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EFFECT OF DICUMAROL UPON PROTEIN SYNTHESIS IN THE RAT

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Abstract—The effect of dicumarol upon protein synthesis at three levels of cellular organization in the rat has been studied. It has been demonstrated that dicumarol administration to rats at such concentrations as to extend the one-stage Quick prothrombin time to greater than 240 sec and to reduce the concentrations of factors II, VII and X levels of 8, 14 and 16 per cent of normal, respectively, did not impair the biosynthesis of serum albumin when studied over a 10- to 20-hr period. Furthermore, dicumarol did not impair the biosynthesis of nascent peptide in liver as revealed by incorporation of radioactivity from amino acids into hepatic polysomes. Finally, no effect of dicumarol at concentrations between 10^{-5} and 2×10^{-4} could be detected upon protein biosynthesis *in vitro* by normal polysomes plus soluble supernatant extract from rat liver. These data do not support the hypothesis that coumarin anticoagulant drugs act by uncoupling oxidative phosphorylation and depressing protein synthesis *in vivo*. Likewise, no effect of dicumarol upon the intrinsic reactions of protein synthesis in the presence of an ATP generating system *in vitro* could be detected.

THE PRECISE mode of action of dicumarol and related coumarin drugs in antagonizing the synthesis of prothrombin and related clotting factors (factors II, VII, IX, X) is still unknown. Martius and Nitz-Litzow[1] suggested in 1954 that the primary action of dicumarol in antagonizing coagulation protein synthesis was the uncoupling of oxidative phosphorylation with a resulting failure of the liver to conserve ATP for anabolic reactions. It has been demonstrated in many laboratories[2] that dicumarol at the level of 10^{-5} M is effective *in vitro* in uncoupling oxidative phosphorylation of isolated mitochondria. The same concentration of warfarin in the plasma of normal human subjects (10^{-5} M) is effective as an anticoagulant.[3] On the other hand, it has been demonstrated by other workers that in the intact animal the uncoupling of oxidative phosphorylation by coumarin drugs at this concentration is not visible in whole cells as determined by the level of adenine nucleotides in the liver of vitamin K-deficient chicks[4] and rats[5] and the P/O ratios of mitochondria isolated from dicumarol treated chicks[6] and rats.[7] Furthermore, vitamin K has not been found to play a role in mitochondrial electron transport in mammals.[8] This paradox continues to obfuscate interpretations of the action of the coumarin anticoagulant drugs, particularly since it has been shown that dicumarol[5] and warfarin[9,10] at 10^{-4} M act to inhibit general protein synthesis in liver slices, possibly because they uncouple oxidative phosphorylation in partially injured cells.

We have, therefore, undertaken the re-investigation of this problem using modern techniques to measure general protein synthesis. Three types of experiments have been

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conducted. First, the serum albumin biosynthesis has been determined by observing the rate *in vitro* of labeling of albumin in blood plasma from radioactive leucine in the presence and absence of dicumarol; second, nascent peptide synthesis in liver polyribosomes has been measured *in vivo* by injecting radioactive amino acids into the portal vein of normal rats in the presence and absence of dicumarol; and third, the effect of dicumarol on the capacity of polyribosomes to carry out protein synthesis *in vitro* in the presence of an ATP generating system has been observed.

MATERIALS AND METHODS

Albino rats weighing 200–300 g of both the Wistar (Doisy Colony) and Sprague–Dawley (Charles River Laboratory) strain were used in these experiments. They were fed Ralston Purina chow during the preparation for the experiment. A solution of dicumarol containing 3 mg/ml at pH 9 was given intraperitoneally to experimental animals. The dose employed was 2·5 mg/100 g daily for 3 successive days. The animals were then subjected to various experiments at intervals after the last drug injection. Oxalated blood samples were obtained by cardiac puncture. One-tenth ml of 0·2 M sodium oxalate was added to 0·9 ml of blood and samples were centrifuged at 2000 rev./min for 10 min. The plasma was taken for one-stage coagulation times. In some experiments, the content of factors II, VII, and X was measured by specific methods. The one-stage method prothrombin time was measured according to the method of Quick and Grossman[11] using Difco thromboplastin. Prothrombin was measured by the method of Hjort;[12] factor X was measured by the method of Denson;[13] and factor VII was measured by the method of Owren and Aas.[14]

