ders quantitation of small sulfamethazine peaks impractical. The chloroform wash of the basic aqueous mixture seems to affect this component most. Overwashing at this point, however, causes a loss of sulfamethazine.

Homogenizing or grinding the tissue prior to extraction tends to improve recovery somewhat but usually generates cleanup difficulties which negate the improved recovery.

Techniques critical to reducing the noise level of the liquid chromatograph include the following:

1. The solvent should be degassed to minimize the effect of small bubbles which are not visible but give detector response. This procedure also helps prevent buildup of air in the pump head, which would result in pulsations.
2. All fittings should be very tight to eliminate even the smallest leaks.
3. Detector electronics and cell windows should be dry, and any air or gas used to purge the reference cell should be scrupulously clean and dry.
4. In some instances, an arrangement to equilibrate the solvent thermally to the temperature of the detector might be helpful.
5. The electrical line to which the chromatograph is connected must be free of interference and large fluctuations. Both the chromatograph and the recorder should be well grounded.
6. The final aqueous solution must be adjusted to pH 8.5–9.0 before evaporation to reduce the volume. If the solution is acidic at this point, the sulfamethazine can be hydrolyzed to sulfanilamide and sulfanilic acid which elute with tissue components.

REFERENCES

(1) The Merck Veterinary Manual," 4th ed., Merck & Co., Rahway, N.J., 1973, p. 466.

(2) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 695.

(3) F. Tishler, J. L. Sutter, J. N. Bathish, and H. E. Hagman, *J. Agr. Food Chem.*, **16**, 50(1968).

(4) R. T. Morrison and R. N. Boyd, "Organic Chemistry," Allyn and Bacon, Boston, Mass., 1959, p. 562.

(5) J. Fellig and J. Westheimer, *J. Agr. Food Chem.*, **16**, 738(1968).

(6) G. B. Selzer and D. Banes, *J. Ass. Offic. Agr. Chem.*, **46**, 703(1963).

(7) S. F. Houston and J. E. Umstead, *J. Ass. Offic. Anal. Chem.*, **51**, 1016(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 2, 1975, from *Philips Roxane, Inc., St. Joseph, MO 64502*

Accepted for publication February 19, 1975.

The authors thank Gene Shipley, D.V.M., Philips Roxane, Inc., for performing the clinical portion of this experiment and Mr. Charles Pidacks, Waters Associates, for his assistance with the column and solvent selection.

[x] To whom inquiries should be directed.

Comparative Pharmacokinetics of Coumarin Anticoagulants XIV: Relationship between Protein Binding, Distribution, and Elimination Kinetics of Warfarin in Rats

AVRAHAM YACOBI and GERHARD LEVY [x]

Abstract □ The relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied. Individual rats eliminated warfarin by apparent first-order kinetics, with a biological half-life of 5.9–41 hr and a total plasma clearance of 2.4–22 ml kg^{-1} hr^{-1}. There is a strong positive correlation between the apparent volume of distribution (V_d) and the elimination rate constant (k_{el}). There was no apparent concentration dependence of warfarin binding to serum proteins over a wide concentration range, but there were pronounced intersubject variations in protein binding, with the free fraction of drug (f) in serum ranging from 0.172×10^{-2} to 1.53×10^{-2}. There are strong positive correlations between f and k_{el}, f and V_d, and f and the kidney–serum concentration ratio of warfarin. Consistent with theory, there is an excellent positive linear correlation between f and total plasma clearance of the drug. The intersubject variation in f is not related to variations in serum albumin or total protein concentration. There is a strong correlation between values of f for serum and liver homogenate in individual animals, consistent with the lack of correlation between f in serum and the liver–serum concentration ratio of warfarin. These results show that the pronounced intersubject variation in the elimination of warfarin observed in this investigation was related to interindividual differences in plasma protein binding of the drug. The differences in protein binding cannot be ascribed to differences in plasma protein concentrations and may reflect configurational differences of proteins or the presence of an endogenous displacing agent at different concentrations.

