investigated further.

For identification of fecal metabolites, the total combined daily powdered feces were extracted with methanol for 48 hr in a soxhlet apparatus. The methanol extract was reduced to 50.0 ml *in vacuo*. Aliquots (100 μl) of this solution were assayed by γ-counting to estimate the percentage of extractable activity.

Volumes (0.5 ml) of the concentrated fecal methanol extract were subjected to TLC analysis in Solvent Systems 1 and 2. Unmetabolized [^{125}I]-4-iodobiphenyl and its hydroxylated metabolites were identified by cochromatography with nonradioactive reference compounds in these solvent systems. The main body of the fecal methanol extract was applied to preparative chromatographic plates and developed in System 1. Bands of [^{125}I]-4-iodobiphenyl and its hydroxylated metabolites were extracted from the plates with appropriate solvents, concentrated, and subjected

[1] Spectra were obtained on Perkin-Elmer 337 IR, Varian A-60-A NMR, and DuPont 21-490 mass spectrometers. γ-Counting was either by liquid scintillation counting on Beckman LS-200 and 150 instruments with a phosphorus 32 window at 5% accuracy or 5-min intervals or with a Searle 1185 γ-system.

[2] Brinkmann 5 × 20 × 0.025-cm silica gel 60 F-254 on glass or Eastman silica chromatogram sheet 6060 for analytical measurements and Brinkmann 20 × 20 × 0.2-cm silica gel 60 F-254 on glass for preparative separations.

[3] Ralston-Purina Co.

0022-3549/81/0500-0546$01.00/0

to GLC–mass spectrometric analysis. Part of the GLC output was collected *via* a three-way collector into scintillation vials and quantitated by liquid scintillation counting. Only the radioactive GLC peaks were analyzed by mass spectrometry.

Assay of [^{125}I]-4-iodobiphenyl and its hydroxylated metabolites was accomplished by estimation of their percentage contributions in TLC patterns developed in Solvent System 1. The results were subsequently confirmed by reverse isotopic dilution in the fecal methanol extracts with corresponding reference compounds.

Acid or base hydrolysis of the fecal polar fraction was done by extracting origin band materials with methanol and reducing the extract to 1.0 ml with nitrogen. An equal volume of 8 *N* HCl or 5% NaOH was added. The mixture was refluxed for 5 hr, and the solution was extracted with ether. Both aqueous and ether solutions were examined with TLC in Solvent System 1 for products.

Enzyme hydrolysis of the fecal polar fraction was performed by taking the methanol extract of the origin band to dryness under a nitrogen stream. The residue was dissolved in 2.5 ml of pH 7.4 phosphate buffer to which 0.1 ml (10,000 Fishman units) of β-glucuronidase–arylsulfatase of snail origin[4] (*Helix pomatia*) was added before it was incubated at 37° for 72 hr. After incubation, the content was centrifuged at 1720×*g* for 30 min, and the supernate was chromatographed in Solvent System 1 for detection of hydrolyzed products.

Silylation of the fecal polar fraction was carried out by reducing the methanol extract of the origin band to complete dryness. The dried residue was reacted with 1.0 ml of *N,O*-bis(trimethylsilyl)acetamide, and the mixture was refluxed in a tightly capped vial for 30 min in a sand bath until a clear solution was observed. This solution was cooled and spotted on TLC plates to be developed in Solvent System 2. The distribution of activity on the chromatogram was reconstructed by liquid scintillation counting of silica gel fractions scraped from consecutive 0.5-cm strips. Radioactivity was extracted from the detected bands, purified, and subjected to GLC–mass spectrometric analysis.

Urine samples (50–100 μl) were assayed daily for radioactivity by γ-counting. Radioactivity distributions of unmetabolized [^{125}I]-4-iodobiphenyl, their hydroxylated metabolites, and the conjugates were estimated after TLC in Solvent System 1. Values for II and III and their glucuronide and sulfate conjugates were confirmed after hydrolysis by reverse isotopic dilution.

