Kinetic and Mechanistic Studies of Blue Tetrazolium Reaction with Phenylhydrazines

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Received July 11, 1977, from the **Department of Chemistry, Southern Methodist University, Dallas, TX 75275,* and the ‡*Food and Drug Administration, Dallas District, Dallas, TX 75204.* Accepted for publication October 25, 1977.

Abstract □ The reaction kinetics of blue tetrazolium with selected arylhydrazines were investigated under pseudo-first-order conditions. The reaction rate constants were obtained at various temperatures, and the enthalpy (8.4–11.2 kcal/mole) and entropy (−38–−45 eu) of activations were calculated. A Hammett plot yielded a straight line with a slope of 0.52. The reaction was inhibited by atmospheric oxygen and iodine. A free radical mechanism is presented.

Keyphrases □ Blue tetrazolium—reaction with various phenylhydrazines, kinetics and mechanism □ Phenylhydrazines, various—reaction with blue tetrazolium, kinetics and mechanism □ Kinetics and mechanism—reaction of blue tetrazolium with various phenylhydrazines

Blue tetrazolium, 3,3′-[3,3′-dimethoxy(1,1′-biphenyl)-4,4′-diyl]bis(2,5-diphenyl-2*H*-tetrazolium) dichloride (I), has become increasingly important as an assay for both quantitative and qualitative reduction by various reducing agents. These agents reduce I to a highly colored formazan whose concentration is then measured spectrophotometrically.

BACKGROUND

This reduction is useful in many areas (1). For example, in histochemical systems, I may be used as a staining technique for light microscopy (2–4) since few organic compounds are yellow in their oxidized form and highly colored in their reduced form. Compound I gives a deep-blue stain, which is desirable for histological (*i.e.*, cytological) demonstration. In biochemical analysis, tetrazolium salts are used as an activity stain in electrophoretic assay (5) because of their low reduction potential. Clinical investigations of I in studies of bacterial infection (6) and carcinomatous tissues (7) also were reported. However, the high toxicity of I restricts this procedure to *in vitro* systems.

Several reports (8–13) noted the utility of I in corticoidal steroid analysis. USP XIX (14) and NF XIV (15) use a slightly modified procedure of Mader and Buck (8) for corticosteroid analysis in which I oxidizes the α-keto moiety of the C_{17} side chain in strongly alkaline solution and is reduced quantitatively to a highly colored formazan. A recent investigation of the steroid–blue tetrazolium reaction (16) showed that I oxidizes the α-keto moiety of the C_{17} corticosteroid side chain *via* the transfer of an electron pair and a proton from the tetrazolium molecule. A bimolecular mechanism involving a cyclic complex, supported by kinetic data, was presented (16).

The blue tetrazolium determination has been applied most extensively to α-keto systems. However, some nonketo compounds such as polyhydric phenols, quinones, and certain active hydrogen compounds are sufficiently reactive to permit quantitative analysis *via* formazan formation (17). A free radical mechanism was proposed based on similar structural reactivity in the blue tetrazolium reaction to the reaction of the same compounds toward oxygen.

This paper investigates the reactivity of a specific class of nonketo active hydrogen compounds—*viz.*, phenylhydrazines, with I. Rate constants for selected phenylhydrazines at various temperatures, the enthalpy and entropy of activation, and Hammett σ and ρ constants obtained from these kinetic results are reported. A mechanism based on the observed experimental data is proposed to gain an understanding of this system and to predict the reactivity of blue tetrazolium with other hydrazine-related compounds.

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EXPERIMENTAL

Apparatus—A UV–visible ratio recording spectrophotometer[2] equipped with water-jacketed cell holders and 1-cm quartz cells, a microbalance[3], a temperature-controlled circulating bath[4], a water-jacketed 40/50 female joint[5], and a gas chromatograph–mass spectrometer[6] containing a 1.2-m column[7] packed with OV-17 on 80–100-mesh Supelcoport were used. A thermocouple[8] was used to determine the difference in temperature between the circulating bath and the cell jackets.

Reagents—Alcohol USP and analytical grade absolute methanol were used as received. Tetramethylammonium hydroxide (II), 1%, was prepared by diluting 5 ml of 10% aqueous[9] II to 50.0 ml with alcohol USP (95% ethanol); 0.25% II in alcohol USP was prepared by diluting 5.00 ml of 1% II with 10.00 ml of alcohol USP. Solutions of I[10] were prepared by dissolving 5.0 mg/ml in absolute methanol.

