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THE EFFECT OF COUMARIN ANTICOAGULANTS ON THE ADENINE NUCLEOTIDE CONTENT AND PROTEIN SYNTHESIS IN RAT LIVER*

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Abstract—Studies were conducted on the mode of action of the inhibition of synthesis of proteins of the prothrombin complex by the coumarin anticoagulants. The comparison of the adenine nucleotide content and the incorporation of ^{32}P-orthophosphate into liver nucleotides of both anticoagulant-treated and control rats was used as index of oxidative phosphorylation in the intact animal. Determinations of adenine nucleotides and ^{32}P-orthophosphate incorporation into liver nucleotides of treated rats gave values similar to the controls. On the basis of these studies, it would not appear that the anticoagulant effect of these agents is the result of uncoupling.

The possible direct effect of these agents on protein synthesis was also studied by the incorporation of ^{14}C-leucine into protein of rat liver slices and cell-free systems. The anticoagulants inhibited the incorporation of ^{14}C-leucine into liver slice proteins. However, the results of ^{14}C-leucine incorporation into RNA and into microsomal or ribosomal protein indicated that bishydroxycoumarin and warfarin had no measurable effect on these reactions.

THE coumarin anticoagulants inhibit the synthesis of several protein factors involved in blood coagulation. In addition, there are various reported actions of these agents which are unrelated to coagulation. Included among these are the depression of cardiac muscle and smooth-muscle contractility, vasodilatation, uricosuria, and antibacterial action.[2] The diversity of action of these agents might be due to an impairment of some fundamental cellular process—that of ATP synthesis. Mitochondrial oxidative phosphorylation, which is responsible for the synthesis of most of the ATP by the cell, is uncoupled by these agents *in vitro*, and this if often cited as the mechanism of action of the anticoagulants.[3] However, it must be emphasized that, to date, there is no evidence that these agents uncouple oxidative phosphorylation *in vivo*. If it is assumed that anticoagulant treatment of rats produces an uncoupling *in vivo*, then such an action may be expected to result in a measurable decrease in the adenine nucleotide content of liver which is the organ responsible for the synthesis of the

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clotting factors. Futhermore, any decrease in the nucleotide content should be related in some way to the diminution of the circulating titer of the proteins of the prothrombin complex, which is readily measured as the prothrombin time. An action of this sort would be considered indirect since the steps involved in protein synthesis are not directly affected. The findings of Green *et al.*[4] do not support the proposal for such an indirect action in that anticoagulant treatment of rats did not alter glycolysis or the other enzyme systems directly or indirectly related to aerobic respiration, which they studied.

The main objective of the present study was to investigate the role of the anticoagulants as uncouplers of oxidative phosphorylation *in vivo*; the second objective was to investigate possible direct effects on the steps of protein synthesis which are at present known. The adenine nucleotide content of the liver of control and treated animals was measured at various time intervals. The incorporation of ^{14}C-leucine was used to follow protein synthesis *in vitro*. The present studies indicate that the liver adenine nucleotide content is unchanged by anticoagulant treatment. The activation of amino acids and the transfer of amino acid to s-RNA and then to microsomal protein was unchanged by the anticoagulent treatment.

MATERIALS AND METHODS

Chemicals and enzymes. The adenine nucleotides were obtained from Pabst Laboratories; guanosine triphosphate and phosphoenolpyruvate from Calbiochem; all were in the form of their sodium salts. The phosphorylated compounds were neutralized before use and stored frozen at $-7°$. In the amino acid incorporation studies an equivalent amount of $MgCl_2$ was added to the nucleotides. The nucleotides were standardized by optical density and enzymatic procedures.

Bishydroxycoumarin (Dicumarol), coumarin, and puromycin were obtained from the Nutritional Biochemicals Corp.; phenindione from Walker Laboratories, Inc.; warfarin from Endo Products, Inc.; salicylic acid from Merck & Co.; 2,3-dichloronaphthoquinone and dinitrophenol from Eastman Kodak Co. Ethyl biscoumacetate (Tromexan), acenocoumarol (Sintrom), and cumachlor (Indaliton, *p*-chlorophenylindandione) were furnished by Geigy Pharmaceuticals. Actinomycin-D was generously provided by Dr. Clement A. Stone of Merck, Sharpe & Dohme. The coumarin derivatives were dissolved in a small volume of 0·1 N NaOH and then diluted and neutralized before use. Only freshly prepared solutions of these agents were used, and control samples containing the solvent were included in all experiments.