Enzymatic hydrolysis was done on 2.0-ml samples of urine, which had been dried under a gentle nitrogen stream. To the residue were added 1.0 ml of pH 7.4 phosphate buffer and 0.1 ml of β-glucuronidase–arylsulfatase[4] (*Helix pomatia*) or β-glucuronidase[4] (bovine liver). The mixture was incubated in a constant-temperature water bath at 37° for 48–72 hr with constant shaking. The hydrolysis was stopped by heating in a steam bath for 5 min, and the protein was removed by centrifugation. The supernate was spotted on TLC plates and developed in Solvent System 1.

The urinary polar fractions, which failed to be hydrolyzed by enzymes, were methylated by dimethylformamide dimethylacetal (7) and quantitated by TLC in Solvent System 2 after derivatization.

The radioactivity of inorganic iodide in the urine was estimated by paper (8) and resin paper (9) chromatography in Solvent Systems 3 and 4, respectively. These values were confirmed by lead iodide reverse isotopic dilution and methyl iodide exchange experiments (9).

RESULTS

Tissue Distribution—Typical tissue radioactivities of iodine 125 for the organ examined are listed in Table I. At all times, the thyroid gland had the highest activity. After 24 hr, there was minor but consistent uptake of radioactivity by fatty tissue as compared to blood levels. Pretreatment of rats with Lugol's iodide solution daily for 2 days prior to administration of [^{125}I]-4-iodobiphenyl suppressed thyroid uptake by 90%. This competitive thyroid uptake experiment indicated that the thyroid activity was due to metabolically liberated inorganic iodide.

Balance Studies—The excretion of radioactivity in feces and urine for three rats was followed for 18 days, with an average total fecal excretion of 57.6% of the administered dose and an average total urinary excretion of 41.2%. In both cases, most radioactivity was eliminated in the first 6 days.

Fecal Metabolites—Methanol extraction of powdered dry feces indicated that 96.7% of feces activity could be extracted by this method. Chromatographic analysis of the concentrated feces methanol extract on TLC plates in Solvent System 1 gave rise to four radioactive bands with R_f values at 0.00, 0.21, 0.38 and 0.64. They were labeled F_A, F_B, F_C, and F_D and found to possess 20.9, 26.4, 8.4, and 2.4% of the radioactivity of the original dose, respectively. Each radioactive band was scraped off the plate, extracted, and subjected to further analysis.

A radioactive band from the condensed hexane extract of F_D cochromatographed with 4-iodobiphenyl (I) at R_f 0.64 (System 1) and 0.62 (System 2) and corresponded to I by GLC–mass spectrometry after separation on a 5% OV-1 column. Reverse isotopic dilution with I indicated 3.8% of the fecal methanol extract (2.2% of the injected dose) was due to unmetabolized [^{125}I]-4-iodobiphenyl, a value in agreement with the 2.4% found for band F_D.

Radioactive band F_C was extracted with chloroform. When this extract was cochromatographed with 2-hydroxy-4′-iodobiphenyl (III), a radioactive spot corresponding to a UV-quenching spot was obtained at R_f 0.38 in System 1 and at R_f 0.50 in System 2. GLC–mass spectrometric analysis of the F_C chloroform extract, similar to that described for F_D, resulted in a mass spectral fragmentation pattern consistent with III: *m/z* (relative intensity) 297 (16%), 296 (100), 170 (6.0), 169 (13), 168 (13), 141 (52), and 115 (42).

When reverse isotopic dilution experiments of the fecal methanol extract were done with III, only 2.6% of the injected dose activity could be identified as III. Based on the similarity of the mass spectrum of band F_C to the spectra of both references II and III, band F_C probably contained other hydroxylated metabolites in addition to III. The reverse isotopic dilution results compared to the TLC results indicate that the 2-hydroxy isomer (III) is about a third of band F_C (Table II).

Cochromatography of an extract of radioactive band F_B with 4-hydroxy-4′-iodobiphenyl (II) gave radioactive spots with R_f values of 0.21 in System 1 and 0.28 in System 2, which corresponded to the reference material. GLC–mass spectrometric analysis of band F_B was also consistent with that of II: *m/z* (relative intensity) 297 (15.4), 296 (100), 267 (2.3), 169 (10.8), 168 (10), 152 (3.8), 151 (3.8), 148 (5.4), 141 (20.8), 139 (18.5), and 115 (18.5). Reverse isotopic dilution experiments with II on the fecal methanol extract indicated that 21.9% of the dose activity was converted to II in the feces.