Arylhydrazine hydrochloride standard solutions contained 0.010 mg of the appropriate phenylhydrazine derivative[11]/ml of alcohol USP, unless otherwise indicated.

Phenylhydrazine Decomposition Study—*In Air*—A 2.5-ml aliquot of phenylhydrazine hydrochloride (0.01 mg/ml of alcohol USP) was mixed with 0.5 ml of 0.5% II, transferred to a cell, and scanned periodically from 300 to 250 nm during 25 min *versus* a reagent blank.

In Nitrogen—The experiment in air was repeated, except that all solutions were purged with nitrogen for 15 min before II was mixed with the phenylhydrazine solution.

Rate Study—All manipulations in the rate study were performed in a nitrogen-filled glove bag. The solutions were purged with nitrogen prior to use; a vacuum was applied to the glove bag during purging. Two water-jacketed ⅁ 40/50 female joints were put in series between the circulating water bath and jacketed cell holder compartment of the spectrophotometer and placed in the glove bag. The solutions were placed in glass-jacketed female joints and allowed to temperature equilibrate for 15 min. Then a 10-ml aliquot of a standard phenylhydrazine hydrochloride solution and a 10-ml blank of alcohol USP were treated with 2.00 ml of I reagent (5 mg/ml) and 0.5 ml of II (0.5%).

Zero time was taken as the time of addition of II to the standard solution. Both solutions were transferred to cells and placed in the spectrophotometer as rapidly as possible, and absorbance readings were taken four times per minute at 525 nm until the reaction essentially reached completion. This procedure was repeated for nine phenylhydrazine derivatives at three different temperatures. Rate constants were calculated as previously described (16).

Determination of Oxidation Products of Phenylhydrazine Salts—At the conclusion of certain kinetic runs, a 5-ml aliquot was drawn from the reaction mixture and subjected to GLC–mass spectral analysis. The mass spectrum obtained for each oxidized phenylhydrazine product was compared with that of an authentic specimen.

Effect of Free Radical Reaction Inhibitor—Three 20.0-ml sets of alcohol USP containing 0, 10.0, and 100 μmoles of iodine and 1.25 μmoles of phenylhydrazine hydrochloride were treated with 2.00 ml of I (5.0 mg/ml) followed by 2.00 ml of 1% II. Each set was run against a reagent blank. Zero time was taken as the time of addition of the 1% II to the standard solution. Both solutions were transferred to cells as rapidly as

[2] Cary model 118.
[3] Mettler model M.
[4] Neslab model RTE-3.
[5] Corning Glass.
[6] DuPont model 321.
[7] Supelco.
[8] Omega Engineering.
[9] Eastman Organic Chemicals.
[10] Dajac Laboratories.
[11] Aldrich Chemical Co.

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Table I—Kinetic Parameters for the Reaction of I with Arylhydrazines, R-$C_6H_4NHNH_2$

R	T, °K	k_0, min^{-1}	$\Delta H^{\ddagger}$, kcal/mole	$\Delta S^{\ddagger}$, eu
3-CH_3	303.53	9.66×10^{-2}	10.04	−38.4
	304.21	4.85×10^{-2}		
	293.29	3.77×10^{-2}		
	293.23	4.86×10^{-2}		
	283.51	2.84×10^{-2}		
	282.92	2.36×10^{-2}		
4-Br	304.02	1.83×10^{-1}	8.31	−42.7
	303.48	1.79×10^{-1}		
	293.38	1.20×10^{-1}		
	293.23	1.17×10^{-1}		
	283.41	6.38×10^{-2}		
	282.92	6.03×10^{-2}		
4-Cl	303.94	1.63×10^{-1}	8.38	−42.7
	303.78	1.67×10^{-1}		
	293.38	9.81×10^{-2}		
	293.58	8.93×10^{-2}		
	283.41	5.76×10^{-2}		
	282.92	5.40×10^{-2}		
3-Br	303.98	2.06×10^{-1}	8.11	−43.1
	304.00	2.05×10^{-1}		
	293.36	1.17×10^{-1}		
	293.58	1.36×10^{-1}		
	283.41	6.99×10^{-2}		
	282.86	7.21×10^{-2}		
3-Cl	303.98	2.01×10^{-1}	7.73	−44.7
	304.00	1.62×10^{-1}		
	293.58	1.05×10^{-1}		
	293.50	1.02×10^{-1}		
	283.41	7.21×10^{-2}		
	282.96	5.99×10^{-2}		
4-CH_3	303.98	6.94×10^{-2}	10.07	−38.7
	304.00	9.58×10^{-2}		
	293.58	3.18×10^{-2}		
	293.42	3.34×10^{-2}		
	283.18	2.30×10^{-2}		
	283.17	2.14×10^{-2}		
3-OCH_3	304.05	8.30×10^{-2}	10.07	−38.4
	304.23	8.83×10^{-2}		
	293.32	4.68×10^{-2}		
	293.37	4.58×10^{-2}		
	282.86	2.42×10^{-2}		
	282.79	2.46×10^{-2}		
H	304.05	7.57×10^{-2}	11.27	−34.8
	304.23	7.30×10^{-2}		
	293.32	3.39×10^{-2}		
	293.34	3.55×10^{-2}		
	282.86	1.59×10^{-2}		
	282.95	1.82×10^{-2}		
4-OCH_3	304.05	9.07×10^{-2}	10.38	−37.3
	304.27	8.87×10^{-2}		
	293.32	4.57×10^{-2}		
	293.32	5.01×10^{-2}		
	282.92	2.18×10^{-2}		
	283.01	2.44×10^{-2}		