Keyphrases □ Coumarin anticoagulants—relationship between protein binding, distribution, and elimination kinetics of warfarin in rats □ Warfarin—relationship between protein binding, distribution, and elimination kinetics in rats □ Plasma protein binding, warfarin—relationship to distribution and elimination, rats

The anticoagulant warfarin is a highly plasma protein bound drug with pronounced intersubject variability in its elimination kinetics in humans and animals (1–7). In this investigation, the relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied in individual rats. A theoretical basis for a relationship between plasma protein binding and the total clearance of warfarin by the body was presented in a preliminary report (8).

EXPERIMENTAL

The studies were carried out on adult male Sprague–Dawley rats, weighing 400–450 g. They were selected from a larger number of animals on the basis of the results of a dicumarol screening test (9, 10) in order to obtain a group of animals with a wide difference

Table I—Biological Half-Life, Apparent Volume of Distribution, and Total Clearance of Warfarin in Individual Rats

Rat	Half-Life, hr	Apparent Volume of Distribution, ml/kg	Total Clearance, ml kg^{-1} hr^{-1}
1	5.91	184	21.6
2	8.13	189	16.1
3	8.80	206	16.2
4	8.93	198	15.4
5	9.24	202	15.2
6	10.3	189	12.7
7	10.3	204	13.7
8	16.7	150	6.2
9	17.7	145	5.7
10	18.6	151	5.6
11	21.1	137	4.5
12	21.9	141	4.5
13	41.3	140	2.4

in warfarin elimination kinetics. The rats received an intravenous injection of ^{14}C-warfarin, 0.6 mg/kg, and blood samples were obtained at frequent intervals.

The rats were killed at a time after injection when the warfarin concentration in plasma was expected to have decreased to about 0.4 μg/ml. They were killed by removing all blood from the aorta under ether anesthesia. Liver and kidneys were removed rapidly, each was cut into five or six slices, and the slices were compressed slightly between paper tissue to remove any remaining blood. The organs were weighed and homogenized with two (liver) or five (kidneys) volumes of ice-cold 0.9% sodium chloride solution, using a homogenizer[1] with a 50-ml Teflon-coated stainless steel sealed container at a rheostat setting of 70 for about 1 min. The blood samples were kept at room temperature for 15 min, and then the serum was separated by centrifugation at 1000×*g* for 10 min.

Warfarin concentrations in serum and plasma were determined by scintillation spectrometry following extraction and TLC, as previously described (7). Warfarin concentrations in liver and kidney homogenates were determined similarly after extracting a mixture of 1 ml of homogenate and 1 ml of 0.3 *N* hydrochloric acid with 10 ml of ethylene dichloride. Recovery of added ^{14}C-warfarin from these homogenates was 85 ± 5% (mean ± *SD*, n = 12) and was independent of concentration in the 0.05–2.5-μg/ml range.

Serum protein binding determinations were carried out by equilibrium dialysis at 25° and subsequently with serum from 12 additional rats at both 25 and 37°. Undiluted serum obtained from the individual animals at the end of the experiment, with no added drug (*i.e.*, with only the concentrations of warfarin and metabolites from the injection of warfarin), was dialyzed for 20 hr against isotonic 0.15 *M* pH 7.4 phosphate buffer in Plexiglas cells separated

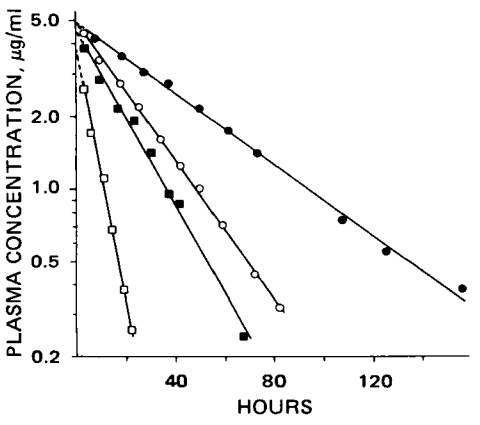

Figure 1—*Concentration of warfarin in the plasma of four rats as a function of time after intravenous injection of ^{14}C-warfarin, 0.6 mg/kg. Key: □, Rat 1; ■, Rat 8; ○, Rat 11; and ●, Rat 13.*

[1] Lourdes Multi-Mix, Lourdes Instrument Corp., Brooklyn, N.Y.

