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THE ACCUMULATION AND DISTRIBUTION OF DICUMAROL IN RAT LIVER SLICES*

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Abstract—Rat liver slices incubated with Dicumarol at concentrations of 1×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M were found to accumulate the anticoagulant so that the concentration in the liver was greater than it would be if it were uniformly distributed between the tissue and the medium. Under the conditions employed, the amount of the anticoagulant taken up by the slices was gradually accumulated and reached a maximum in about 30 min. The amount of drug accumulated by the slices was proportional to the amount of drug added to the medium, and amounted to about 60 per cent of the Dicumarol added to the medium. Various experiments with liver slices and isolated cell fractions showed that Dicumarol was bound by the soluble, mitochondrial, microsomal, and nuclear fractions. Extension of the binding studies to a variety of purified proteins and synthetic polymers showed that some, but not all, of these substances were capable of binding Dicumarol, which indicated that there was some specificity involved in the binding.

VARIOUS factors involved in blood clotting are synthesized in the liver. Although bishydroxycoumarin (Dicumarol) and related anticoagulants inhibit the synthesis of the clotting factors, the intracellular site of action and the mechanism of action are not known. Liver slices have been used in attempts to elucidate the action of the coumarin anticoagulants,[1–3] but little is known about the time course of uptake and the distribution of these agents in the tissue in studies *in vitro*. The purpose of the present report is to describe the time course of uptake of Dicumarol by rat liver slices and its binding by various cell fractions.

MATERIALS AND METHODS

Preparation of tissues samples and incubation procedures. Slices were prepared from the livers of rats weighing 300–450 g (Wistar strain), which had been fasted overnight. The rats were sacrificed by decapitation after which the livers were perfused with ice-cold 0·9% NaCl via the hepatic portal vein in order to remove residual blood cells and plasma proteins. The liver was removed, chilled in 0·9% NaCl, and 0·5-mm slices were prepared in a cold room at about 0° with a McIlwain tissue chopper.[4] The slices were rinsed with cold saline 2 or 3 times in order to remove material released from the cut cells. Slices were weighed and then incubated in Krebs–Ringer phosphate solution[5] at 37° in a Dubonoff metabolic shaker with air as the gas phase.

Isolation of cell fractions. Cell fractions were isolated essentially as described elsewhere by Schneider and Hogeboom.[6] Nuclei were prepared essentially as described

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by Busch *et al.*[7] Ribosomes were prepared according to the procedure described by Korner.[8]

Dialysis procedures. Nucleohistone, arginine-rich histone, lysine-rich histone, ovalbumin, catalase, and the phosvitin used in the dialysis studies were obtained from Worthington Biochemical Corp., Freehold, N.J. Polyglutamic acid and polylysine were obtained from Mann Research Laboratories Inc., New York, N.Y. Dialysis experiments with cell fractions or purified proteins were carried out either with 3·0-ml quantities of material using a dialysis bag or 1·0-ml quantities using the dialysis chamber described by Karush.[9] Dialysis experiments were carried out with the use of Visking casing at about 2° with continual mechanical agitation.

Radioactivity determinations. ^{14}C-methylene-labeled Dicumarol (1·8 mc/m-mole) was obtained from Calbiochem. Radioactivity determinations were made on samples dispersed with Duponal and agar as described by Campbell *et al.*[10] and counted on a Nuclear-Chicago thin window gas flow counter. Corrections were not made for self absorption.

RESULTS

Time course of uptake of Dicumarol by liver slices. The time course of disappearance of Dicumarol from the medium is illustrated in Fig. 1. The slices (1·0 g) were incubated