On the basis of TLC Systems 1 and 2, band F_A, which consistently remained at the origin, contains highly polar metabolites such as sulfates, glucuronides, and mercapturic acid derivatives.

Repeated attempts to hydrolyze band F_A materials with acid, base, and β-glucuronidase–arylsulfatase enzymes failed to create new radioactive TLC bands of higher R_f values in either System 1 or 2. Derivatization of F_A materials finally led to the formation of a product with *N,O*-bis(trimethylsilyl)acetamide. Upon chromatography of this product, a radioactive band at R_f 0.81 in System 2 was obtained, and it was estimated that about half of the original F_A materials had reacted (9.8% of the injected dose). Efforts to elucidate the structure of this F_A derivative by GLC–mass spectrometry were not successful.

Urinary Metabolites—Analysis of the urinary metabolites started with cochromatography of the urine with fecal methanol extracts in Solvent System 1. This chromatogram indicated that most of the urinary metabolite activity (~32.0% of the injected dose) lay under the origin

Table I—Tissue Distribution of Radioactivity after Intraperitoneal Administration of [^{125}I]-4-Iodobiphenyl (55 μCi/0.5 ml of Injection) to Rats

Tissue	Radioactivity, cpm/mg					
	3 hr	6 hr	24 hr	48 hr	72 hr	96 hr
Thyroid	79	63	2426[a]	5377	7255	3025
Fat	4	17	96	78	59	53
Pituitary	10	17	53	25	47	17
Adrenals	8	13	30	19	22	13
Kidneys	22	11	11	15	21	14
Ovaries	6	8	22	13	23	9
Intestine	3	17	42	10	23	14
Liver	46	9	14	10	20	12
Vagina	2	5	23	7	15	6
Uterus	2	6	48	11	12	7
Blood	39	10	23	9	19	5
Lungs	9	5	10	6	10	6
Brain	3	4	4	4	3	3
Muscle	1	2	15	3	6	3
Spleen	39	3	6	3	5	3

[a] In separate experiments, in which rats were pretreated with Lugol's solution prior to administration of 3.9 μCi of [^{125}I]-4-iodobiphenyl, 24-hr thyroid activities were 9.7 and 21.6 cpm/mg. These values were compared to 137.4, 199.2, and 258.9 cpm/mg for nonpretreated rats at the same dose of I.

[4] Sigma Chemical Co.

Table II—4-Iodobiphenyl and Metabolites Expressed as Percent of Dose

Compound	Feces		Urine	
	TLC[a]	RID[b]	TLC	RID
4-Iodobiphenyl (I)	2.4[c] (1.8–2.8)[e]	2.2 (2.11–2.26)	None detected	—[d]
4-Hydroxy-4′-iodobiphenyl (II)	26.4 (24.0–27.2)	21.9 (21.3–22.5)	4.9 (4.0–5.4)	—
Total other monohydroxy iodobiphenyls	8.4 (7.0–9.2)	—	1.6 (1.2–1.8)	—
2-Hydroxy-4′-iodobiphenyl (III)	—	2.6 (2.53–2.63)	—	—
II β-glucuronide or sulfate	None detected	—	18.2 (16.1–19.8)	17.7 (17.2–18.3)
Total other monohydroxy iodobiphenyl β-glucuronide or sulfate	None detected	—	6.4 (5.9–7.5)	—
III β-glucuronide or sulfate	None detected	—	—	1.9 (1.82–1.93)
Methylated urinary polar metabolites	—	—	3.0 (1.6–4.0)	—
Silylated fecal polar metabolites	10.3 (8.0–13.7)	—	—	—
Inorganic iodide	—	—	7.3[f] (6.91–7.87)	8.2[g] (8.10–8.26)

[a] TLC values are averages of data from three rats. [b] Reverse isotopic dilution values. [c] Numerical values are expressed as percentage of administered dose. [d] Not determined. [e] Numbers in parentheses express the range of values. [f] Determined by resin paper chromatography (Ref. 9). [g] Determined by lead iodide reverse isotopic dilution method (Ref. 9).

band, representing highly polar materials. Only 4.9% of the dose activity could be accounted for in the urine as free phenolic II, and an additional 1.6% was III. No unmetabolized I could be detected in urine.