Table II—Concentrations of Warfarin in Liver, Kidneys, and Serum of Individual Rats at Approximately Similar Serum Concentrations

Rat	Warfarin Concentration, μg/g or ml			Concentration Ratio	
	Liver	Kidneys	Serum	Liver–Serum	Kidney–Serum
1	0.977	—[a]	0.288	3.39	—[a]
2	0.976	—[a]	0.590	1.65	—[a]
3	0.764	0.229	0.244	3.13	0.94
4	1.31	—[a]	0.517	2.53	—[a]
5	0.863	—[a]	0.494	1.75	—[a]
6	0.933	—[a]	0.408	2.29	—[a]
7	0.895	0.293	0.313	2.86	0.94
8	0.897	0.218	0.297	3.02	0.73
9	0.851	—[a]	0.491	1.73	—[a]
10	1.07	0.254	0.369	2.89	0.69
11	0.710	0.214	0.380	1.87	0.56
12	1.13	0.225	0.393	2.88	0.57
13	0.942	0.267	0.375	2.51	0.71
Mean	0.948	0.243	0.397	2.50	0.73
SD	0.156	0.028	0.101	0.59	0.16

[a] Not determined.

by a cellophane membrane[2]. Liver binding was determined similarly, using liver homogenate diluted with 0.15 *M* pH 7.4 phosphate buffer to yield a final concentration of 1 g of liver/10 ml. The liver homogenates were spiked with ^{14}C-warfarin, 0.3 μg/ml of homogenate.

Total protein concentrations in serum were determined by the method of Gornall *et al.* (11), using rat albumin as the standard. The fraction of albumin and other serum proteins was determined by electrophoresis[3], and concentrations of these proteins were determined from the product of the fraction and total protein concentration. The coefficient of variation of the serum albumin concentration determinations averaged 3.7% in six determinations on each of five samples.

For the correlation studies between certain pharmacokinetic parameters and serum protein binding, additional data from control experiments of other studies[4] were included. The fraction of warfarin *(F)* in the liver or kidneys relative to the amount of warfarin (free and protein bound) in the entire body was calculated from Eq. 1:

$$F = W_t C_s{}^0 C_t (C_s \times \text{dose})^{-1} \qquad \text{(Eq. 1)}$$

where W_t is the weight of the organ, C_t is the drug concentration

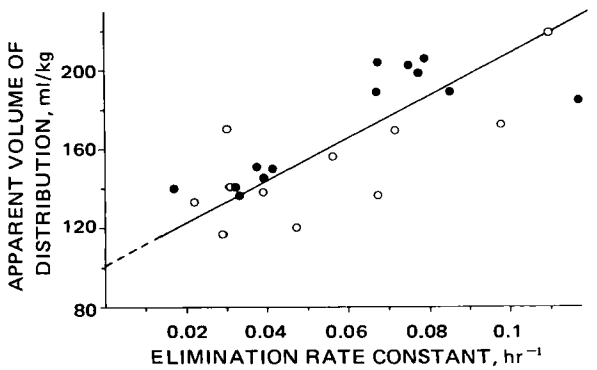

Figure 2—*Relationship between the apparent volume of distribution and the apparent first-order elimination rate constant of warfarin in individual rats that received an intravenous injection of ^{14}C-warfarin, 0.6 mg/kg (r = 0.761, p < 0.001). Key: ●, data from this study; and ○, data from another study (to be published).*

[2] Cut from dialysis tubing, average pore radius 24 Å, VWR Scientific, Rochester, N.Y.

[3] Gelman serum protein electrophoresis system, Gelman Instrument Co., Ann Arbor, Mich.

[4] To be published.

Table III—Effect of Concentration on Protein Binding of Warfarin in Rat Serum[a]

Rat A		Rat B	
Concentration, μg/ml	Free Fraction (f) × 100	Concentration, μg/ml	Free Fraction (f) × 100
0.325	0.364	0.285	1.06
0.620	0.328	0.588	1.20
1.22	0.347	1.25	1.13
3.70	0.387	2.55	1.14

[a] Serum from unmedicated animals was spiked with ^{14}C-warfarin.

in the organ, C_s^0 is the zero-time drug concentration in plasma, and C_s is the drug concentration in the serum when the liver and kidneys were removed. Other experimental details and the methods of pharmacokinetic analysis were described previously (7).