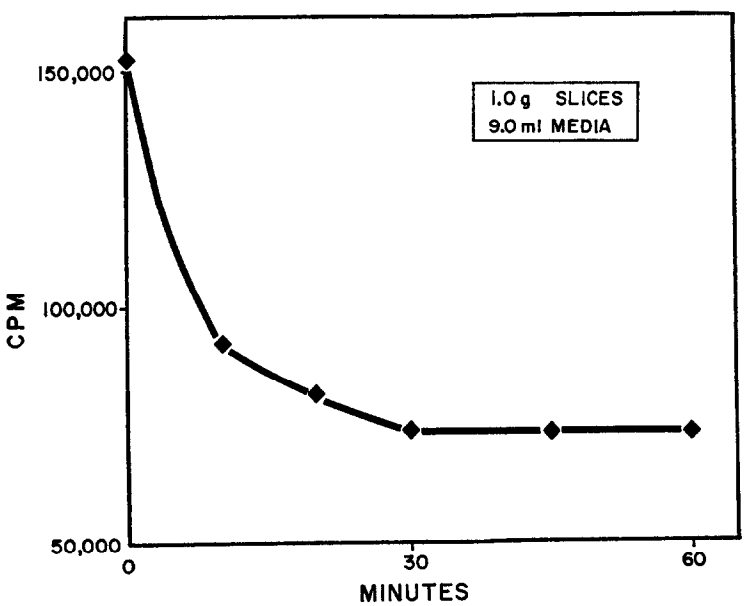

FIG. 1. Time course of disappearance of Dicumarol from the medium liver slice studies. Rat liver slices (1·0 g) were incubated in 9·0 ml Krebs–Ringer bicarbonate medium containing 100 μg Dicumarol plus ^{14}C-labeled Dicumarol. The slices were incubated in a Dubonoff metabolic shaker at 37° for the indicated periods of time after which aliquots of the media were taken for radioactivity determinations.

in 9·0 ml of medium containing nonradioactive Dicumarol which, if uniformly distributed, would correspond to 3×10^{-5} M, and radioactive Dicumarol. Aliquots of the medium were taken at the indicated times and radioactivity determinations were made. It will be noted that there was a gradual disappearance of radioactive

material from the medium for about 30 min, after which an equilibrium was attained. Balance studies showed that the Dicumarol which had disappeared from the medium was accounted for in the slices. Similar time courses were obtained at 0°, and when smaller or greater quantities of Dicumarol were used.

Amount of Dicumarol taken up by the tissue. In other experiments, the quantity of Dicumarol added to the incubation medium was varied, and the amount taken up by the tissue was determined after equilibrium had been established. As a routine, the amount in the tissue was calculated from the difference between the total amount used and the amount remaining in the medium. The results of such an experiment are presented in Fig. 2; Dicumarol was used in amounts which, if uniformly distributed,

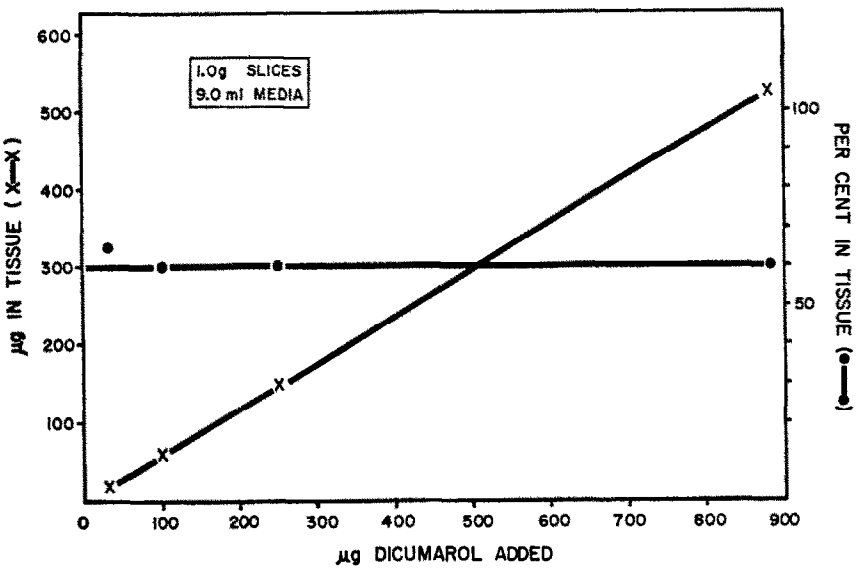

FIG. 2. Accumulation of dicumarol in rat liver slices. The slices were incubated as described in Fig. 1 with the indicated amount of Dicumarol for 90 min at which time aliquots of the media were taken for radioactivity determinations. The amount of Dicumarol in the tissue was determined by the difference between the amount added to the medium and the amount remaining after 90 min.

ranged from 1×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M. Over this range, the amount of Dicumarol taken up by the tissue was proportional to the amount added; about 60 per cent of the Dicumarol added to the medium was taken up by the tissue. Due to the limited solubility of Dicumarol in the medium, higher concentrations were not feasible so that the level required for saturation of the binding sites in the tissue could not be determined. Even at the highest concentration studied, there was no indication of saturation of available binding sites.