possible, and absorbance readings were made each minute at 525 nm for 60 min. The alcohol had been swept with nitrogen for 15 min to minimize oxygen-induced degradation of phenylhydrazine.

RESULTS AND DISCUSSION

Several modifications of the general procedure had to be made in the rate studies of the arylhydrazine–I reaction. The official procedure (14) for the quantitation of corticosteroids calls for very dilute standard solutions, which are very basic [apparent pH ~14 (12)]. The absorptivity of the formazan and its formation rate from freshly prepared solutions of arylhydrazine salts were much higher than solutions that had stood approximately 1 hr before analysis.

UV absorption scans from 200 to 350 nm of arylhydrazine solutions of apparent pH 14 were taken in the presence and absence of atmospheric oxygen. Figure 1 shows a rapid change in the UV spectrum of phenylhydrazine when run under ordinary atmospheric conditions for 25 min. Only slight changes were observed in the UV spectrum (Fig. 1) of a similar solution of phenylhydrazine that had been purged with nitrogen. This result indicates that the decomposition of the hydrazine molecule in basic solution is accelerated by atmospheric oxygen. Hydrazine decomposition was reported previously (18–21) under these conditions and

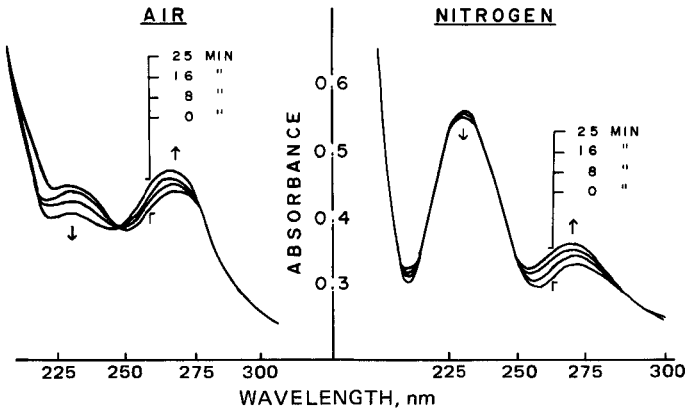


Figure 1—*Effect of oxygen on the UV spectra of phenylhydrazine in basic solution.*

shown to involve the oxidation of the hydrazine moiety *via* a free radical mechanism.

To ensure that all oxidation of phenylhydrazine was from its reaction with the tetrazolium moiety:

1. Fresh hydrazine solutions were prepared prior to taking each set of rate measurements.
2. Hydrazine solutions were made 50% more concentrated.
3. All solutions were purged with nitrogen.
4. All dilutions, pipetting, and mixings were performed under a nitrogen atmosphere.
5. All solutions were allowed to temperature equilibrate prior to mixing.
6. The 0.25% II was used.
7. Four absorbance readings per minute were recorded.

