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Effect of Bilirubin on the Distribution, Elimination and Anticoagulant Action of Dicumarol in Gunn Rats[1,2] (38547)

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The coumarin anticoagulants have been implicated in a number of drug interactions involving the displacement of these highly bound compounds from their binding sites on plasma proteins (1, 2). An extensively investigated example is the interaction of coumarin drugs with concomitantly administered phenylbutazone (3–5), which involves not only displacement and its pharmacokinetic consequences but also inhibition of drug metabolism (6, 7). It is likely that various endogenous substances may also displace coumarin drugs from plasma proteins, particularly when the concentration of these endogenous substances is elevated by disease. Bilirubin deserves primary consideration in this context since this substance itself is extensively bound to plasma proteins and its concentration in plasma is increased substantially in various disease states. In the investigation described here, the effect of bilirubin on the distribution, elimination, and anticoagulant action of dicumarol has been determined in jaundiced and nonjaundiced Gunn rats, and the effect of bilirubin on the plasma protein binding of dicumarol has been determined *in vitro*.

Materials and Methods. Animals. Jaundiced and nonjaundiced male and female Gunn rats weighing 175–480 g were used for the *in vivo* studies. Several litters were obtained from Dr. J. Krasner at Buffalo Children's Hospital and from Dr. C. T. Hanson of the National Institutes of Health. One jaundiced and one nonjaundiced rat of the same sex, selected at random from each litter, were paired. Pairs 2 and 3, 4 and 5, 8 and 12, and 9, 10 and 13, respectively, were obtained from four litters. Serum for the dialysis rate studies was obtained from male Sprague–Dawley rats weighing 300–350 g, obtained from Blue Spruce Farms, Altamont, NY.

Determination of the distribution, elimination, and anticoagulant effect of dicumarol. Details of the experimental methods have already been described (5, 8). Briefly, the rats received an ip injection of dicumarol, 4.0–25.1 mg/kg, including usually about 40 μCi of ^{14}C-dicumarol per kg of body wt. From five to ten serial blood samples were obtained from the tail artery and the dicumarol concentration and prothrombin complex activity in the plasma were determined. The dicumarol assay was not affected by the presence of bilirubin. The apparent first order elimination rate constant (k_{el}) for dicumarol was calculated from the least-squares slope of plots of log dicumarol concentration in the plasma versus time (slope = $-k_{el}/2.3$). The apparent volume of distribution (V_d) was estimated by dividing the injected dose by the extrapolated zero time plasma concentration of dicumarol. Prothrombin complex activity (PCA) was determined from the clotting time (t) of plasma samples diluted 1:10 with barium sulfate inactivated plasma from adult male Sprague–Dawley rats, according to the relationships $t = 15.5 + 85.4/\text{PCA}$ for the male Gunn rats and $t = 15.5 + 49.1/\text{PCA}$ for the female Gunn rats. The rate of synthesis of PCA (R_{syn}), the rate constant for degradation of PCA (k_{deg}), the slope (m) and the extrapolated intercept on the concentration axis (C_{max}) of the apparently linear portion of a plot of R_{syn} versus log dicumarol concentration in plasma were calculated as previously described (9).

Determination of bilirubin in plasma or serum. Bilirubin concentrations were deter-

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mined in 20 μl samples of plasma (obtained before drug administration) or serum by a micro-modification of the method of Jendrassik and Grof (10). The reproducibility of the procedure was excellent; the coefficient of variation for 10 assays of 10 mg/100 ml plasma was less than 1 %.

