The Effect of Dicumarol on the Transvascular Exchange of T-1824-Labeled Protein in Rabbits

By THOMAS E. NELSON, JR., M.S., ELIA CASTRONOVA, B.S. AND CHESTER HYMAN, PH.D.

The effect of Dicumarol on the peritoneal transvascular transfer of T-1824-labeled plasma protein was investigated in the intact rabbit by a specially designed method. A significantly increased transfer rate was found in animals given two injections of Dicumarol 48 and 24 hours prior to testing; but determinations made 3 hours after a single injection demonstrated variable susceptibility to the drug. Prothrombin levels were depressed in every case.

The hemorrhagic tendency frequently associated with the depression of various components of the hemostatic mechanism appears to be more than coincidental and has prompted several investigators1,2 to speculate as to the probable role of the coagulation mechanism in the maintenance of normal integrity of the vascular wall. Early investigation3 of the sweet clover disease suggested that the hemorrhagic manifestation might be caused by the prothrombin depression. On the other hand, Bollman and Preston,3 Diem4 and Koller5 felt that decreased prothrombin alone could not explain the bleeding, and others have pointed out that a single large dose of Dicumarol, and the resulting severe prothrombin depression, did not cause bleeding. In addition, measurements of capillary resistance by the RumpLeeds method are not necessarily higher in cases of idiopathic hypoprothrombinemia.6

Searching for a more exact measure of capillary injury in Dicumarol therapy, Neumayr and Schmid7 determined the capillary permeability in human beings using the technique of Landis and associates.8 They found that Dicumarol increased the transcapillary escape of plasma protein, but that the change was apparently independent of the altered prothrombin time. They therefore suggested that Dicumarol acted through two separate mechanisms. Similarly, others found an increased permeability after a single dose of Dicumarol, prior to any detectable decrease in prothrombin level.9 In contrast to this, A. Linke9 reported that the intensity of the permeability augmentation approximately parallels the effect of Dicumarol or Tromexan on prothrombin.

Coumarin anti-coagulant therapy has now gained widespread acceptance in the treatment of thromboembolic disease, but even today hemorrhagic complications occur in at least 5 per cent of cases.10 It therefore seemed advisable to reinvestigate the problem of Dicumarol induced capillary permeability changes with the aid of new and improved procedures.11

In the present research the permeability of the blood-ascitic fluid barrier was studied in animals with hemorrhagic tendencies, using the method of Courtice and Steinbeck12 modified to yield quantitative data in individual animals.

METHODS

Young female rabbits weighing approximately 2.25 Kg. were used throughout. Under pentobarbital anesthesia, one femoral artery was exposed to facilitate blood sampling, and a 1 ml. control blood sample was withdrawn into a heparin-rinsed tuberculin syringe. Immediately thereafter, 75 ml. of normal rabbit serum* diluted 1:1 with 0.9 per cent

* Rabbit serum obtained through the courtesy of the El Monte Rabbitry, El Monte, California.
saline were then injected into the peritoneal cavity. Simultaneously, 10 mg. of the dye T-1524 was injected through an ear vein and all subsequent samples timed from this injection. Arterial blood samples were taken every 30 minutes over a period of 180 minutes. Every 15 minutes during the first hour, and at 30 minute intervals thereafter, 1.5 ml. samples of ascitic fluid were withdrawn through an indwelling perforated 20 gage hypodermic needle. At the end of 180 minutes the animals were sacrificed with pentobarbital and the ascitic fluid remaining was collected and measured.

Both blood and ascitic fluid samples were centrifuged; the latter because they contained a few white cells and fibrin reticulum which would otherwise interfere with colorimetric dye determination. Plasma samples were then diluted 1:19 and the centrifuged ascitic fluid samples were diluted 1:1 with saline before reading in a Colman Colormeter (filter 580 μ).