To determine if any glucuronide or sulfate conjugates were present in the urinary polar fraction, the polar fraction at the origin was extracted with methanol, dried, and hydrolyzed in buffer solution with β-glucuronidase–arylsulfatase enzyme (*Helix pomatia*). TLC of the hydrolyzed products in System 1 showed that 18.2 and 6.4% of the injected dose in the urinary polar fraction corresponded in R_f to II and III, respectively. Thus, in the urine, a total of 23.1% of the dose activity could be accounted for as II and its glucuronide or sulfate conjugates. Similarly, 8.0% of the dose activity was transformed into metabolites corresponding in R_f to III and its conjugates.

Confirmation of the TLC-derived quantitation of the two free phenolic metabolites was sought by two separate reverse isotopic dilution experiments using the urinary polar radioactive fraction that had been hydrolyzed enzymatically. Compounds II and III were used as the reference compounds. Based on these results, it was calculated that II accounted for 17.7% (18.2% by TLC) and III accounted for 1.9% (TLC indicated 6.4%) of the original dose. The remaining 4.5% was speculated to be due to other monohydroxy iodobiphenyls in a manner similar to the corresponding fecal metabolite band.

Differentiation between glucuronide and sulfate conjugates of the phenolic metabolites was made by independent enzyme hydrolysis of the urinary polar fraction methanolic extracts by β-glucuronidase–arylsulfatase from *Helix pomatia versus* bovine β-glucuronidase. Comparisons by TLC indicated that of the 18.2% of II released by enzyme hydrolysis, 16.1% was from sulfate conjugates while only 2.2% was due to glucuronide conjugates. Similarly, of the 6.4% released as material cochromatographing with III, 5.1% was from sulfate and only 1.3% was from glucuronide conjugates.

Treatment of the polar band left at the origin after enzyme hydrolysis with dimethylformamide dimethylacetal revealed that about half (3.1% of dose) of the activity could react. The product, when chromatographed in System 1, showed a radioactive band at R_f 0.70. When chromatographed in System 2, this band appeared at R_f 0.60. Due to the minute quantity that could be isolated from this radioactive band, attempted GLC–mass spectrometric analyses to characterize its structure were not successful.

The various metabolites of I found in the rat feces and urine are summarized in Table II, together with their relative percentages of the administered dose.

DISCUSSION

While biotransformations of 4-iodobiphenyl (I) have not been reported previously, those of 4-chlorobiphenyls have been studied extensively (10–14). Results of the present study agree with the literature in that hydroxylation at the *para*-position and subsequent partial conjugation are the major metabolic pathways.

No hydroxylation at position 2′ was found for a number of animal species administered 4-chlorobiphenyl (10–14). However, the present finding of hydroxylation at this position for iodobiphenyl agrees with such transformation of biphenyl *per se* (15–18).

The presence of metabolites hydroxylated at positions 2′ and 4′ of I is consistent with the formation of 2′,3′- and of 3′,4′-arene oxide intermediates of I in the initial biotransformation of this compound. The reaction product of the urinary polar fraction after enzyme hydrolysis and subsequent reaction with dimethylformamide dimethylacetal was speculated to be a methyl derivative of a mercapturic acid metabolite of I. A mercapturic acid metabolite would be a logical arene oxide derivative, and the electron-withdrawing effect of the attached iodophenyl moiety would stabilize the arene oxide which, in turn, would allow more time for glutathione conjugation (19). The findings of West *et al.* (20), who reported isolation of *N*-acetyl-*S*-biphenyl-L-cysteine from rat urine after dosing with biphenyl, support such conjugation. Also supporting this conjugation is the work of Wood and his associates (21, 22) who were able to isolate [^{131}I]-4-iodophenylmercapturic acid after oral administration of [^{131}I]iodobenzene to rats.

The fecal polar fraction resisted hydrolysis, indicating the absence of glucuronide or sulfate metabolites, but it reacted with *N,O*-bis(trimethylsilyl)acetamide. This fraction also could contain mercapturic acid conjugates or their precursors.