The L-leucine-1-^{14}C (specific activity 4–8 mc/m-mole) supplied by Calbiochem was dissolved in 0·15 M KCl and stored frozen. Carrier-free ^{32}P-orthophosphate was obtained from Oak Ridge National Laboratories and neutralized before use.

Myokinase, pyruvate kinase, and ribonuclease were obtained from Calbiochem. Adenylic acid deaminase and adenyl pyrophosphatase were prepared as described previously.[5] Inorganic phosphate was determined according to Fiske and SubbaRow.[6]

Administration of drugs and blood-sampling techniques. Drugs were usually administered by the intracardiac route, but in some experiments the intraperitoneal route was used. Blood samples were obtained by cardiac puncture as described by Burhoe[7] and collected in 0·1 volume of 0·1 M oxalate. Prothrombin times were determined by the one stage method[8] with Simplastin (Warner Chilcott) to start the reaction.

Preparation of liver samples for adenine nucleotide analysis. The anticoagulant-treated animals were anesthetized with ether, and blood samples for prothrombin times were taken by heart puncture. While the rat was anesthetized, the liver was exposed by an abdominal incision, after which about 0·5 g of the central lobe was removed and plunged into liquid nitrogen. The time elapsed between the liver excision and the transfer to liquid nitrogen did not exceed 2–3 sec. The frozen sample was then pulverized in a cold room (– 20°), extracted with perchloric acid, and assayed for adenine nucleotides as described previously.[9]

^{32}P-Orthophosphate incorporation into nucleotides was determined by adsorption to Norit, as described by Tsuboi and Price.[10] Specific activities were determined after hydrolysis, as described by Crane and Lipmann.[11]

Preparation of tissue samples and incubation procedures. Albino Wistar strain rats of either sex weighing between 100 and 250 g were obtained from Carworth Farms. As a routine they were fasted overnight, sacrifice by a blow on the head, and exsanguinated. The liver was excised and rinsed twice in the medium which was selected for those studies. All manipulations were carried out as quickly as possible at 0–3° unless otherwise noted.

Liver slice preparation and incubations. In the preparation of liver slices, blood and debris were removed from the tissue by rinsing in cold Krebs Ringer phosphate solution[12] containing 130 mg glucose/100 ml. The washed liver was sliced with a McIlwain tissue chopper (Brinkman Instrument Co.) set at 0·25-mm thickness. The slices were washed in cold Krebs-Ringer phosphate solution, blotted to remove excess fluid, and weighed. The individual slices weighed from 25–100 mg depending on the size of the liver lobe used. Adjacent slices of approximately the same size and weight were used for control and experimental samples. They were incubated in 10-ml Erlenmeyer flasks containing 3 ml Krebs-Ringer phosphate solution at 37° in air with moderate shaking (120/min) on the Dubnoff metabolic shaking incubator. Additions of anticoagulants, ^{14}C-leucine and other agents to the Krebs solution were made as indicated in Results. Incorporation experiments with ^{14}C-leucine were terminated with 0·1 volume of 50% TCA.

Isolation of cell fractions amd incubation procedures. For the preparation of cell fractions, the liver was rinsed twice with cold Medium A[13], and its weight was estimated by volume displacement. The liver was passed through a tissue press and homogenized in 2·3 volumes of cold Medium A in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 15,000 *g*. The supernatant fluid was used as a "crude" system for amino acid incorporation studies. The 15,000 *g* supernatant fluid was further fractionated as described by Keller and Zamecnik[13] to obtain the "pH 5 enzyme fraction" and the microsomal fraction. Microsomal fractions were used on the day of preparation. Ribosomes were prepared according to the method of Korner.[14]