Isotopic experiments. Three preparations of isotopic amino acids were used. The first was L-leucine-U-^{14}C (specific activity, 6·6 mc/mm). The second was L-leucine-4, 5-^{3}H (specific activity, 500 mc/mm); and the third was an algae protein hydrolysate providing uniformly labeled ^{14}C-amino acids (specific activity, 600 μc/mg, equivalent to about 75 mc/mm). Two types of experiments *in vivo* were performed. In the first instance, L-leucine-U-^{14}C (5 μc) was injected intraperitoneally into control and dicumarol-treated rats 4 hr after the last dicumarol injection. Ten to 20 hr later, blood samples were taken for serum albumin isolation and for clotting assays. Animals with serious hemorrhage which suffered from shock during this period were rejected. Albumin was isolated from 5 ml of blood by the method of Debro *et al.*[15] as modified by Campbell *et al.*[16] The purity of each albumin preparation was checked by electrophoresis on cellulose acetate strips. All samples showed one band with the proper mobility. The purified albumin was precipitated with 10% TCA containing 0·1% cold leucine shaken, and allowed to stand for 30 min after precipitation. It was filtered through millipore filters (pore size, 0·45 μ) and washed three times with 5 ml of 5% TCA. The millipore filters were then dried and counted in a Model 4000 Packard scintillation spectrometer using PPO/POPOP as phosphors.

Ribosomes were prepared by the method of Wettstein *et al.*[17] The method involves the homogenization of rat liver in 0·25 molar sucrose, centrifugation at 27,000 *g* for 10 min to remove cell debris and mitochondria, treatment of the post mitochondrial supernatant with sodium desoxycholate which liberates bound polyribosomes from their membrane and centrifugation of the free polysomes through a discontinuous sucrose gradient for 4 hr at 105,000 *g*. The ribosomal pellet was subjected to centrifugation with gradients of 0·5 M to 1·1 M sucrose in tris buffer containing 25 mM

KCl and 5 mM magnesium chloride. They were centrifuged for 25,000 rev./min at 4° for 2·5 hr in a Spinco SW-25 rotor. Immediately after centrifugation, the gradients were analyzed by piercing the bottom of the plastic tube and pumping out the solution through continuous flow recording spectrophotometer. After passing through the spectrophotometer, the gradient was collected in a series of tubes which were analyzed for radioactivity.

In the experiments in which labeling of nascent peptide *in vivo* was observed, rats which had previously received dicumarol and nontreated controls were anesthetized with sodium amytal and a laparotomy performed. The gut was moved to the side of the abdominal cavity and wrapped in saline-soaked gauze. The whole portal vein was exposed and 0·5 ml of a solution of 5 μc/ml of ^{14}C-labeled amino acids from algae hydrolysate was injected into the portal vein. Three min after the injection the liver was removed and chilled on ice. Pairs of livers from identically treated rats were pooled and ribosomes prepared and studied as indicated above.

For the study of protein synthesis *in vitro*, ribosomes were prepared as indicated above. Supernatant enzyme was prepared with a 30 mM tris buffer, pH 7·4, containing 3 mM magnesium acetate to which 0·25 M sucrose was added. Post-mitochondrial supernatant was centrifuged for 3 hr at 105,000 *g* and supernatant drawn off with a Pasteur pipette. This supernatant supplied the necessary enzymes, cofactors, free amino acids, the transfer-RNAs. The assay medium contained the following substances at their respective concentrations: tris–HCl buffer, 30 mM (pH 7·4); magnesium acetate, 5 mM; KCl, 80 mM; Mercaptoethanol, 5 mM; GTP, 0·4 mM; ATP, 1·0 mM; glutathione (GSH), 2·5 mM; phosphocreatine, 20 mM; 100 i.u. penicillin per ml, 3 μg of crystalline creatine phosphokinase and a mixture of 20 amino acids in suitable concentrations.[17] Labeled leucine in the amount of 0·05 ml which contained either 5 μc/ml of L-leucine-U-^{14}C (specific activity of 6·6 μc/μm) or 25 μc/ml of leucine-4, 5-^{3}H (specific activity of 500 μc/μm), supernatant enzyme in the amount of 0·5 ml and ribosomes containing approximately 0·3 mg of RNA, were added to provide a final volume of 1·4 ml.

In some experiments different concentrations of dicumarol were added. The final mixture was incubated at 37° for 60–90 min. Aliquots in the amount of 0·1 ml were removed at intervals to chart the course of the reaction, and protein synthesis was stopped by dilution of the 0·1 ml with 0·9 ml of cold 0·1% leucine. An equal volume of 10% trichloroacetic acid was then added and the precipitated solution heated at 90° for 20 min to eliminate any charged tRNAs. The precipitates were collected on millipore filters, washed with 10% TCA containing 0·1% leucine, dried, and counted in a Packard scintillation spectrometer.