Protein binding of dicumarol. The effect of bilirubin on the protein binding of dicumarol in rat serum was assessed on the basis of the dialysis rate of dicumarol across a cellophane membrane. Pooled serum, obtained from seven rats, containing 0, 5.6 or 11.2 mg bilirubin per 100 ml and 9.5 to 9.7 mg ^{14}C-dicumarol per liter initially, was dialyzed against the same serum without dicumarol but with the same bilirubin concentration. The initial pH of all serum samples was adjusted to 7.4 with 1 *M* phosphate buffer, 2 ml per 40 ml serum. Dialysis was carried out at 37° in 2 ml capacity Plexiglas cells which were wrapped in aluminum foil and rotated at 15 rpm in a water bath. One-tenth ml samples were removed from each side at 0, 8, 14 and 28 hr for analysis of dicumarol. One-tenth milliliter was also used for analysis of bilirubin at times zero and 28 hr.

Dialysis rates of dicumarol were determined from the drug concentrations in the receiving compartment which never exceeded 1 % of the concentration in the originating compartment. The fraction of dicumarol not bound to serum proteins (f) was estimated from the following relationship, which applies under sink conditions:

$$\text{dialysis rate} = f \cdot k \cdot c$$

where c is the drug concentration in the originating compartment and k is the apparent first-order dialysis rate constant for dicumarol. To determine the value of k, a dialysis rate experiment similar to those described above was carried out with a solution of ^{14}C-dicumarol, 4 mg/liter, in 0.1 *M* phosphate buffer at pH 7.6 (i.e., without serum or bilirubin) in the originating compartment and 0.1 *M* phosphate buffer at pH 7.6 in the receiving compartment. A pH of 7.6 was needed to avoid solubility problems with dicumarol. Samples were obtained at 10, 25, 45, 75, 115 and 175 min. From this experiment, where $f = 1$, a k value of 0.408 hr^{-1} was obtained.

Results. The jaundiced Gunn rats had an average plasma bilirubin concentration of 7.0 mg/100 ml while the bilirubin concentration in the nonjaundiced rats was 0.3 mg/100 ml or less (Table I). The results of the distribution and elimination study are summarized in Table I. The V_d for dicumarol was 32 % larger on the average in the jaundiced animals ($P < 0.005$). The average k_{el} for dicumarol was 0.0766 hr^{-1} and 0.0504 hr^{-1}, respectively, in the jaundiced and nonjaundiced rats. This is equivalent to a half-life of 9.0 and 13.8 hr, respectively, and the difference is statistically significant ($P < 0.001$).

Bilirubin had no apparent effect on the slope of the anticoagulant effect-log dicumarol concentration regression line (m), but it displaced this line toward a lower concentration range as is evident from the statistically significant ($P < 0.01$) decrease in the C_{max} in jaundiced as compared to non-jaundiced animals (Table II). Litter pairs 2–5 had to be excluded from this part of the study; pairs 2 and 3 yielded very variable results which could not be interpreted and pairs 4 and 5 could not be sampled long enough because they received an unusually large dose of drug. Experiments on six jaundiced and 10 nonjaundiced rats were carried out to determine a value for k_{deg}. From these animals, only two of each group were available for the experiment summarized in Table II, and their individual k_{deg} values were used to calculate R_{syn} values. For the other animals listed in Table II a k_{deg} of 4.59 and 3.80 $days^{-1}$ for jaundiced and nonjaundiced animals, respectively, was used. These two values were the means obtained in the separate experiments for the determination of k_{deg}. However, since the two means were found to be not statistically significantly different, R_{syn} values were also calculated on the basis of a k_{deg} of 4.10 $days^{-1}$, which is the mean value for both groups of animals. The m and C_{max} values obtained with R_{syn} data resulting from both methods of calculation were very similar (Table II).

There is a statistically significant correlation between the jaundiced and nonjaundiced

TABLE I. EFFECT OF BILIRUBIN ON DISTRIBUTION AND ELIMINATION OF DICUMAROL IN LITTERMATE PAIRS OF JAUNDICED (J) AND NON-JAUNDICED (NJ) GUNN RATS.