Binding by cell fractions. The binding of Dicumarol by cell fractions was studied by using various approaches: (1) incubation of Dicumarol with liver slices until equilibrium had been attained, after which cell fractions were isolated; (2) incubation of liver homogenates with Dicumarol, followed by isolation of cell fractions by differential centrifugation; (3) incubation of Dicumarol with particulate cell fractions, followed by centrifugation and by (4) dialysis equilibrium studies. In the present studies, the distribution of Dicumarol was followed by radioactivity because of the greater

sensitivity of this method rather than by the spectrophometric determination.[11] In general, all of the cell fractions studied, viz. nuclear, mitochondrial, microsomal, and soluble fractions of rat liver, bound Dicumarol to a high degree.

Table 1 summarizes the results of a typical experiment in which liver slices were incubated with Dicumarol after which the cell fractions were isolated and analyzed

TABLE 1. BINDING OF DICUMAROL BY CELL FRACTIONS*

Cell fraction	Total radioactivity (cpm)
Nuclei	21,390
Mitochondria	9330
Microsomes	17,640
Soluble fraction	14,450

* Rat liver slices (1·0 g) were incubated with Dicumarol ($7{\cdot}5 \times 10^{-5}$ M, if uniformly distributed) containing ^{14}C-labeled Dicumarol for 30 min at 37°. The slices were rinsed once, after which the cell fractions were isolated and the radioactivity of each fraction was determined.

for radioactivity. It will be noted that all of the fractions that were isolated by centrifugation bound Dicumarol. From the experiment presented, it is not clear whether Dicumarol was bound to proteins in the soluble fraction or simply in solution. However, in separate dialysis experiments the soluble fraction also was found to bind Dicumarol.

Similar results were obtained when homogenates were incubated with Dicumarol for 30 min at 0°, after which the cell fractions were isolated by differential centrifugation and analyzed for radioactivity. Although each fraction bound Dicumarol, it should be pointed out that these fractions are not homogeneous. The nuclear fraction, for example, may contain intact cells and other contaminants.

In order to diminish the possible effect of contaminating cell fractions, other experiments were carried out in which more rigorous procedures were employed. For example, nuclei were prepared as described by Busch *et al.*[7] and mitochondria were washed twice before use. The nuclear and mitochondrial fractions, prepared in a more rigorous fashion, bound Dicumarol in both centrifugation and dialysis experiments, which substantiated the above observations with less homogeneous preparations. Ribosomes also bound Dicumarol to as great an extent as microsomes.

Binding by mitochondria. Oxidative phosphorylation, which is carried out by the mitochondrial fraction of the cell, is uncoupled by Dicumarol and other anticoagulants *in vitro*.[12] In view of this effect, the binding of Dicumarol by mitochondria was investigated further by using preparations that were washed twice. In separate studies, the binding of Dicumarol by the mitochondria was found to take place rapidly compared to the experiments with slices. A 5-minute incubation period was found to be more than adequate for the binding to take place and this time period was usually used for the studies with mitochondria. The results of a typical experiment in which the quantity of mitochondria was varied are shown in Table 2. As indicated, the per cent bound increased with increasing amounts of mitochondria, but with respect to the amount bound per milligram of mitochondrial protein, there was a decrease.

The opposite type of experiment was also carried out in which the quantity of mitochondria was kept constant and the amount of Dicumarol was varied (Table 3). It will be noted that with increasing amounts of Dicumarol, increasing amounts of Dicumarol were bound, without evidence of saturation over the range of concentrations studied. Little, if any, of the Dicumarol bound to the mitochondria could be removed by repeated centrifugation and resuspension in 0·25 M sucrose.