The results of the kinetic studies of the blue tetrazolium reaction with selected phenylhydrazines are shown in Table I. Pseudo-first-order rate constants were observed for all phenylhydrazines during the time interval used in rate calculations. The specific rate constants, k_i, were obtained by performing a least-squares regression on the equation:

$$\ln (1 - A_i/A') = -k_r t_i + E \qquad \text{(Eq. 1)}$$

where A_i is the absorbance at time t_i, A' is the theoretical or optimum absorbance, k_r is the observed rate constant, and E is the intercept. Because of the strong reducing ability of the hydrazine molecule, the reaction essentially reached completion within 20 min for most phenylhydrazines studied. To obtain a maximum number of data points before the reaction reached maximum absorbance, four measurements were made each minute. In each case, a correlation coefficient of 0.99 or greater was observed.

Figure 2 is a typical absorbance plot observed for phenylhydrazines under modified conditions. First-order kinetics were observed in the straight line of negative slope, k_r, and an intercept of zero.

Figure 3 is a Hammett plot of log (k_G/k_H) *versus* σ_G for the reaction of I with substituted phenylhydrazine derivatives at 20°, where k_G and k_H are the specific rate constants for a substituted and unsubstituted phenylhydrazine and σ_G is the substituent constant (22). The ρ value (slope) for this curve is approximately +0.52 (r = 0.8), which reflects the small influence substituents have on the reaction rate. For example, *m*-bromophenylhydrazine, the most reactive substituted phenylhydrazine in this study, reacts only approximately three times faster than phenylhydrazine itself at 20°.

The *meta*- and *para*-isomers of nitrophenylhydrazine and cyanophenylhydrazine also were treated with I. However, suitable kinetic parameters could not be obtained for these compounds since the cyano and nitro substituents are not stable under the extreme basic conditions employed in the blue tetrazolium reaction.

The enthalpy and entropy of activation results are also shown in Table I. These values were obtained by performing a least-squares regression on the equation:

$$\ln (k_r h/kT) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R \qquad \text{(Eq. 2)}$$

where k_r is the observed rate constant, h is Planck's constant, k is Boltzman's constant, T is absolute temperature, R is the gas constant, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively. Again, first-order kinetics were followed, and a correlation coefficient of 0.99 or greater was observed in each case.

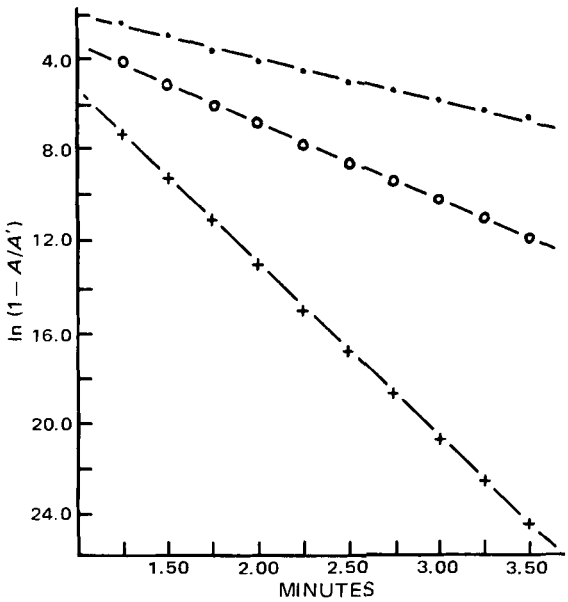


Figure 2—*Reaction rate of phenylhydrazine with I at various temperatures. Key: ●, 10°; ○, 20°; and +, 30°.*

The $\Delta H^{\ddagger}$ values for the phenylhydrazines (8.4–11.2 kcal/mole) were approximately half (17.3–19.8 kcal/mole) of those observed for corticosteroids (16), whereas the $\Delta S^{\ddagger}$ values for the phenylhydrazines (−38–−45 eu) were considerably more negative than those (−8–−12 eu) observed for corticosteroids. This result indicates a more facile transfer of a reduction unit and a more ordered transition state in the I reaction with phenylhydrazine salts as compared with corticosteroids.

GLC–mass spectral analysis of the various reaction mixtures indicated that arylhydrazine salts R–$C_6H_4NHNH_2$ were oxidized to the corresponding arene derivatives R–C_6H_5.