RESULTS

The biological half-life, the apparent volume of distribution, and the total plasma clearance of warfarin in 13 rats are listed in Table I. Figure 1 shows the time course of plasma concentrations in the animals with the shortest and longest half-lives of warfarin and in two animals with intermediate half-life values. There is a positive and highly statistically significant correlation between the apparent volume of distribution and the apparent first-order elimination rate constant for warfarin in individual rats (Fig. 2).

The average serum warfarin concentration when the animals were killed was about 0.4 μg/ml (Table II). The concentrations of warfarin in the liver were about 2.5 times higher on the average, while the concentrations in the renal tissues were 56–94% of the serum concentrations (Table II). The livers contained about 44% of the total amount of warfarin in the body on the average (F_{liver} = 0.44); the kidneys contained only 2.7% on the average. There is no significant correlation between F_{liver} and the elimination rate constant of warfarin.

The extent of serum protein binding of warfarin, expressed as the free fraction (f_{serum}), varied widely between animals, with f_{serum} values ranging from 0.00172 to 0.0153 at 25°, equivalent to 98.47–99.83% protein binding. The f_{serum} value in any one animal was essentially constant over a wide concentration range, irrespective of whether the animal was one with a relatively high or low f_{serum} value (Table III). There is an excellent correlation between f_{serum} values at 25 and 37° for the serum from any one animal (Fig. 3); the average ratio of f_{serum} values, 37°/25°, was 1.32.

There are highly statistically significant positive correlations between f_{serum} and the apparent volume of distribution (Fig. 4) and between f_{serum} and the elimination rate constant of warfarin (Fig. 5) in individual rats. The f_{serum} values obtained at 25° were multiplied by 1.32 to obtain equivalent values at 37° since f_{serum} determinations in subsequent studies were done at the higher temperature.

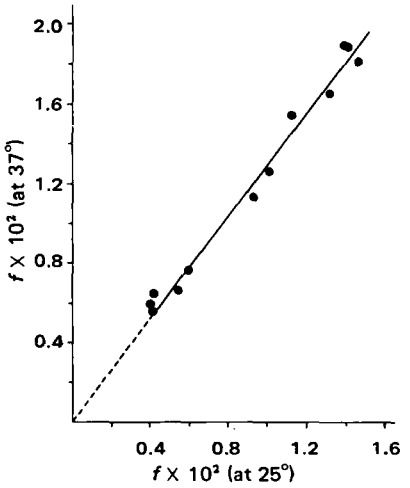

Figure 3—*Effect of temperature on serum protein binding of warfarin. The graph shows the relationship between the free fraction of warfarin at 25 and 37° in the same samples of serum, with a warfarin concentration of 0.3–2.1 μg/ml (r = 0.989, p < 0.001). The average ratio of the free fraction at 37 and 25° is 1.32.*

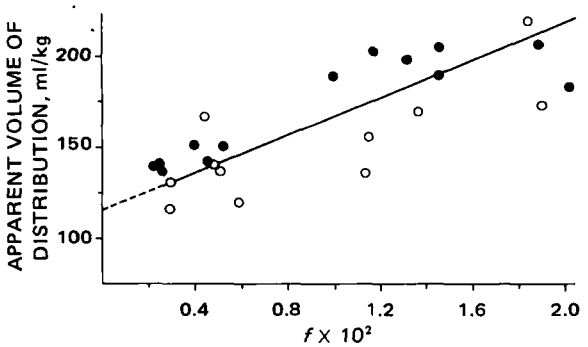

Figure 4—*Relationship between the apparent volume of distribution of warfarin and the fraction of free drug in serum of individual rats (r = 0.787, p < 0.001). Symbols are as given in Fig. 2. The ● values in this figure and in Figs. 5–7 are the experimental values multiplied by 1.32 to obtain f at 37°.*

A theoretical pharmacokinetic equation was formulated recently which predicts a linear correlation between the total plasma or serum clearance of warfarin and f_{serum}, with a zero intercept (8). The experimental data are in excellent agreement with this theory, showing a strong and statistically highly significant positive linear correlation between total clearance and f_{serum}, with the intercept of the regression line close to the origin (Fig. 6).