TABLE 2. BINDING OF DICUMAROL BY RAT LIVER MITOCHONDRIA*

Mitochondria	Radioactivity bound		Dicumarol bound	
(mg protein)	(cpm)	(%)	(μg)	(μg/mg protein)
1·6	3100	17	9·2	5·7
3·2	4600	25	13·7	4·3
6·4	6600	36	19·7	3·1
9·6	8200	45	24·4	2·5
12·8	9400	51	28	2·2
16·0	9900	54	29	1·8

* Varying amounts of Dicumarol were incubated in a medium used for studies of oxidative phosphorylation containing Dicumarol ($7·5 \times 10^{-5}$M) and ^{14}C-labeled Dicumarol (approx. 18,500 cpm) for 5 min at 0°, after which each sample was centrifuged to sediment the mitochondria. Radioactivity determinations were made on the supernatant fluid of each sample. The amount of Dicumarol bound was calculated from the difference between the total used and the amount remaining in the supernatant fluid.

TABLE 3. EFFECT OF CONCENTRATION OF DICUMAROL ON BINDING OF DICUMAROL BY RAT LIVER MITOCHONDRIA*

Unlabeled Dicumarol added	Dicumarol in supernatant fluid		Dicumarol bound	
(μg)	(cpm)	(μg)	(μg)	(μg/mg protein)
0	8350			
5	7940	3·8	1·2	0·6
15	7250	10·4	4·6	2·1
30	6500	18·7	11·3	5·2
60	6600	38	22	10
120	5920	58	58	24

* Aliquots (2·2 mg) of mitochondria on a protein basis, were incubated in 1·5 ml of a medium used for the study of oxidative phosphorylation for 5 min at 0°. The medium contained the indicated amount of Dicumarol plus ^{14}C-labeled Dicumarol (approx. 10,450 cpm). The mitochondria were removed by centrifugation and radioactivity determinations were made on the supernatant fluid.

Although mitochondria were found to take up Dicumarol from the medium, it was not clear whether the anticoagulant was bound to the membranes and cristea of the mitochondria or whether it was taken up and concentrated within the mitochondria. In order to study this point, mitochondria were disrupted by osmotic shock and also by freezing and thawing. The treated samples were centrifuged and the sediment was tested for its binding ability. The residue obtained by such disruption, which most likely consisted of mitochondrial membranes and cristae, also bound Dicumarol (Table 4).

Binding of Dicumarol by proteins. The finding that Dicumarol was bound by each of the cell fractions studied prompted the extension of the binding studies to purified proteins in order to determine whether there was some degree of specificity, or whether this was a general nonspecific binding. Most of these studies were carried out with protein concentrations of about 10 mg/ml (1·0 ml) vs. Dicumarol (1·0 ml) at a

TABLE 4. BINDING OF DICUMAROL BY MITOCHONDRIA

Treatment	Radioactivity in residue (cpm)
None	13,800
(a) Frozen	12,600
(b) Osmotic shock	10,950
(c) Osmotic shock residue	12,720

Aliquots (12 mg) of twice-washed mitochondria were treated as follows: (a) frozen twice at − 17°; (b) osmotic shocked mitochondria were prepared by suspension of mitochondria in distilled water for 1 hr at 0°; (c) shocked residue was prepared as for (b), but was centrifuged and resuspended in 0·25 M sucrose. The samples were incubated in 3·0 ml of a medium used for studies of oxidative phosphorylation containing Dicumarol (5×10^{-5} M) and ^{14}C-labeled Dicumarol (approx. 21,800 cpm) for about 5 min at 0°, after which the samples were centrifuged and radioactivity determinations were made on the supernatant fluid.

concentration of $7{\cdot}5 \times 10^{-5}$ M containing labeled Dicumarol. The studies were carried out at pH 7·4 and 2° with mechanical agitation until equilibrium had taken place, which usually occurred in about 24 hr. Under these conditions, bovine serum albumin and arginine-rich histone bound Dicumarol to a high extent, i.e. two-thirds or more. The following proteins bound Dicumarol to an intermediate extent (one-third to two-thirds): lysine-rich histone, catalase, and globulin. The following bound little (less than one-third) or none of the Dicumarol: nucleohistone, fibrinogen, polyglutamic acid, polylysine, ovalbumin, and phosvitin. On the basis of these results, it appears that there is some specificity involved in the binding of the anticoagulent. In the present studies no attempts were made to determine equilibrium constants or the number of binding sites per molecule for the proteins that bound the anticoagulant.