These data were used to provide a quantitative estimate of capillary permeability as reflected in the rate of movement of labeled protein from the vascular compartment into the ascitic fluid. This rate, when corrected for the concentration gradient across the barrier, proved to be relatively reproducible. In addition to the data determined, the actual computations required estimation of the reabsorption of fluid from the peritoneal cavity and correction for the amounts of labeled protein reabsorbed from this area via the lymphatics. These corrections were applied as follows: the total quantity of labeled protein which had moved across the barrier in any period of time was taken to be equal to the difference between the quantities found in the ascitic fluid at the beginning of the period and the quantity found at the end of the period. To this was added the quantity removed from the peritoneal cavity during this same period. The quantity present at the moment of sampling could be determined from the product of the concentration of dye in the sample and the estimated volume of ascitic fluid. The volume of ascitic fluid at any time was interpolated from the initially injected volume and the volume remaining at the end of the experiment by assuming a constant rate of ascitic fluid reabsorption. In addition, correction for the volumes of ascitic fluid removed in the previous samples was made. The quantity of labeled protein moved by the lymphatics during any given period was calculated on the assumption that the fluid reabsorbed during that period contained labeled protein at a concentration midway between that of the preceding and subsequent samples. Thus, the total amount of protein moving from the vascular to the ascitic compartment during any period of time could easily be determined from the data using the several assumptions implicit in the calculations here outlined. The individual values so obtained were plotted on rectilinear coordinate paper as a function of time and a best-fit curve drawn through the points. The curve was then divided into 30 minute increments, each beginning 15 minutes before and ending 15 minutes after the representative plasma sample times. The increase in amount of labeled protein during each 30 minute interval was then divided by the concentration difference between the two fluids, and the quotient plotted against time on semi-logarithmic paper. A line drawn through the points and extrapolated to the time of dye injection established the apparent initial protein transfer rate. In addition, the exponential rate of disappearance of dye from the circulation was calculated from the plasma concentrations in each case, using the method of Paldino, Sosnow, and Hyman. These data are reported as "disappearance rates."

Three series of animals were studied. In the control group the labeled protein exchange was measured with no previous treatment. Animals of the second group were given two intravenous injections of 22 mg. of Dicumarol/Kg./day, 48 and 24 hours prior to testing, and those of the third group were given a single dose of 22 mg./Kg. 3 hours prior to measuring the transfer rate. In order to avoid variations in intestinal absorption, the Dicumarol was administered intravenously. We have found that the insoluble crystalline form of Dicumarol can be suspended in gelatin and that it can then safely be injected intravenously into the rabbit. This suspension was made by grinding the crystalline preparation in a mortar with Baxter Oxypolygelatin.* Prothrombin levels were determined on several arterial blood samples taken from each animal immediately before and during the experiment. The Ware and Straubell modification of the Owren method was used, and in the absence of a rabbit prothrombin standard, all results are here expressed as per cent of the individual animal's own control level.

RESULTS

Reproducibility of the method was established in a series of control animals and is represented in table 1A. Calculated ascitic fluid absorption rates and "disappearance rates" are included in the table to show the lack of correlation between these factors and protein transfer rates. The points on figure 1 represent the geometric mean of comparable points obtained in 10 individual experiments. The semicircle was not experimentally determined, but represents the mean of the individual extrapolated intercepts. Similar data obtained from 10 animals given Dicumarol for two days prior to the deter-

* Supplied through the courtesy of the Don Baxter Laboratories, Glendale, California.
TABLE 1.—Summary of labeled protein transport data in normal and Dicumarol treated animals

<table>
<thead>
<tr>
<th>Intercept</th>
<th>T-1824 Disappearance rate</th>
<th>Ascitic fluid absorption rate</th>
<th>Prothrombin level</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/hr</td>
<td>mg/ml conc. diff.</td>
<td>per cent/min.</td>
<td>ml/min.</td>
</tr>
<tr>
<td>1.54 0.301</td>
<td>0.110</td>
<td>1.32 0.304</td>
<td>0.110</td>
</tr>
<tr>
<td>1.28 0.354</td>
<td>0.064</td>
<td>1.28 0.390</td>
<td>0.061</td>
</tr>
<tr>
<td>1.24 0.266</td>
<td>0.100</td>
<td>1.26 0.323</td>
<td>0.183</td>
</tr>
<tr>
<td>1.32 0.260</td>
<td>0.183</td>
<td>1.304 ± 0.104</td>
<td>0.319 ± 0.038</td>
</tr>
</tbody>
</table>

Mean: 1.304 ± 0.104 0.319 ± 0.038

B 48 hour Dicumarol administration

| 3.60 0.334 | 0.100 <5 | 2.80 0.296 | 0.155 <5 |
| 3.44 0.277 | 0.100 <5 | 3.64 0.269 | 0.155 <5 |
| 4.10 0.300 | 0.255 22 | 2.64 0.296 | 0.133 <5 |
| 3.60 0.338 | 0.100 10 | 2.30 0.360 | 0.155 40 |
| 2.56 0.300 | 0.072 30 | Mean: 3.26 ± 0.588 0.301 ± 0.036 |

Mean: 3.26 ± 0.588 0.301 ± 0.036

C 3 hour Dicumarol administration

| 5.20 0.300 | 0.156 84 | 1.28 0.200 | 0.128 68 |
| 10.00 0.292 | 0.183 47 | 1.76 0.292 | 0.183 70 |
| 5.08 0.270 | 0.155 66 | 1.80 0.300 | 0.134 72 |
| 1.72 0.302 | 0.083 56 | Mean: 5.20 0.292 | 0.183 47 |

Mean: 5.20 0.292 | 0.183 47

determination are represented in figure 1 and table 1B.