RESULTS

The 3-day schedule of dicumarol injections into Wistar and Sprague–Dawley rats gave Quick one-stage clotting times in excess of 240 sec. The content of factors in the plasma of each series of animals 24 hr after the last dose of dicumarol is shown in Table 1. Under these conditions, little difference in the response to the drug was noted between these two strains. The one-stage prothrombin time in untreated normal rats was 22·1 $\pm$ 0·8 (S.E.M.) sec for St. Louis University Colony Wistar rats and 22·9 $\pm$ 0·6 (S.E.M.) for Sprague–Dawley rats. As shown in Table 2, the specific activities of

TABLE 1. EFFECT OF DICUMAROL TREATMENT UPON ONE-STAGE PROTHROMBIN TIME AND THE PLASMA CONTENT OF FACTORS II, VII AND X OF RATS*

Strain of rats	No. rats	One-stage time (sec)	Factor II (%)	Factor VII (%)	Factor X (%)
Wistar (Doisy)	6	>240	6·6 ± 2·1	14·2 ± 5·5	10·3 ± 4·5
Sprague–Dawley (Charles River)	5	>240	8·1 ± 2·0	16·9 ± 4·1	12·7 ± 3·1

* Dicumarol in amounts of 2·5 mg/100 g of body weight was injected intraperitoneally once daily for 3 days and the animals studied 24 hr after the last dose. Wistar rats were obtained from the Doisy Colony at St. Louis University and Sprague–Dawley rats were purchased from Charles River Laboratories. The content of coagulation factors is expressed in per cent of the normal value for rat plasma. Normal one-stage prothrombin times were 22·1 ± 0·8 sec for Wistar rats and 22·9 ± 0·6 sec for Sprague–Dawley rats. Variance is reported in terms of standard error of the mean.

TABLE 2. INCORPORATION OF LEUCINE-U-^{14}C INTO SERUM ALBUMIN IN NORMAL AND DICUMAROL-TREATED RATS*

Experiment	R_X	Time of leucine-^{14}C administration before sacrifice (hr)	Purified serum albumin	
			Radioactivity (dis./min/ml)	Specific radioactivity (dis./min/mg)
1	N	−20	906	254
	N	−20	635	176
	D	−20	1150	328
	D	−20	650	242
2	N	−10	560	210
	N	−10	220	73
	D	−10	535	164
	D	−10	660	214

* Experimental rats were injected with isotopic leucine 4 hr after the last dicumarol injection and killed either 10 or 20 hr later. Vitamin K dependent coagulation factors were observed to be depressed below 15 per cent of normal levels for 24 hr after the last dicumarol injection (see text), and the one-stage prothrombin time was prolonged beyond 240 sec. Control rats, dosed with isotope in the same manner, were normal in all respects. The concentration of albumin in the final extract was determined by measuring absorbancy at 280 mμ and specific activities reported in terms of disintegrations per minute per milligram.

serum albumin were not lower in the dicumarol-treated and normal animals 10 and 20 hr after injection of leucine-U-^{14}C.

The results of the study of incorporation of labeled amino acids into nascent peptide in the liver of dicumarol-treated and normal rats are shown in Fig. 1. The radioactivity measurements represent the mean of three, closely agreeing experiments. No significant differences between the drug-treated and untreated animals were noted. Typical

polysomes were isolated from both normal and dicumarol-treated rats which showed a similar distribution of radioactivity; if anything, the ratio of radioactivity to absorbance was higher in the dicumarol-treated rats.