Since Dicumarol can partition into an organic solvent such as ethylenedichloride,[11] some caution must be used in extending these observations to binding by cell fractions such as mitochondria and microsomes, which may contain as much as 40 per cent lipid. With cell fractions that contain much lipid, it is possible that some of the Dicumarol may have been associated with the lipid. Despite this possibility, it is clear that some proteins are capable of binding the anticoagulant, whereas other proteins are not. If proteins that bind Dicumarol are present in the circulation or in organs, the pharmacological potency and action of the drug would be affected.

DISCUSSION

From the present studies, it is evident that Dicumarol was accumulated by rat liver slices so that the amount present in the tissue was greater than that in the medium.

The accumulation was gradual, reaching equilibrium in about 30 min (Fig. 1). In view of the heterogeneous nature of the tissue slices, the term "equilibrium" is used in a broad sense in that the amount taken up reached a maximum and appeared to be at an equilibrium. The time required to reach equilibrium may be due in part to the time required for the anticoagulant to penetrate to the interior of the 0·5-mm slices and in part to the time required for entry into the cell. In this experiment, about 100 μg Dicumarol was present in the medium initially. If the Dicumarol were distributed uniformly throughout the medium and the tissue, this would correspond to about 3×10^{-5} M. After 30 min, about one-half of the Dicumarol disappeared from the medium to a level of about 5 μg/ml (about 45 μg total). The tissue, on the other hand, contained about 50 μg Dicumarol/g tissue. Although Dicumarol was reported by Christensen to be metabolized *in vivo*,[13] the rate does not appear to be rapid enough to alter the results of these short-term experiments; therefore, Dicumarol was followed by radioactivity determinations.

When a lesser or greater quantity of Dicumarol was added to the medium, the amount taken up by the tissue was proportional to the amount added (Fig. 2). Even with the highest quantity of Dicumarol that was feasible at neutral pH, there was no indication of a saturation of binding sites. Presumably, with the amount of tissue used, there was an excess of binding sites. Since the rate of uptake of Dicumarol at 0° appeared to be as rapid as at 37°, it did not appear that cellular energy was required for the uptake. As far as could be determined, under these experimental conditions the amount of Dicumarol taken up at 0° was the same as that taken up at 37°.

The amount of Dicumarol present in the liver slices after equilibrium was attained was in the same range as that reported in the liver of treated animals. For example, Weiner *et al.*[11] found 109 μg Dicumarol/g dog liver 24 hr after the i.v. administration of 50 mg/kg. Twenty-four hr after the last of 4 oral doses of 50 mg/kgeach, the liver content was 90 μg/g. Twenty-four hr after the last of 2 oral doses of 0·1 mg/g each, Green *et al.*[14] found 18–45 μg Dicumarol/g rat liver. Serum albumin binds Dicumarol to a high degree.[11] Since in the above studies the plasma levels of Dicumarol were about double those of the liver, it is possible that the tissues analyzed may have contained blood with bound Dicumarol, thus providing values of anticoagulant in the tissue which would be greater than the actual intracellular value. Christensen[13] attempted to eliminate this possible source of error by removing as much of the blood from the animal as possible by cardiac puncture. By using this approach, Christensen found 61·3 μg Dicumarol/g rat liver 1 hr after the i.v. administration of 27 mg/kg, which is in the same range found in the studies cited above. Thus, the amount of Dicumarol found in the liver slices in the present and in previous studies *in vitro*[2] was in the range found in the liver of animals treated with Dicumarol.