The mean intercepts of the two groups are significantly different in contrast to the means of the “simple dye disappearance rates,” which in both groups are comparable to the values reported by Paldino and associates for untreated control rabbits.

The third group of animals, table 1C, given a single dose of Dicumarol three hours prior to determination, show an inconsistent response, although the prothrombin values were all substantially depressed.

**DISCUSSION**

The method described in this report was developed for use in experiments which forbid the use of extensive surgical procedures, and is thus applicable to the intact animal. As a direct measure of the movement of protein from the blood into a specific body cavity, it has proven sufficiently sensitive to measure variations in transcapillary exchange not demonstrable by simple dye disappearance tests. When applied to a group of normal animals under closely standardized conditions, the results indicated high reproducibility.

Apparently, this measure of protein transfer can be influenced by factors acting from either side of the barrier: thus, addition of small amounts of histamine to the ascitic fluid in-
creased the transference rate to values as high as 13.6 mg./hr./mg. dye/ml. concentration difference. Because of this extreme sensitivity we found it advisable to further standardize our tests by using the same batch of frozen pooled serum on all animals. An artificial ascitic fluid, obtained by diluting serum 1:1 with saline, maintained a constant protein concentration during the course of the entire experiment. This suggests that our assumption concerning the concentration of labeled protein in the reabsorbed fluid is probably valid. An initial volume of 75 ml. was arrived at empirically and is sufficiently large to assure a satisfactory residual volume after 180 minutes. This is important in order to insure satisfactory mixing and to avoid the formation of isolated pools.

Our data clearly show that intravenous administration of Dicumarol in large doses over a period of two days significantly increases the initial transvascular transfer of labeled plasma protein. It is a relatively consistent increase and is only infrequently accompanied by hemorrhage or grossly observable vascular damage, although in many instances, the prothrombin was depressed to less than the 10 per cent level reported as critical in rabbits.4 The short-term experiments, on the other hand, show no consistent augmentation of the transfer rate. As an incidental observation, we found that the prothrombin levels were reduced in all cases, in contrast to the findings of Kushinsky and Ludwig.15 This is probably due in part to the difference in route of administration of the drug, since the concentration in the blood determines the development and duration of prothrombin depression.20 Part of this discrepancy may reflect the increased sensitivity of the prothrombin assay procedures17 used in this study.

In these experiments we were unable to find any correlation between the degree of prothrombin depression and the increase in transvascular protein transfer, which appears to substantiate the observations of others4,7,10,12 that Dicumarol acts by several separate mechanisms.

**Summary**

A method is described for the measurement of transvascular transfer of labeled plasma protein in intact rabbits. It is especially suitable for use where the impairment of the coagulation mechanism prohibits surgical procedures. It consists in the establishment of an artificial ascitic fluid pool which may be readily sampled to determine the actual amount of labeled protein transferred from the blood to the peritoneal cavity.

Dicumarol was found to increase significantly the transfer of plasma protein into the peritoneal cavity. Treatment with Dicumarol over a period of two days produced a consistent increase in transfer rate in contrast to the erratic results obtained 3 hours after a single dose. In all cases, the prothrombin levels were depressed below normal, but there appears to be no correlation between the degree of increased rate and the severity of prothrombin depression.

**Acknowledgment**

These experiments were performed in laboratories generously provided by the Alan Hancock Foundation.

**Summario in Interlingua**

Es describite un metodo pro le mesuration del excambio transvascular de etiquettate proteina plasmatic in conilios intacte. Le metodo es specialmente recommendabile pro uso in casos in que le presentia de un disturbate mechanismo coagulative prohibi le application de methodos chirurgic. Illo consiste in le institution de un artificial reservoir de fluido ascitic que permitte un facilitasse prension de specimen pro le determination del quantitate de proteina etiquettate transferite ab le sanguine a in le cavitate peritoneal.

Il esseva constatate che Dicumarol augmenta significativamente le transferimento de proteina plasmatic a in le cavitate peritoneal. Le tractamento con Dicumarol durante un periodo de duo dies produceva un regular augmento del nivello de transition. In contrasto con isto, resultatos erratic esseva obtenite tres horas post le administration de un dose unic. In omne casos le nivellos de prothrombina esseva deprimite a infra le norma, sed il non pare exister un correlation inter iste depression e le augmento del nivello de transition.
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