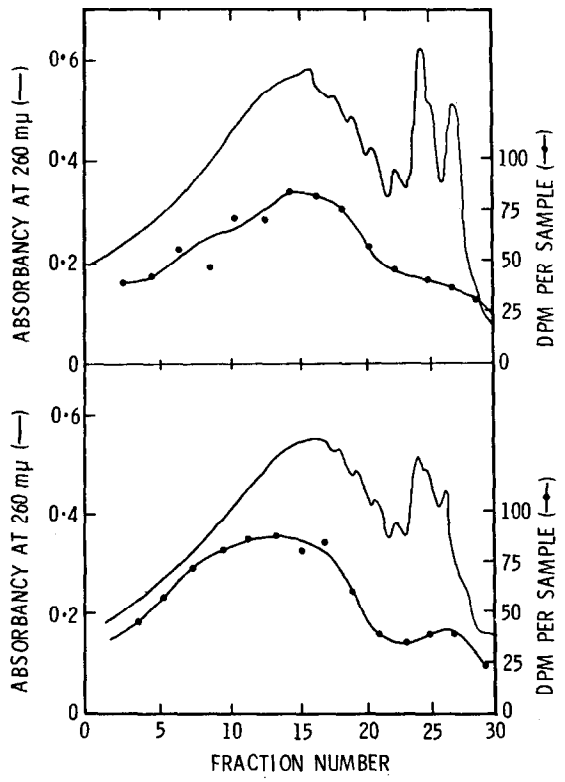

FIG. 1. Incorporation *in vivo* of isotopic amino acids into nascent peptide in livers of normal and dicumarol-treated rats. Two and five-tenths μc of uniformly labeled amino acids-^{14}C was given intraportally. The polysomes were analyzed on 1·1–0·5 M linear sucrose gradients. The top panel shows the results with normal rats and the bottom with anticoagulated rats.

The study of protein synthesis *in vitro* using polyribosomes and a solution faction from liver revealed no difference between the drug-treated and the normal ribosomes. As shown in Fig. 2, the incorporation of either leucine-U-^{14}C or leucine-3,5-^{3}H proceeded normally over a 90-min period regardless of the combinations of dicumarol-treated ribosomes with normal supernatant, normal ribosomes with dicumarol supernatant, and other combinations which revealed no essential alterations from normal. The maximum incorporation of radioactivity from L-leucine-U-^{14}C was 8000 dis./min/ml; with L-leucine-4, 5-^{3}H, it was 40,000 dis./min/ml. This shows that general protein synthesis is not blocked by the presence of dicumarol *in vitro* despite inhibition of prothrombin biosynthesis which represents a very small per cent of the total synthesis. The last figure, Fig. 3, shows the results of the effect of dicumarol added to a protein synthesizing system *in vitro* at concentrations from 10^{-5} M to 2×10^{-4} M, i.e. (5 μg to 100 μg) in the presence of an ATP generating system. Again,

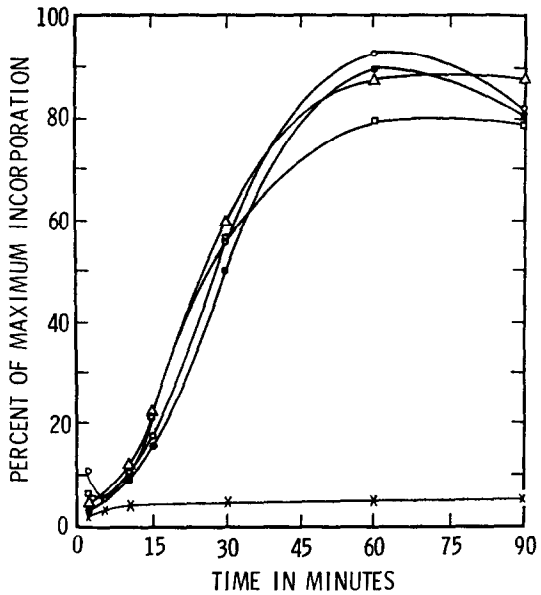

FIG. 2. Protein synthesis *in vitro* by polyribosomes from normal and dicumarol-treated rats. Polyribosomes and supernatant enzymes were prepared from normal and anticoagulated rats and combined in various ways. Normal ribosomes and normal supernatant: ○—○; normal ribosomes and dicumarol supernatant: ●—●; dicumarol ribosomes and normal supernatant: □—□; dicumarol ribosomes and dicumarol supernatant: △—△; no ribosomes and normal supernatant: ×—×.

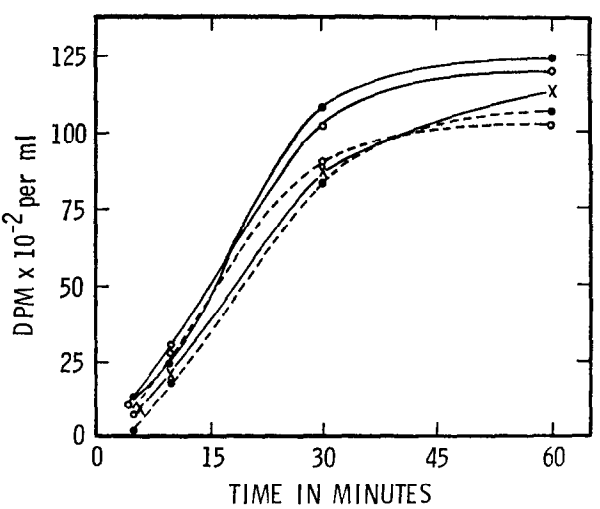

FIG. 3. Effect of addition of dicumarol *in vitro* upon protein synthesis by polyribosomes from normal rats. Dicumarol was dissolved in dilute alkali and adjusted with HCl to pH 7·6. Amounts added were: None, —●—; pH control (KOH plus HCl), —×—; 0·012 mM, --○--; 0·024 mM, --●--; 0·24 mM, —○—.

there was no effect of dicumarol on the biosynthesis of total proteins by normal ribosomes under these conditions.