Since the tissue slices accumulated Dicumarol, it was of interest to determine which cell fractions might be involved in binding the anticoagulant. The present studies extend the studies of Green *et al.*[14] in that especially prepared fractions were used in order to utilize samples as homogeneous as could be obtained. In addition, several approaches were used to study the binding of Dicumarol by various cell fractions. Each of the fractions prepared for the present studies bound Dicumarol to a high degree. Nuclei that were free of intact cells, twice-washed mitochondria, microsomes, and ribosomes all bound the anticoagulant. Furthermore, the binding *in vitro* of the anticoagulant (Table 1) was rather similar to the distribution observed by Green *et al.*[14]

when the cell fractions were prepared from treated animals. From these observations, it can be concluded that any one of these intracellular structures is a possible site for the action of Dicumarol.

The binding of Dicumarol by the mitochondria is of special interest, because the effect of the anticoagulants has been attributed to an uncoupling of oxidative phosphorylation which is carried out by the mitochondria. The data in Table 4 show that the greater portion of the anticoagulant was bound by the residue of disrupted mitochondria, which in intact mitochondria would carry out oxidative phosphorylation. The data in Table 3 show that the amount of Dicumarol bound per milligram of mitochondrial protein is proportional to the amount of Dicumarol present. The data in Table 2 show that the total amount of Dicumarol bound is proportional to the amount of Dicumarol added; however, the amount bound per milligram of protein decreases with increasing amounts of mitochondria. In view of this, the term "molarity" in studies of this sort may be misleading, since the amount bound depends upon the stoichometric relationship between the quantity of Dicumarol added and the quantity of mitochondria used. This has not usually been taken into consideration. The importance of this was shown by Green *et al.*[15] who pointed out that, *in vitro*, uncoupling was diminished when the amount of mitochondria used was increased. A possible explanation for this is that some of the binding on the mitochondria may have been to sites which are not involved in oxidative phosphorylation. This possibility is not unlikely in view of the complexity of the mitochondria. This would also account for their finding that the mitochondria obtained from treated animals were not uncoupled even though anticoagulant was associated with the mitochondria. In the present studies, it was found that Dicumarol bound to mitochondria was not readily removed by washing them. Thus it seems likely that the Dicumarol associated with the mitochondria in the livers of the treated animals studied by Green *et al.* also was retained by the mitochondria during their isolation.

The finding that the Dicumarol was bound by all of the cell fractions studied, as well as by some purified proteins, suggests that there is some ubiquitously distributed group or groups available for binding. Since a number of purified proteins bound Dicumarol, it is evident that they possessed moieties which intereact with Dicumarol. Although the nature of the group or groups is not known, it is of interest to speculate on some possibilities. At neutral pH, Dicumarol possesses a negative charge which would favor interaction with positively charged groups such as are found in arginine-rich histone, which bound Dicumarol, rather than with negatively charged groups such as are found in polyglutamic acid, which did not bind Dicumarol. Although lysine would possess a positive charge at neutral pH, lysine-rich histone was less effective in binding Dicumarol than arginine-rich histone. Furthermore, polylysine bound little or no Dicumarol, which would suggest that these residues may not be involved in binding; of course, other groups may also be involved in the binding. For example, Overman *et al.*[16] found that benzene rings on Dicumarol were necessary for anticoagulant activity *in vivo*, and it is possible that these lipophilic rings may also be involved in an interaction with hydrophobic side chains in a protein or possibly with lipid components. As pointed out by Goldstein,[17] aromatic rings can contribute van der Waal forces in binding of organic compounds to proteins. Of course, until the critical site involved in anticoagulant action is known with greater certainty, this cannot be analyzed in full detail.

On the basis of the present studies *in vitro*, it is evident that Dicumarol is concentrated in the tissue to values greater than would be found if it were uniformly distributed between the medium and the slices. The amount accumulated in the slices *in vitro* was in the same range as that reported by other investigators in the livers of animals, that had been given Dicumarol. Binding studies with isolated cell fractions showed that nuclei, mitochondria, microsomes, and ribosomes bound the anticoagulant, which would suggest that the action of the drug could result from an interaction with any of these fractions. Further studies are in progress to determine the site and mechanism of action of Dicumarol.

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