The above cell fractions were used to study the effect of anticoagulants on ^{14}C-leucine incorporation into s-RNA and into protein. The fractions tested were pre-incubated with various agents (dissolved in 0·15 M KCl and neutralized) in a constant volume at 37° in 13 × 100 mm test tubes under 95% N_2–5% CO_2 with moderate shaking (120/min) in a Dubnoff metabolic shaker. The reactions were started by the addition of the following as a mixture: Tris, 30 μmoles at pH 7·5; phosphoenolpyruvate, 10 μmoles; pyruvatekinase, 40 μg; ATP, 1 μmole; guanosine triphosphate, 0·3

μmole; pH 5 fraction, 2 mg; microsomes, 6 mg protein; or ribosomes, 2 mg protein; L-leucine-1-^{14}C, 0·06 μmole (100–200,000 cpm); and 0·15 M KCl added to a final volume of 1·0 ml. Reactions were stopped at desired times with an equal volume of 10% TCA.

Radioactivity determinations. In ^{14}C-leucine incorporation studies, the reactions were stopped with TCA, and the precipitates were washed as described by Siekevitz.[15] The washed precipitates were dissolved in formic acid, and suitable aliquots were plated out for counting. The dried samples (usually 1–6 mg) were counted in a Nuclear-Chicago model D-47 gas flow, thin-window (Micromil) instrument. The specific activities of the proteins are expressed as counts per minute per milligram dry weight; self-absorption corrections were not made. The specific activity of the TCA zero-time sample was usually low, near 1 cpm.

RESULTS

Effects of warfarin on liver adenine nucleotides

The administration of anticoagulants such as warfarin and Dicumarol to rats prolonged the prothrombin time after a lag of serveral hours, during which period the agent was removed from the circulation and accumulated in the liver to exert its effect, and the clotting factors present in the circulation were metabolized. Occurrence of prolongation indicated that the agent was at its site of action and had produced its known effect. Studies were carried out to determine whether there was an associated decrease in the liver adenine nucleotide content prior to or during the peak prolongation of the prothrombin time. For this reason the livers of treated and untreated rats were analyzed for their nucleotide content at various times after the administration of the anticoagulants.

TABLE 1. THE EFFECT OF WARFARIN ON THE ADENINE NUCLEOTIDE CONTENT OF RAT LIVER

(hr)	AMP (μmoles/g)	ADP (μmoles/g)	ATP (μmoles/g)	Total (μmoles/g)	Prothrombin time (sec)
Control (12)	0·94 ±0·08	1·20 ±0·18	2·08 ±0·16	3·95 ±0·13	12·6
6 (9)	1·13 ±0·13	1·39 ±0·27	1·88 ±0·16	4·31 ±0·30	12·8
16 (6)	1·13 ±0·18	1·21 ±0·30	1·50 ±0·25	3·84 ±0·18	41·1
24 (6)	1·17 ±0·19	1·06 ±0·31	2·52 ±0·28	4·75* ±0·41	52·4† (3)
48 (3)	1·28 ±0·28	1·37 ±0·33	1·61 ±0·31	4·26 ±0·19	90·0† (1)

Warfarin (10 mg/kg) was given intraperitoneally. Animals were sacrificed at the indicated times, and their livers analyzed for adenine nucleotides. Values include standard errors.

* P values = 0·03; all other values show no significant difference from controls.

† Average does not include those times greater than 20 min; the number of these is in parenthesis.

A series of rats was given warfarin (10 mg/kg i.p.); the controls received solvent by the same route. The results of a typical experiment relating the liver adenine nucleotide content to the progressively prolonged prothrombin time are summarized in Table 1. At 16 hr the prothrombin time was increased and continued to be prolonged

at 24 and 48 hr. Statistical analysis of the adenine nucleotide data indicated that there was no significant difference between the experimental and control values either before the effect had been produced or when the prothrombin time was prolonged. The single value showing significance in the total nucleotides at 24 hr (Table 1) was not observed in other experiments.

Effect of Dicumarol on the liver adenine nucleotide content

Dicumarol (25 mg/kg) was administered to rats by intracardiac injection; the controls received an equal volume of 0·2% sodium bicarbonate solution (which was about the same pH as Dicumarol) by the same route. After 16 and 24 hr the prothrombin times were prolonged; however, the liver nucleotides were the same as the controls (Table 2, Exp. 1). In other experiments, Dicumarol administered by the intracardiac route in single doses of 5, 10, or 50 mg/kg gave results similar to those obtained at 25 mg/kg (Table 2), insofar as the nucleotide content was no different from controls, and prothrombin times were in the range that might be called therapeutic. Furthermore, analyses of liver nucleotides at various time intervals over a range of 3 – 72 hr after Dicumarol treatment (at each dosage mentioned above) yielded values unchanged from the controls.