There is no relationship between individual f_{serum} values and either the albumin or total protein concentration in the serum of a total of 42 rats (Fig. 7). Binding studies carried out with diluted, ^{14}C-warfarin-spiked liver homogenates from seven rats yielded f_{liver} values that correlate strongly and highly statistically significantly (r = 0.946, $p < 0.005$) with f_{serum} values in the same animals (Table IV). To rule out the possibility that this correlation is an artifact due to retained blood in the liver, f_{serum} values in 1:100 diluted serum were determined (Table V). The significance of these data will be considered under *Discussion*.

There is no correlation between the liver–serum concentration ratio of warfarin (Table II) and f_{serum}, but there is a strong and highly statistically significant correlation between the kidney–serum concentration ratio and f_{serum} in individual rats (r = 0.918, $p < 0.005$).

DISCUSSION

The results of this investigation indicate that plasma protein binding is the major determinant of the pronounced interindividual differences in warfarin elimination kinetics in the rats studied.

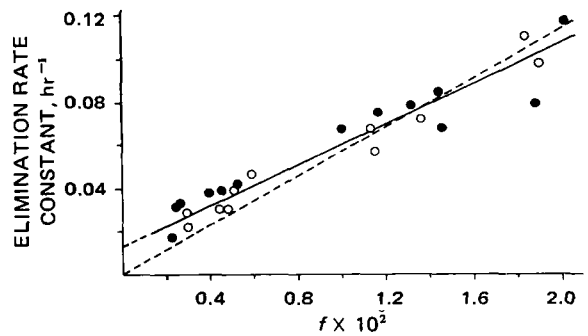

Figure 5—*Relationship between the apparent first-order elimination rate constant of warfarin and the fraction of free drug in the serum of individual rats (r = 0.970, p < 0.001). Symbols are as given in Fig. 2. The continuous line was fitted to the data by the double regression method (13); the stippled line was forced through the origin.*

Table IV—Protein Binding of Warfarin in Serum and Liver Homogenate of Individual Rats

Rat[a]	Free Fraction[b] × 100	
	Serum	Liver[c]
3	1.43	18.0
7	1.11	20.8
8	0.396	9.80
10	0.300	9.04
11	0.198	9.22
12	0.191	7.26
13	0.172	7.46

[a] Determinations of free fraction in liver homogenate of Rats 1, 2, 4, 5, 6, and 9 were not made. [b] At 25°. [c] A 1:10 homogenate of liver containing about 0.1 μg/ml (final concentration) of added ^{14}C-warfarin.

It was suggested earlier (8) that:

$$\text{total plasma clearance} = k''f_{serum} \qquad \text{(Eq. 2)}$$

where k'' is an intrinsic clearance constant equal to the product of k' (an intrinsic elimination rate constant) and V_w (a physiological space consisting of plasma water and other water exclusive of dissolved macromolecules capable of binding warfarin). The results of this investigation are in excellent agreement with this prediction (Fig. 6).

These results imply that the activity of the warfarin-metabolizing enzyme system(s) did not vary significantly between different rats under the experimental conditions. A subsequent study[4], in which the activity of the warfarin-metabolizing enzyme system(s) was altered by pretreatment of the animals with an enzyme inhibitor, did show a change in k'' value (*i.e.*, a decrease in the slope of the total clearance *versus* f_{serum} plot) as a result of such treatment.