Littermate pair no.	Apparent volume of distribution (V_d) (l/kg)		Elimination rate constant (k_{el}) (hr^{-1})		Bilirubin concentration in plasma of J Rats[a] (mg/100 ml)	Dose of dicumarol (mg/kg)
	J	NJ	J	NJ		
1	[b]	[b]	0.0727	0.0525	6.6	20.0
2	0.105	0.119	0.0752	0.0411	3.9	4.0
3	0.128	0.0828	0.0638	0.0456	5.0	4.0
4	0.143	0.112	0.1043	0.0481	5.6	25.1
5	0.153	0.098	0.0610	0.0536	7.6	25.1
6	[b]	[b]	0.1053	0.0447	8.4	6.0
7	0.0963	0.0611	0.0644	0.0584	6.1	5.4
8	0.0996	0.0811	0.0692	0.0402	8.3	5.4
9[c]	0.107	0.105	0.0684	0.0411	7.8	6.0
10[c]	0.113	0.0873	0.0519	0.0535	9.0	6.0
11[d]	0.180	0.109	0.0950	0.0345	4.0	4.0
12[d]	0.134	0.116	0.0865	0.0702	7.7	5.4
13[c, d]	0.236	0.167	0.0780	0.0711	11.1	6.0
Mean	0.136	0.103	0.0766	0.0504	7.0	
Statistical significance of difference[e]	$P < 0.005$		$P < 0.001$			

[a] Bilirubin concentrations in the NJ rats were ≤ 0.3 mg/100 ml.
[b] V_d could not be estimated due to prolonged absorption.
[c] Rats from N.I.H.
[d] Male rats.
[e] By paired t tests.

member of each littermate pair with respect to V_d ($r = 0.79$, $P < 0.01$), m($r = 0.83$, $P < 0.01$), and C_{max} ($r = 0.77$, $P < 0.02$) but not k_{el} ($r = -0.13$). There is no statistically significant relationship between the plasma bilirubin concentration in the jaundiced rats and their k_{el}, V_d, m, and C_{max} values.

It is estimated from the results of the dialysis rate experiments that dicumarol is about 99.97% bound, on the average, to serum proteins under the experimental conditions. The free fraction was increased by 34% in the presence of 5 mg bilirubin/100 ml while 11 mg bilirubin/100 ml increased the free fraction by 81% (Table III). During these experiments the bilirubin concentration decreased by only about 10% due to degradation.

Discussion. Previous studies in this series with isolated perfused rat liver systems and intact animals have shown that a decrease in the plasma protein binding of dicumarol, produced by decreasing the concentration of plasma proteins, administering a displacing agent, or by other factors, will increase the rate of elimination of the drug and increase the anticoagulant effect observed at a given dicumarol concentration in the plasma (5, 11–13). The results of this study show that an endogenously produced substance, bilirubin, in the concentration range encountered in jaundiced animals and humans, can significantly decrease the plasma protein binding of dicumarol and thereby increase the apparent volume of distribution and the elimination rate of the drug. In addition, the relationship between anticoagulant effect and plasma concentration is shifted to a lower concentration range so that a particular plasma concentration of dicumarol elicits a more pronounced effect in jaundiced animals. The lack of a significant correlation between the concentration of bilirubin in the jaundiced animals and either V_d, k_{el}, or C_{max} is probably due to the rather narrow

TABLE II. EFFECT OF BILIRUBIN ON THE RELATIONSHIP BETWEEN ANTICOAGULANT EFFECT AND PLASMA CONCENTRATION OF DICUMAROL IN JAUNDICED (J) AND NON-JAUNDICED (NJ) GUNN RATS.