TABLE 2. THE EFFECT OF DICUMAROL ON RAT LIVER ADENINE NUCLEOTIDE CONTENT

	AMP (μmoles/g)	ADP (μmoles/g)	ATP (μmoles/g)	Total (μmoles)/g	Prothrombin time (sec)
	Expt. 1. Intracardiac Dicumarol (25 mg/kg)				
16 hr					
Controls (6)	1·52 ± 0·19	2·63 ± 0·27	2·04 ± 0·24	6·19 ± 0·42	14·1
Dicurmarol (6)	1·46 ± 0·08	2·40 ± 0·16	2·11 ± 0·25	5·98 ± 0·23	30·0
24 hr					
Controls (6)	1·64 ± 0·13	2·10 ± 0·32	1·53 ± 0·17	5·28 ± 0·40	14·8
Dicumarol (6)	1·73 ± 0·14	2·27 ± 0·17	1·40 ± 0·13	5·40 ± 0·14	46·0
	Expt. 2. Dietary Dicumarol (10 mg/kg per day) for 5 days				
Controls (12)	1·41 ± 0·58	2·33 ± 0·68	1·41 ± 0·50	5·14 ± 1·02	14·0
Dicumarol (11)	1·75 ± 0·42	2·72 ± 0·28	0·95 ± 0·38	5·34 ± 0·22	57·9*

Anticoagulent was administered as described in the text. The animals were sacrifieced at the indicated times, and their liver nicleotides were determined. The prothrombin times represent the average for each group.

* Average does not include values > 10 min.

Alternatively, Dicumarol (10 mg/kg per day) was fed to rats over a 5-day period in an amount of food known to be consumed daily. The control animals were fed the same diet without Dicumarol. Although Dicumarol caused a prolongation of prothrombin time (Table 2, Exp. 2) the adenine nucletide content of the experimental animals was not considered significantly different ($P = 0·07$) from the untreated animals. Furthermore, experiments in which the rats were kept on the Dicumarol diet for shorter periods of time gave similar results. It is of interest that the total liver

weights expressed as a percentage of body weight were unchanged from controls after the 5-day Dicumarol diet. The mean weight ± standard error for control livers was 2·83 ± 0·09% of the body weight; that for livers of the Dicumarol-fed rats was 2·80 ± 0·11%. Further more, levels of glycogen and inorganic phosphate in livers of treated animals were no different from the control values.

Effect of Dicumarol on the incorporation of ^{32}P into liver nucleotides

In order to evaluate the turnover of liver adenine nucleotide, the incorporation of ^{32}P-orthophosphate into nucleotides was investigated. Neutralized ^{32}P-orthophosphate (10^7 cpm) was given by the intracardiac route to control and Dicumarol-treated rats at 16 hr after receiving the anticoagulant. Five minutes after the ^{32}P-orthophosphate was given, the liver sample was removed and frozen in liquid nitrogen, after which the nucleotides were extracted, adsorbed to charcoal, and assayed for labile phosphate. Approximately 10% of the injected counts were found in liver, i.e. 10^6 cpm—6×10^5 cpm as inorganic ^{32}P-orthosphosphate and 3×10^5 cpm as hydrolyzable phosphate; most of this was adenine nucleotides. The nonhydrolyzable fraction contained about 10 per cent of the counts present in the labile phosphate fraction. There was no difference between the treated and control animals in either the total ^{32}P-orthophosphate incorporated or the specific activity of the hydrolyzable phosphate fraction (Table 3). Although this does not represent a vigorous study of nucleotide turnover, the incorporation of ^{32}P into nucleotides which were adsorbed to charcoal is a reflection of the rate of nucleotide turnover. Thus, on this basis, Dicumarol does not measurably affect the turnover of the liver nucleotides. Although various anticoagulants do uncouple oxidative phosphorylation in studies with isolated mitochondria, the present studies *in vivo* are not consistent with uncoupling as the mechanism of action of these agents.