The experimental data shown in Figs. 4 and 5 yield the empirical relationships:

$$V_d = a + bf_{serum} \qquad \text{(Eq. 3)}$$

$$k_{el} = a' + b'f_{serum} \qquad \text{(Eq. 4)}$$

The experimental value of a' in Eq. 4 does not differ significantly from zero, as should be the case if the rate of biotransformation is a function of only the free drug concentration. Combination of Eqs. 3 and 4 yields:

$$V_d k_{el} = aa' + (ab' + a'b)f_{serum} + bb'f_{serum}^2 \qquad \text{(Eq. 5}a\text{)}$$

or:

$$\text{total clearance} = k_1 + k_2 f_{serum} + k_3 f_{serum}^2 \qquad \text{(Eq. 5}b\text{)}$$

Equation 5*b* suggests a parabolic rather than a linear relationship between total clearance of warfarin and f_{serum}. The curvature of the parabola is, however, very slight over the range of the f_{serum} values observed in this study (Fig. 8). It is not feasible, therefore, to distinguish on the basis of the experimental data between the linear relationship represented by Eq. 2 (which is based on theoretical considerations) and the parabolic relationship represented by Eq. 5*b* (which was obtained empirically by assuming that Eqs. 3 and 4 describe the experimental data in Figs. 4 and 5). The apparently linear relationship between V_d and k_{el} (Fig. 2) is consistent with the reasonably good fit of the experimental data to Eqs. 3 and 4. When a' is assumed to be near zero, combination of the two equations by eliminating f_{serum} yields:

$$V_d = a + \frac{b}{b'}k_{el} \qquad \text{(Eq. 6)}$$

which describes the experimental data in Fig. 2 quite well.

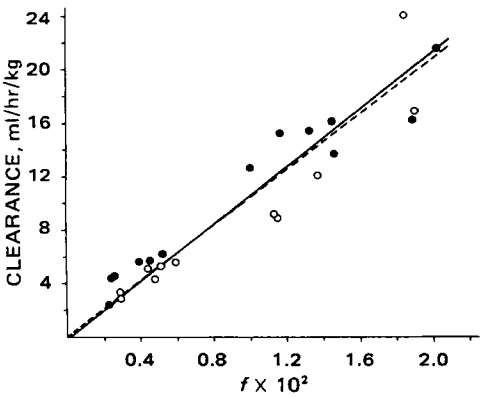

Figure 6—*Relationship between total plasma clearance of warfarin and free fraction of drug in the serum of individual rats (r = 0.947, p < 0.001). Symbols are as given in Fig. 2; regression lines are as given in Fig. 5.*

Table V—Effect of Dilution on Protein Binding of Warfarin in Rat Serum

Rat	Free Fraction[a] × 100	
	Undiluted Serum	Serum Diluted 1:100
1-V	0.560	26.7
4-V	0.660	33.3
9-V	1.65	57.9
12-V	1.89	58.3

[a] Undiluted serum contained 0.3–0.7 μg of ^{14}C-warfarin/ml; diluted serum contained 0.6–0.7 μg/ml. Binding studies were carried out at 37°.

Surprisingly, the striking interindividual differences in the serum protein binding of warfarin are not due to differences in the concentration of serum proteins (Fig. 7). The serum concentrations of albumin and total protein observed in this study are in good agreement with the concentrations reported by other investigators (12). The correlation between the extent of warfarin binding to serum proteins and liver in any one animal is another very significant observation. It is not an artifact due to retention of blood in the excised livers. Even if the excised livers retained as much as 10% of their weight of blood, the diluted liver homogenate (one part of liver in 10 parts of diluted homogenate) would contain only about 0.5% plasma.

The free fraction values obtained with a 1% aqueous solution of serum are much higher than the f_{liver} values obtained with the di-

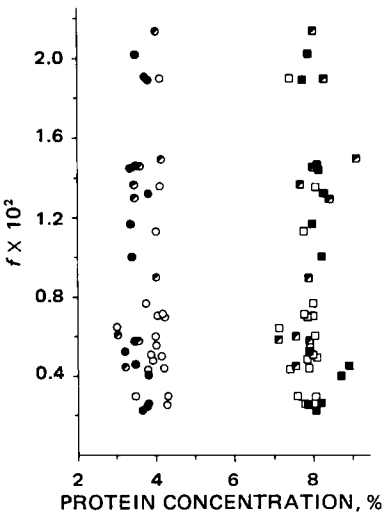

Figure 7—*Relationship between serum protein binding of warfarin and the concentrations of albumin (circles) and total protein (squares) in the serum of individual rats. Solid symbols represent data obtained in this investigation; open and half-open symbols represent data from two other studies carried out in this laboratory (to be published).*