Even though no deiodination was detected in metabolic studies of 4,4′-diiodobiphenyl in rabbits (23), results from the present studies indicated that a substantial amount of inorganic iodide could be found from the metabolism of I in rats. The absence of dehalogenation by rabbits also was pointed out by Block and Cornish (10) in their studies of 4-chlorobiphenyl. Thus, a species difference in dehalogenation of biphenyl compounds may be indicated.

REFERENCES

(1) H.-J. Herms and V. Taenzer, in "Drug Design, Vol. VI," E. J. Ariens, Ed., Academic, New York, N.Y., 1975, p. 262.

(2) R. E. Counsell and R. Ice, in *ibid.*, p. 171.

(3) J. E. Sinsheimer, T. Wang, S. Röder, and Y. Y. Shum, *Biochem. Biophys. Res. Commun.*, **83**, 281 (1978).

(4) H. R. Schmidt, C. M. Savoy, and J. L. Abernethy, *J. Am. Chem. Soc.*, **66**, 491 (1944).

(5) M. Oki and H. Iwamura, *Bull. Chem. Soc. Jpn.*, **34**, 1395 (1961).

(6) L. G. Dring, R. L. Smith, and R. T. Williams, *Biochem. J.*, **116**, 425 (1970).

(7) J. P. Thenot and E. C. Horning, *Anal. Lett.*, **5**, 519 (1972).

(8) R. Grasbeck, B. A. Lamberg, and F. Bjorksten, *Acta Endocrinol.*, **34**, 113 (1960).

(9) Y. Y. Shum and J. E. Sinsheimer, *J. Pharm. Sci.*, **68**, 258 (1979).

(10) W. D. Block and H. H. Cornish, *J. Biol. Chem.*, **234**, 3301

(1959).
(11) S. Safe, O. Hutzinger, and D. Ecobichon, *Experientia*, **30**, 720 (1974).
(12) S. Safe, O. Hutzinger, and D. Jones, *J. Agric. Food Chem.*, **23**, 851 (1975).
(13) O. Hutzinger, D. M. Nash, S. Safe, A. S. W. DeFreitas, R. J. Norstrom, D. J. Wildish, and V. Zitko, *Science*, **178**, 312 (1972).
(14) S. Safe, N. Platonow, and O. Hutzinger, *J. Agric. Food Chem.*, **23**, 259 (1975).
(15) P. J. Creaven, D. V. Parke, and R. T. Williams, *Biochem. J.*, **96**, 879 (1965).
(16) R. Ammon, H. Berninger, and I. Berninger, *Qual. Plant. Mater. Veg.*, **16**, 263 (1968).
(17) H. Berninger, R. Ammon, and I. Berninger, *Arzneim.-Forsch.*, **18**, 880 (1968).
(18) P. Raig and R. Ammon, *ibid.*, **20**, 1266 (1970).
(19) D. M. Jerina, *Lloydia*, **37**, 212 (1974).
(20) H. D. West, J. R. Lawson, I. H. Miller, and G. R. Mathura, *Arch. Biochem. Biophys.*, **60**, 14 (1956).
(21) G. C. Mills and J. L. Wood, *J. Biol. Chem.*, **219**, 1 (1956).
(22) M. M. Barnes, S. P. James, and P. B. Wood, *Biochem. J.*, **71**, 680 (1959).
(23) S. Safe, D. Jones, and O. Hutzinger, *J. Chem. Soc., Perkin Trans. I*, **4**, 357 (1976).

Optical Studies on Interaction of Biliary Contrast Agents with Native and Modified Human Serum Albumin

K. J. FEHSKE and W. E. MÜLLER ˣ

Received July 24, 1980, from the *Pharmakologisches Institut der Universität Mainz, D-6500 Mainz, West Germany.* Accepted for publication October 24, 1980.

Abstract □ The interaction of two homologous series of biliary contrast agents with native human and bovine serum albumin and with modified human serum albumin was investigated using circular dichroism and equilibrium dialysis. For most derivatives, extrinsic Cotton effects were observed for the interaction with both albumins. In some cases, these effects were strongly affected by only small changes in the chemical structure of the drugs. These large differences in extrinsic Cotton effects can be explained by definite effects of the chemical structures on the binding site selectivity of some drugs. For example, iopodate preferentially binds to the warfarin binding site of human serum albumin, while an ethyl group into the propionic acid side chain reduces the affinity for the warfarin site but strongly increases the affinity for the diazepam binding site of human serum albumin.