TABLE 3. EFFECT OF DICUMAROL ON THE INCORPORATION OF ^{32}P-ORTHOPHOSPHATE INTO RAT LIVER NUCLEOTIDES

	Hydrolyzable phosphate adsorbed to Norit		
	Radioactivity		Specific activity
	(cpm)	(cpm/g)	(cpm/μmole P_i)
Control (8)	$2{\cdot}96 \times 10^5$	29,000	5,870
Dicumarol (8)	$3{\cdot}12 \times 10^5$	30,600	6,360

Dicumarol (25 mg/kg) was injected intracardially. After 16 hr, $^{32}P_i$ orthophosphate (10^7 cpm) was given by the same route. Five minutes later, liver samples were removed, plunged into liquid nitrogen, extracted with PCA, and the nucleotides adsorbed on Norit. Average prothrombin times (sec): controls, 13; experimental 37.

Nucleotides in tissue slices

One of the major problems encountered in experiments on the intact animal was the wide variation in the adenine nucleotide content in both the control and treated subjects, which could mask any small differences due to the agent. For this

reason it was decided to test the effects of anticoagulants on rat liver slices *in vitro*, since both the control and experimental slices could be obtained from the same liver and in this way the nucleotide differences attributed to animal variations could be circumvented. The effect of Dicumarol on the adenine nucleotide content of liver slices was studied along with the effect on respiration. However, the ATP as well as total adenine nucleotide content rapidly declined to about 10 per cent of the values found *in vivo*. Although Dicumarol depressed the ATP levels further, this may not be relevent to the situation *in vivo*, and further studies were not pursued. The decrease in ATP content of rat liver slices also has been noted by Kayne *et al.*[16]

The incorporation of ^{14}C-*leucine into liver slice protein*

The coumarin anticoagulants reversibly inhibit the synthesis of at least four blood-clotting proteins. Since the adenine nucleotide content was not affected by anticoagulant treatment, experiments were conducted to test the direct effect of these agents on protein-synthesizing systems.

At the tissue level, rat liver slices were incubated with ^{14}C-leucine in the presence and absence of Dicumarol, after which the liver proteins were isolated, and radioactivity measurements were made as described in Methods. The amino acid was incorporated into protein in a linear fashion throughout the 3-hr incubation (Fig. 1).

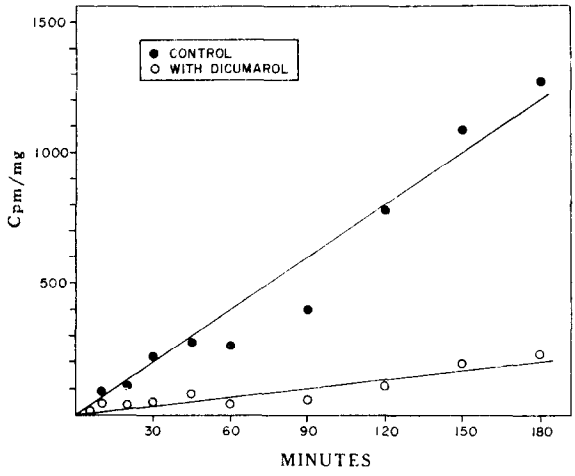

FIG. 1. The effect of Dicumarol on ^{14}C-leucine incorporation into protein of rat liver slice. Rat liver slices (ca. 75 mg) were incubated for various times at 37° in Krebs-Ringer phosphate solution containing ^{14}C-leucine (80,000 cpm) in the absence and presence of Dicumarol (10^{-4} M). The reactions were stopped with TCA, and the precipitates were washed and radioactivity determinations made as described in Methods.