Littermate pair no.	Slope of effect-log concentration regression line (m) (%/day)		Intercept of extrapolated regression line at maximum effect (C_{max}) (mg/liter)	
	J	NJ	J	NJ
1	−215	−492	9.8	13.5
6	−483	−761	14.6	16.4
7	−480	−540[a]	23.4	30.0[a]
8	−988	−1150	17.6	21.8
9	−336	−251	17.0	32.1
10	−254	−199	24.0	33.4
11	−190	−281	16.8	37.7
12	−525	−410	11.9	17.1
13	−527	−343	8.7	14.0
Mean	−444	−492	16.0	24.0
Statistical significance of difference[b]	$P > 0.4$		$P < 0.01$	
Mean of individual data calculated by alternate method[c]	−417	−518	15.8	24.0
Statistical significance of difference[b]	$P > 0.1$		$P < 0.01$	

[a] Estimated value due to excessive scatter of data

[b] By paired t test.

[c] The individual m and C_{max} values were calculated from R_{syn} data based on a k_{deg} value of 4.10 days^{-1}, which is the mean value for a total of 16 J and NJ rats.

TABLE III. EFFECT OF BILIRUBIN ON PROTEIN BINDING OF DICUMAROL IN RAT SERUM.[a]

Experiment number	Bilirubin concentration[b] (mg/100 ml)	Dicumarol concentration[b] (mg/liter)	pH[b]	Dicumarol dialysis rate (mg/liter/hr $\times 10^3$)[c]	Estimated unbound fraction of dicumarol[d] ($\times 100$)
1a	0	9.4	7.3	1.03	0.027
1b	0	9.5	7.3	1.02	0.026
2a	5.2	9.4	7.3	1.35	0.035
2b	5.3	9.6	7.3	1.41	0.036
3a	10.5	9.3	7.2	1.82	0.048
3b	10.7	9.3	7.3	1.83	0.048

[a] Serum from adult male Sprague–Dawley rats. The dialysis rate experiments were carried out at 37°.

[b] Average of initial and final (28 hr) values.

[c] Rate of increase of drug concentration in the receiving compartment.

[d] Based on a dialysis rate constant of 0.048 hr^{-1} in aqueous solution at pH 7.6. Fraction $\times$ 100 is numerically equal to the percentage. For example, 0.027% of the dicumarol in the plasma in experiment 1a was not bound to proteins.

range of bilirubin concentrations in these rats.

The method used for the estimation of the effect of bilirubin on the serum protein binding of dicumarol was designed to overcome the lack of stability of bilirubin in protein-free aqueous solutions, which made it impossible to utilize equilibrium dialysis procedures. The use of serum on each side of the dialysis membrane, with the same bilirubin concentration on each side, made it possible to carry out a 28 hr experiment with a bilirubin degradation of only about 10%. In addition, there is no change in the concentration of dialyzable constituents other than dicumarol due to the dialysis procedure *per se*. Serum rather than plasma was used to prevent possible effects of heparin, citrate, or oxalate on the binding of dicumarol. The estimate of the extent of protein binding of dicumarol in bilirubin-free serum is in general agreement with the results of equilibrium dialysis studies with ^{14}C-dicumarol of high specific activity (to be published).

The statistically significant correlation be-

tween jaundiced and nonjaundiced members of littermate pairs with respect to V_d, m, and C_{max} indicates that these characteristics may be subject to hereditary influence. Since changes in dicumarol distribution affect m and C_{max} (5, 13), the observed correlations probably represent different expressions of a single effect, namely a hereditary influence on drug distribution. It is surprising that there was no significant correlation between jaundiced and nonjaundiced members of littermate pairs with respect to k_{el}, since previous studies have shown that there is a very pronounced relationship between the distribution and elimination kinetics of dicumarol (5, 11–13) and warfarin (14) in groups of nonjaundiced rats.

Summary. Jaundiced Gunn rats were found to have a significantly larger apparent volume of distribution and higher rate constant for elimination of dicumarol than their nonjaundiced littermates. Anticoagulant effect-log plasma dicumarol concentration lines were parallel in the two groups of animals, with the line for the jaundiced rats shifted to a lower concentration range. *In vitro* studies showed that bilirubin can displace dicumarol from serum protein binding sites. There are indications of a genetic influence on the distribution and anticogulant effect of dicumarol in Gunn rats.

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