DISCUSSION

The effect of dicumarol upon general protein synthesis at three levels of cellular organization in the rat has been studied with negative results. The finding that serum albumin synthesis in the intact rat is unaffected at doses of dicumarol that block the synthesis of prothrombin and two related vitamin K-dependent clotting proteins supports the work of Pool and Borchgrevink,[9] who found no effect upon the incorporation of glycine into liver and serum proteins of rats who had received anticoagulating doses of warfarin. Hill *et al.*[18,19] observed, furthermore, that vitamin K deficient rats showed a normal capacity for the synthesis of serum proteins, liver proteins, total microsomal protein *in vitro*, and the induction of hepatic tryptophane pyrrolase. Our finding that nascent peptide in hepatic polyribosomes is synthesized normally in the presence of anticoagulating doses of dicumarol which are of the order of 10^{-4} M in body water is strong evidence for an adequate supply of intracellular ATP and is supportive of other studies in which the distribution of adenine nucleotides in liver of rats treated with dicumarol was found to be normal.[5] Of interest is the paradoxical finding of Pool and Borchgrevink[9] and Couri and Wosilait[5] that coumarin drugs at concentrations of 10^{-4} M inhibit general protein synthesis in liver slices. Although they did not study the P/O ratios of mitochondria isolated from liver slices incubated in the presence of warfarin to determine whether or not oxidative phosphorylation was adequate to sustain protein synthesis, it is possible that coumarin drugs under these conditions *do* uncouple oxidative phosphorylation. It is also possible that other factors exist to inhibit the uncoupling action of dicumarol *in vivo*. One such factor could be the availability of intracellular proteins capable of binding the drug to the extent that it would not impair the integrity of the mitochondrial membrane. Ernster *et al.*[20] has concluded that the effect of dicumarol and related anticoagulants in uncoupling oxidative phosphorylation in isolated mitochondria is not an anti-vitamin K effect, but is a more general effect upon components of the electron transport chain. It may be concluded that the coumarin anticoagulant drugs are not specific competitive antagonists of vitamin K in any system. In mitochondria, free dicumarol *in vitro* has a deleterious effect on the membrane or membrane-bound components of the electron transport chain in the absence of vitamin K. *In vivo*, dicumarol appears to counteract the prothrombin synthetic action of vitamin K in an allosteric rather than competitive manner.[21]

We found that dicumarol had no effect upon protein synthesis catalyzed by polyribosomes in the presence of an ATP generating system. This eliminates any effect of dicumarol upon the intrinsic events of protein synthesis, including activation of amino acids to their tRNA derivatives, binding of these acylated-tRNA molecules to the ribosomes, and the manifold activities of the polyribosomes in accomplishing protein synthesis. Although no studies were carried out with synthetic messengers such as Poly U, the data eliminate an effect of dicumarol upon native mRNA. Couri and Wosilait[5] observed no effect of 10^{-4} M dicumarol upon the incorporation of ^{14}C-leucine into total rat liver tRNA.

We have recently demonstrated the biosynthesis of prothrombin in microsomes from normal rats[22] and have shown, furthermore, that this specific biosynthesis is not

antagonized by warfarin *in vitro*.[23] This biosynthesis is presumably carried out by polyribosomes bound to membranes since prothrombin, like albumin, is a protein manufactured by the liver for export.[24,25] Studies by Blobel and Potter[26] have shown that in a postmitochondrial supernatant of liver prepared at 17,000 *g* for 10 min, approximately 50 per cent of the ribosomes are free and 50 per cent are bound. Under our conditions of preparation, i.e. 27,000 *g* for 20 min,* about 80 per cent of the ribosomes in the supernatant is free. If dicumarol were to have a differential effect upon free ribosomes, this would certainly be visible in our studies.

These results provide strong indirect evidence for a specific effect of the coumarin anticoagulant drugs on the biosynthesis of prothrombin and the related vitamin K-dependent proteins. Recent studies suggest that the effect is mediated by a regulatory protein which binds both vitamin K and the coumarin drugs and catalyzes the initiation of coagulation factor synthesis.[27]

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* Anita Aspen, private communication.