In the presence of 10^{-4} M Dicumarol the incorporation was also linear with time but was inhibited 75 per cent or more. This inhibitory effect was observed as early as 10 min and was well pronounced after 20 min or more of incubation. The inhibition of ^{14}C-leucine incorporation occurred earlier than the depression of respiration.[17]

TABLE 4. EFFECT OF DICUMAROL ON ^{14}C-LEUCINE INCORPORATION INTO PROTEIN OF RAT LIVER SLICE

Incubation	Total radioactivity (cpm)	Specific activity (cpm/mg)	Incorporation (%)
Control	3665	329	100
Dicumarol 10^{-7} M	3390	339	103
Dicumarol 10^{-6} M	2530	180	55
Dicumarol 10^{-5} M	1950	115	35
Dicumarol 10^{-4} M	480	50	15
DNP 10^{-6} M	3390	176	54
DNP 10^{-4} M	340	20	6
95% N_2–5% CO_2	40	3	1
Zero time	5		0

Rat liver slices (ca. 70 mg) were incubated as described for 90 min with ^{14}C-leucine (80,000 cpm) and the addition noted. The reactions were stopped with TCA, and the precipitates were washed and analyzed as described in Methods.

TABLE 5. EFFECT OF ANTICOAGULANTS AND OTHER AGENTS ON ^{14}C-LEUCINE INCORPORATION INTO PROTEIN OF RAT LIVER SLICE

Addition	Concentration (M)	Radioactivity (cpm)	Specific activity (cpm/mg)	Incorporation (%)
Experiment 1				
None		1192	115	100
Dicumarol	5×10^{-5}	389	38	33
Warfarin	10^{-3}	188	17	15
Tromexan	7×10^{-4}	291	17	15
Phenindione	7×10^{-4}	216	18	16
Cumachlor	7×10^{-4}	152	17	15
Experiment 2				
None		2047	144	100
Coumarin	10^{-3}	638	40	28
Salicylate	7×10^{-4}	1661	132	92
Actinomycin	(200 μg)	1007	92	64
Puromycin	5×10^{-4}	44	4	3
2,3-Dichloronaphthoquinone	7×10^{-4}	130	13	9
Sintrom	7×10^{-4}	72	6	4
Zero time		5	0·5	0

Rat liver slices (ca. 100 mg) were incubated as described for 2 hr with ^{14}C-leucine (125,000 cpm) and the additions noted. Reactions were stopped with TCA, precipitates washed, and radioactivity measurements made as described in Methods. Each value represents the average of duplicate experiments.

The effect of concentration of Dicumarol on ^{14}C-leucine incorporation is summarized in Table 4. At 10^{-7} M no inhibition was noted, while at 10^{-6} M there was about 45 per cent inhibition. Dinitrophenol, an uncoupler of oxidative phosphorylation, was similarly inhibitory. Anaerobiosis abolished the incorporation of leucine into protein, which would indicate that glycolysis could not support the synthesis.

Various anticoagulants including bishydroxycoumarin (Dicumarol), warfarin, ethyl biscoumacetate, cumachlor, acenocoumarol, and phenindione also were found to inhibit ^{14}C-leucine incorporation into liver slice proteins (Table 5). These agents were used in a concentration range which affected the respiration of rat liver slices and oxidative phosphorylation.[17] Coumarin, which has no anticoagulant activity, was included in this group to determine whether the absence of the 4 hydroxyl substituent would effect the leucine incorporation. The inhibition noted with coumarin would indicate that the structural requirement of the 4 hydroxyl substituent for anticoagulant activity is not necessary in this system. Salicylate, which in high concentration is an uncoupler of oxidative phosphorylation and a weak anticoagulant, showed little or not effect. The inhibition obtained with 2,3-dichloronaphthoquinone is of interest in that this compound was reported as a vitamin-K antagonist.[18]

The incorporation of ^{14}C-leucine into protein of rat liver slices prepared from Dicumarol-treated rats

Since Dicumarol readily inhibited ^{14}C-leucine incorporation into liver slice protein, it was of interest to determine whether slices obtained from anticoagulant-treated rats had an altered rate of incorporation. In a group of six rats (litter mates, each 120 g), three were given Dicumarol (10 mg/kg) by the intracardiac route, and the remaining three were given the solvent by the same route. After 18 hr the prothrombin times were prolonged (av. 37 sec), and liver slices were prepared from each animal as described in Methods. The incorporation of ^{14}C-leucine into protein was measured in duplicate incubations. Statistical analysis of the data showed that ^{14}C-leucine incorporation in the slices obtained from treated animals (502 ± 33 cpm/mg) was not different from the controls (542 ± 24 cpm/mg). Thus it appears that pretreatment of the rat with the anticoagulent does not measurably affect the incorporation of ^{14}C-leucine in the *in vitro* system. The possibility that Dicumarol was leached out of the tissue during preparation and incubation of the slices cannot be excluded.