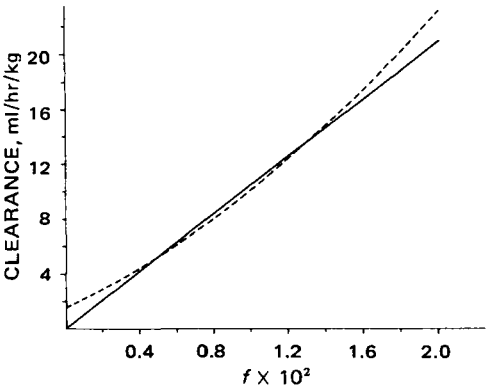

Figure 8—*Comparison of the directly determined relationship between total plasma clearance of warfarin and free fraction in serum (continuous line from Fig. 6) and the relationship as calculated from the regression equations for the data in Figs. 4 and 5 (stippled line).*

luted liver homogenates (Tables IV and V), showing that the liver tissues are responsible for the warfarin binding in liver homogenates. The fact that the liver–serum concentration ratio of warfarin is 2.5 (Table II), *i.e.*, considerably above unity, is consistent with this conclusion. Also consistent is the observation that the liver–serum concentration ratio of warfarin shows no correlation with f_{serum}; a parallel change in f_{serum} and f_{liver} would minimize any changes in the liver–serum concentration ratio as a function of f_{serum}. No direct studies of warfarin binding to kidney tissues were performed, but the fact that the kidney–serum concentration ratios correlate strongly with f_{serum} suggests that the f_{kidney} values do not change in parallel with the f_{serum} values in individual rats.

Since there is no correlation between f_{serum} and serum protein concentrations, one or more other factors must be responsible for the pronounced differences in the serum protein binding of warfarin. All animals were of the same strain and were obtained from the same supplier. They received identical food and housing. Whatever accounts for any one animal's f_{serum} value is unlikely to vary appreciably with time since the kinetics of warfarin elimination in any given animal are highly reproducible with time (9). Among the reasons potentially responsible for interindividual differences in protein binding of warfarin are *(a)* differences in the structure of certain plasma and liver proteins, and *(b)* the presence of an endogenous displacing agent at different concentrations in different animals. These and other mechanisms, as well as possible genetic influences on them, are being explored.

REFERENCES

(1) R. A. O'Reilly, P. M. Aggeler, and L. S. Leong, *J. Clin. Invest.*, **42**, 1542(1963).

(2) R. A. O'Reilly and P. M. Aggeler, *Proc. Soc. Exp. Biol. Med.*, **128**, 1080(1968).

(3) R. J. Lewis, M. Spivak, and T. H. Spaet, *Amer. J. Med.*, **42**, 620(1967).

(4) K. Pyörälä, E. A. Nikkilä, P. Vuopio, and G. Myllylä, *Acta Med. Scand.*, **183**, 437(1968).

(5) D. B. Hunninghake and D. L. Azarnoff, *Arch. Intern. Med.*, **121**, 349(1968).

(6) K. Pyörälä, *Ann. Med. Exp. Biol. Fenn.*, **46**, 23(1968).

(7) A. Yacobi, L. B. Wingard, Jr., and G. Levy, *J. Pharm. Sci.*, **63**, 868(1974).

(8) G. Levy and A. Yacobi, *ibid.*, **63**, 805(1974).

(9) A. Yacobi and G. Levy, *J. Pharmacokinet. Biopharm.*, **2**, 239(1974).

(10) L. B. Wingard, Jr., and G. Levy, *J. Pharmacol. Exp. Ther.*, **184**, 253(1973).

(11) A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751(1949).

(12) J. Guénard, F. Knappen, W. Frölke, I. Weisse, H. Köllmer, and H. Stotzer, *Arzneim.-Forsch.*, **24**, 1542(1974).

(13) W. J. Jusko and G. Levy, *J. Pharm. Sci.*, **58**, 58(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 27, 1975, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication February 24, 1975.

Supported in part by Grant GM 20852 from the National Institute of General Medical Sciences, National Institutes of Health.

[x] To whom inquiries should be directed.