The inhibition of oxygen on the reaction rate and the low $\Delta H^{\ddagger}\rho$ (23) values indicate that the mechanism for the oxidation of phenylhydrazine salts resembles the free radical mechanism proposed previously (17) for active hydrogen compounds. Further evidence for the free radical mechanism was obtained by the study of iodine inhibition on the reaction rate of phenylhydrazine and I. The reaction of blue tetrazolium with 1.25 μmoles of phenylhydrazine containing 10.0 and 100 μmoles of iodine yielded absorbances of 1.222 and 0.692 units, respectively, while the absorbance of a similar solution without iodine was 1.282 units for the same time. The reaction of blue tetrazolium with phenylhydrazine in the two solutions containing iodine was inhibited by 4.7 and 46%. Since iodine is a radical scavenger that inhibits radical reactions and since the reaction rate of phenylhydrazine with blue tetrazolium is inhibited by iodine, the evidence indicates that the oxidation of phenylhydrazine by blue tetrazolium proceeds by a free radical mechanism.

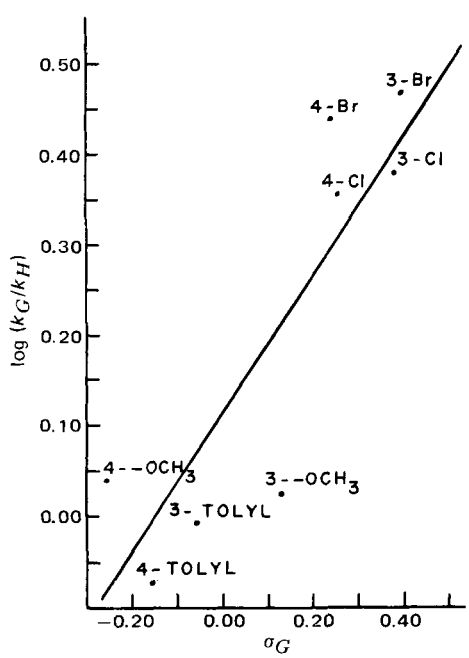
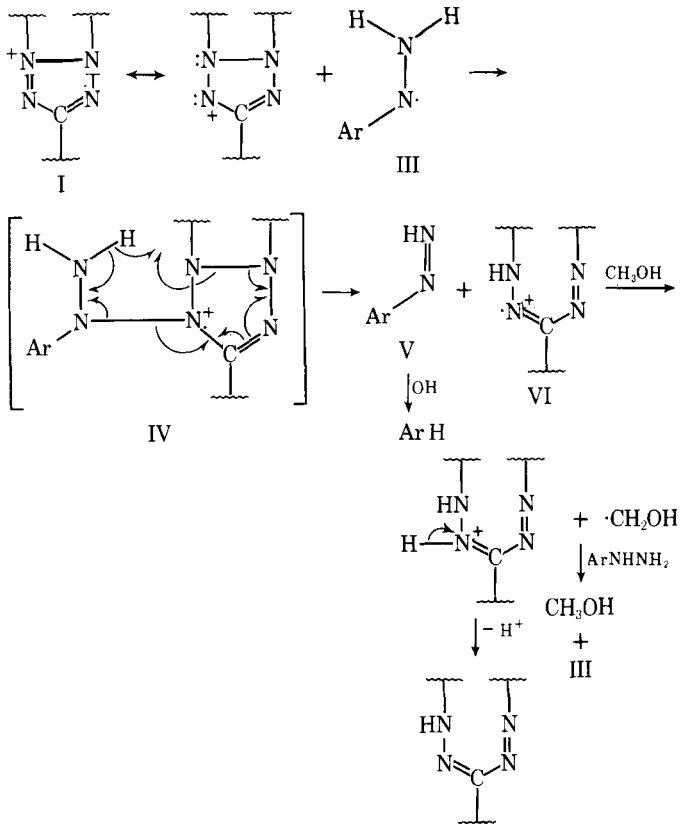


Figure 3—*Effect of substituents on the reaction of phenylhydrazine with blue tetrazolium at 20°.*



Scheme I

A pictorial description of such a mechanism is illustrated in Scheme I. Initially, a free radical complex, IV, is formed between either the free radical of arylhydrazine, III, and I or vice versa (only the former is shown in Scheme I). Complex IV subsequently decomposes to the aryldiimide V and the free radical derivatives of formazan VI. During the decomposition of IV, a hydrogen atom from the β-nitrogen atom and an electron from the α-nitrogen atom are transferred from the arylhydrazine portion of IV to I. Further decomposition of diimide V yields the corresponding arene derivative; this decomposition in basic solution is very rapid (24). The fate of radical VI is not known accurately, but it might abstract a hydrogen atom from methanol to yield a formazan. The resulting $\cdot CH_2OH$ radical then propagates the chain reaction by reacting with phenylhydrazine to produce III.