The incorporation of ^{14}C-leucine into protein by the mitochondrial supernatant fraction

In an attempt to determine a more specific site of action of the anticoagulants, experiments were conducted in a cell-free protein-synthesizing system. The 10,000-*g* supernatant fluid of rat liver homogenate was used as a crude incorporating system. Incorporation was relatively rapid within the first 3 min and then decreased markedly, which is consistent with the work of others. In none of these experiments was a consistent effect of Dicumarol found. Concentrations of Dicumarol from 10^{-7} M to 10^{-4} M were investigated, and no distinct effect of the agent was evident. The difference between experimental and control preparations is not considered to be significant.

Similar studies were carried out on 10,000-*g* supernatant fluid prepared from livers of two vitamin K-deficient chicks. The incorporation of ^{14}C-leucine into protein by the fraction obtained from the deficient chicks was the same as that found in normal chicks (145 vs. 142 cpm/mg respectively). These values represent averages of the specific

activities obtained for duplicate 30 min incubations. Thus, using a different approach to study the synthesis of the clotting factor, we found no measurable differences in the synthetic capacity between normal livers and livers of animals which were hypoprothrombinemic either from anticoagulant treatment or vitamin K deficiency.

The incorporation of ^{14}C-*leucine into s*-RNA

The incubation of the pH 5 fraction supplemented with an ATP-generating system, cofactors, and ^{14}C-leucine resulted in the incorporation of the labeled amino acid into s-RNA (Table 6). Dicumarol ($10^{-5} - 2 \times 10^{-4}$ M) had no effect on the incorporation of ^{14}C-leucine into s-RNA. In other experiments, warfarin was similarly without effect on this system. It is inferred that Dicumarol and warfarin had no effect on the activation of ^{14}C-leucine, since activation of the amino acid precedes its transfer to s-RNA. Studies concerned with the time course of incorporation of label into s-RNA showed that near maximal incorporation occurred in the first few minutes. Furthermore, it was found that 10^{-4} M Dicumarol did not alter the time course nor the maximal level of incorporation attained when followed over a range of 3–60 min of incubation.

TABLE 6. THE EFFECT OF DICUMAROL ON THE INCORPORATION OF ^{14}C-LEUCINE INTO RAT LIVER S-RNA

	Radioactivity (cpm)	Specific activity (cpm/mg)	Incorporation (%)
Control	242	105	100
Dicumarol (1×10^{-5} M)	229	92	87
Dicumarol (5×10^{-5} M)	220	85	81
Dicumarol (1×10^{-4} M)	266	100	95
Dicumarol (2×10^{-4} M)	203	88	84
−ATP-generating system	26	11	11
Control after TCA 90°, 15 min.	28	12	11

The pH 5 fraction containing s-RNA was preincubated with Dicumarol for 10 min 37° under 95% N_2–5%CO_2. Reactions were started with ATP-generating system, cofactors, and ^{14}C-leucine (80,000 cpm). After 10 min, reactions were stopped with TCA; precipitates were washed, and radioactivity was measured as described in Methods.

The incorporation of ^{14}C-*leucine into protein by the microsomal fraction*

The incorporation studies of ^{14}C-leucine incorporation into protein by the rat liver microsomal fraction are summarized in Table 7. The incorporation was maximal within the 10-min incubation period. The presence of Dicumarol (or warfarin) at 10^{-4} M and lower had no effect on this incorporation. In other experiments, it was noted that Dicumarol had no effect on the incorporation after other periods of incubation ranging from 3 to 60 min. The microsomal system functioned as well when incubated in air as it did under anaerobic conditions, and Dicumarol had no effect in either case. The labeled product was stable to hydrolysis in 5% TCA at 90° for 15 min, indicating that the labeled material did not include appreciable amounts of

s-RNA. In some experiments ribosomes were used in place of the microsomes; the results of these studies were similar to those obtained with the microsomal fraction.