Keyphrases □ Biliary contrast agents—interaction with native and modified human serum albumin, optical studies on warfarin and diazepam binding sites of albumin □ Albumin, human serum—interaction of biliary contrast agents with native and modified albumin, optical studies of warfarin and diazepam binding sites □ Binding sites—warfarin and diazepam binding sites on human serum albumin molecule, interaction of biliary contrast agents with albumin □ Structure–activity relationships—interaction of biliary contrast agents with native and modified human serum albumin, optical studies of warfarin and diazepam binding sites

Contrast agents for the intravenous or oral visualization of the biliary tract are, in general, highly bound to albumin in the blood of humans and laboratory animals (1). Because of their strong binding to albumin, their low lipophilicity, and their low volumes of distribution, plasma protein binding of biliary contrast agents seems to be an important determinant of the pharmacokinetics of these drugs (2, 3). Furthermore, since the toxicity of the biliary contrast agents increases with increasing plasma protein binding, binding mechanisms leading to high albumin binding and high toxicity may have similarities (1, 4).

Therefore, detailed knowledge of the degree of albumin binding of the biliary contrast agents and of the binding mechanisms is of great interest. Thus, it must be determined which moieties of the contrast medium molecules are important for high binding and to which sites of the human serum albumin molecule the substances are bound. While some quantitative data on affinity constants and numbers of binding sites for two groups of homologous biliary contrast agents were published previously (5), this study describes qualitatively their interaction with human serum albumin, using optical methods and modified albumin derivatives.

EXPERIMENTAL

Materials[1]—The human serum albumin[2] had an electrophoretic purity of 100%. All other chemicals were obtained from commercial suppliers.

Preparation of Albumin Derivatives—The modification of human serum albumin with 2-hydroxy-5-nitrobenzyl bromide (XII·Br) was reported in detail elsewhere (6). Human serum albumin was dissolved in 10 *M* urea adjusted to pH 4.4 by acetic acid, and a 1100-fold molar excess of XII·Br was added (XII–albumin). Another albumin sample was treated similarly but without the reagent and was called urea–albumin (control). Albumin modified with *o*-nitrophenylsulfenyl chloride (XIII·Cl) was prepared with a 22-fold molar excess of the reagent in 20% acetic acid (7) to yield XIII–albumin. With both reagents, the lone tryptophan residue of human serum albumin was modified completely (7).

For the preparation of tetranitromethane- (XIV) modified human serum albumin derivatives (XIV–albumin), a 4-, 15-, or 64-fold molar excess of tetranitromethane was added to human serum albumin dissolved in 0.05 *M* tromethamine buffer and adjusted to pH 8.0, resulting in the modification of 2.2, 5.1, or 7.8 tyrosine residues per albumin molecule, respectively. The modification was described in detail elsewhere (7).

The degree of tryptophan and tyrosine residue modification of the albumin derivatives was determined spectrophotometrically and by amino acid analysis (6, 7).

Circular Dichroism Measurements—Circular dichroism measurements were performed with a spectropolarimeter[3] calibrated with *d*-camphorsulfonic acid. All solutions were prepared in 67 m*M* phosphate buffer (pH 7.4). Results are expressed as molar ellipticities $[\theta]$ (degree square centimeter per decimole), calculated with reference to the albumin concentration, using a molecular weight of 69,000. The optical path length was 10 mm, and the albumin concentration was 30 or 13.1 μM. Difference spectra were obtained by subtracting the effect of the corresponding albumin derivative as the blank.

Equilibrium Dialysis Experiments—The binding of the ^{125}I-labeled

[1] Biliary contrast agents were the gifts of Dr. U. Speck (Schering AG, Berlin), except iopanoic and iophenoxic acids, which were gifts of Dr. G. H. Mudge (Dartmouth Medical School).
[2] Behringwerke, Marburg, West Germany.
[3] Cary 61.

0022-3549/81/0500-0549$01.00/0