In all cases, the theoretical absorbance of formazan, based on transfer of one reduction unit from arylhydrazine to I (10), was observed. Therefore, this method might be feasible for the quantitation of pharmaceuticals containing the hydrazine moiety. The analysis of hydralazine and isoniazid by this proposed method is under investigation.

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Pharmacokinetics of Iodoxamic Acid in Rhesus Monkey: Biliary Excretion, Plasma Protein Binding, and Enterohepatic Circulation

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Received August 4, 1977, from the **Department of Pharmacy, School of Pharmacy, and the* ‡*Departments of Radiology and Surgery, School of Medicine, University of California, San Francisco, CA 94143.* Accepted for publication October 19, 1977.

Abstract □ The previously reported steady-state method allowed estimation of the capacity-limited pharmacokinetics of the cholangiographic agent, iodipamide. To circumvent the long time period required to establish each steady-state level, a dynamic method was applied to the study of the rate processes involved in the hepatic uptake and biliary excretion of a new cholangiographic agent, iodoxamic acid, in rhesus monkeys. The dynamic method has the advantage that the pharmacokinetic parameters involved in capacity-limited hepatic uptake or biliary excretion can be obtained from a single infusion experiment. The V_{max} was 1.03 ± 0.25 μmoles/kg/min (mean ± *SD*); K_m varied from animal to animal and ranged from 1.5 to 16.4 μ*M*. Protein binding was estimated using equilibrium dialysis. The Freundlich isotherm yielded a linear plot when the natural logarithm of unbound iodoxamic acid concentration in plasma was plotted against the natural logarithm of its blood concentration. The plasma protein binding data also could be fitted to the Langmuir isotherm, presuming two independent classes of binding.

Keyphrases □ Iodoxamic acid—biliary excretion, plasma protein binding, and enterohepatic circulation in rhesus monkeys □ Excretion, biliary—iodoxamic acid in rhesus monkeys □ Binding, plasma protein—iodoxamic acid in rhesus monkeys □ Enterohepatic circulation—iodoxamic acid in rhesus monkeys □ Pharmacokinetics—iodoxamic acid in rhesus monkeys □ Radiopaque media—iodoxamic acid, biliary excretion, plasma protein binding, and enterohepatic circulation in rhesus monkeys

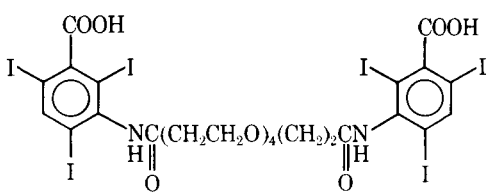
Cholecysto-cholangiographic agents bind significantly to plasma proteins (1–3), and this binding influences their biliary excretion. The role of serum albumin in the hepatic excretion of iodipamide was studied (4), and the binding of iodipamide to albumin retarded iodipamide transfer from plasma to the bile, probably because of competition between albumin and the anion binding protein of the liver.

Previous studies (5, 6) with a steady-state infusion method demonstrated that iodipamide is highly bound to plasma protein and has low blood clearance. Therefore, the unbound fraction of iodipamide is probably the major determinant in its renal and hepatic elimination (7). Although the steady-state method allows extensive analysis of the pharmacokinetics of iodipamide (5, 6), it has the disadvantages that: (*a*) a lengthy time is required to establish each steady-state level; (*b*) the time it takes to reach the steady state increases as one approaches saturation; and (*c*) a series of steady states is required in different experiments in the same animal, but the physiological status of an animal might change between experiments.

To circumvent some of these problems, a dynamic method was used to study the rate processes involved in the biliary excretion and hepatic uptake of a new cholecysto-cholangiographic agent, iodoxamic acid[1] (I), in rhesus monkeys. This method has the advantage that the pharmacokinetic parameters involved in the capacity-limited hepatic uptake or biliary excretion can be obtained from a single experiment.

EXPERIMENTAL

Studies were performed on three healthy rhesus monkeys (one male and two females), 4.5–9.1 kg, restrained in plastic chairs. They were



I

[1] Cholovue 40, 3% injection of the meglumine salt, E. R. Squibb and Sons, Inc., Princeton, N.J.

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