In order to investigate the possibility that Dicumarol might exert some effect *in vivo* that cannot be observed *in vitro*, the pH 5 fraction and microsomes were obtained from Dicumarol-treated (18 hr) and control rats. The incorporation of ^{14}C-leucine

TABLE 7. EFFECT OF DICUMAROL ON ^{14}C-LEUCINE INCORPORATION INTO RAT LIVER MICROSOMES

Additions	Radioactivity (cpm)	Specific activity (cpm/mg)	Incorporation (%)
None	474	149	100
Dicumarol			
(5×10^{-5} M)	488	157	105
(1×10^{-4} M)	481	146	98
RNase (2 μg)	26	7	5
Zero time	9	3	0

Microsomes, pH 5 fraction, and Dicumarol were preincubated for 10 min at 37° under 95% N_2–5% CO_2 in the Dubnoff metabolic shaker. Reactions were started by the addition of the ATP-generating system and ^{14}C-leucine (80,000 cpm). After 10 min, reactions were stopped with TCA, precipitates were washed, and radioactivity was measured as described in Methods.

into s-RNA and protein was the same in both cases. In experiments in which the pH 5 fraction was obtained from the Dicumarol-treated animal and the microsomes from the untreated animal (or vice versa), the incorporation did not appear to be altered.

DISCUSSION

Although the oral anticoagulants in different chemical classes uncouple oxidative phosphorylation *in vitro*, there is no information in the literature which clearly shows that the uncoupling effects observed *in vitro* can account for their anticoagulant action *in vivo*. The present studies were initiated to determined whether the coumarin anticoagulants produced an alteration in adenine nucleotide content of treated animals, as might be expected if uncoupling had taken place as a result of the treatment. From the observations made in the present studies, the ATP content of anticoagulant-treated rats was no different from that of control animals. This was found to be the case at the peak effect on prothrombin time, as well as before the peak effect. The latter point is of special significance, since the anticoagulants do not inhibit the endogenous factors that are in the circulation in excess and must be metabolized; therefore, an effect on the nucleotide content would most likely precede the effect on prothrombin time. Perhaps of most significance are the dietary experiments, which were carried out to keep the blood level of anticoagulants elevated and also to prolong the prothrombin times to such an extent that they would be in the hazardous range. Even under these conditions the ATP levels of treated animals were no different from control animals. It might be pointed out that in individual animals in which the prothrombin time was greater than 10 min, the adenine content appeared to be in the normal range.

Thus, observations made in the present studies do not support the view that uncoupling of oxidative phosphorylation is responsible for the anticoagulant action of Dicumarol and warfarin.

In view of this lack of support, other possible sites of action were considered. Pool and Robinson[19] reported the synthesis of factor VII (and its inhibition by warfarin) in liver slices, which indicated the feasibility of the slice approach. The present studies extended these observations to various anticoagulants which included members of classes of chemicals other than the coumarins—e.g. indandione and salicylates. Factor VII production was not measured, but ^{14}C-leucine incorporation into liver protein was measured. The clotting factors, in general, have a rapid turnover compared to liver proteins, which indicates the feasibility of the short-term slice studies. For example, factor VII was reported to have a relatively rapid turnover ($t_{\frac{1}{2}}$ of about 2·5 hr) while the proteins of various cell fractions of liver ranged from 4 to 8 days. The finding that liver slices prepared from anticoagulant-treated animals incorporated ^{14}C-leucine at a rate comparable to the control slices does not support the findings obtained when the anticoagulant was added *in vitro*. The possibility that some of the anticoagulant present in the tissue was leached out during the preparation and incubation of the slices to subinhibitory levels must be considered.

The finding that ^{14}C-leucine incorporation into liver slice protein was depressed by the anticoagulants prompted a number of studies to determined their possible effects in amino acid activation and transfer to s-RNA and ribosomes. Thus far these studies have given negative results. It seems likely that if these agents inhibited amino acid activation or transfer to s-RNA, they would be more toxic than clinical results reveal. Although no measurable effect was observed on the transfer to the ribosomes, this site cannot be excluded as a possibility. At present the ribosomal fraction undoubtedly contains a mixture of messengers, and it is not known what fraction of these is involved in the synthesis of the clotting factors. It is possible that an effect was masked by other activities going on concomitantly.

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