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THE EFFECT OF ANTICOAGULANTS ON THE HIGH-ENERGY PHOSPHATE CONTENT OF RAT AURICLES*

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Abstract—Various oral anticoagulants and nitrophenols were found to depress the amplitude of contraction of isolated rat auricles. Associated with the depression of contractility, there was a depression of the creatine phosphate content of the auricles. In the case of Warfarin and 2,4-dinitrophenol, the depression of contraction was reversed by washing out the drug. After the contraction amplitude was restored, the creatine phosphate content was also restored to the normal range.

IN ADDITION to their well-known effects on the synthesis of factors involved in blood clotting, various reports have shown that the oral anticoagulants also display a variety of other effects. For example, on the tissue level they depress the respiration of liver slices,[1] while on the subcellular level they uncouple oxidative phosphorylation,[2] and inhibit vitamin K_1 reductase.[3] On the heart, Dicumarol was reported to increase coronary flow,[4] and in another study it was reported to depress the contractility of papillary muscle.[5] Uricosuric effects are also produced,[6, 7] indicating an effect on the kidney. Finally, among microorganisms, some of the anticoagulants were reported to exhibit antibacterial action.[8]

In view of this array of effects, it seemed possible that a common mechanism might be responsible for the effects produced in different tissues, especially since such processes as respiration and oxidative phosphorylation are affected by the anticoagulants. The present study was directed toward an analysis of the effects of the oral anticoagulants on the high-energy phosphate content of tissue since effects of these on metabolism would be reflected in the amount of high-energy phosphates present. Such effects might be the common basis for the aforementioned diversity of actions. In view of the previously cited experiments on the heart, cardiac tissue was employed to test this idea. Rat auricles were selected because they could be rapidly frozen when effects were noted on contractility, and then thawed and assayed for high-energy phosphates. In the present studies it was found that the amplitude of contraction was depressed by the anticoagulants, and this was accompanied by a decrease in the creatine phosphate content; however, the adenine nucleotide content was not affected.

METHODS

Male Wistar strain rats, weighing approximately 300 g, were decapitated. The heart was rapidly removed and transferred to Krebs bicarbonate solution[9] containing

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0·01 M glucose through which was bubbled 95% O_2 and 5% CO_2. Essentially, the procedure described by Furchgott and deGubareff[10] was followed for the isolation of the auricles, but in the present studies spontaneously beating, double-auricle preparations were employed. The tip of one auricle was secured to a muscle holder with a thread, and a second thread was tied to the tip of the other auricle to enable subsequent attachment to a muscle lever. The pair of auricles was then mounted in a 20-ml chamber filled with Krebs solution through which the O_2–CO_2 gas mixture was slowly passed. A water bath and thermoregulator maintained the chamber at 26°. Contractions were recorded on a kymograph.

At the end of a 20- to 30-min equilibration period, the control solutions were replaced with media containing the drug to be tested. The relative insolubility of many of the agents tested precluded additions of aliquots of concentrated samples.

Control preparations were set up in the same fashion and allowed to contract spontaneously for equivalent periods of time in the absence of drug.

Ethyl biscoumacetate (Tromexan), acenocoumarol (Sintrom), and cumachlor (Indaliton, *p*-chlorophenylindandione) were furnished by Geigy Pharmaceuticals. Dicumarol was obtained from the Nutritional Biochemicals Corp.; Phenindione from Walker Laboratories, Inc.; Warfarin from Endo Products, Inc.; and the nitrophenols from the Eastman Kodak Company.

Analysis of inorganic phosphate and phosphocreatine

At the end of the experimental period, the control and the drug-treated auricles were frozen in liquid nitrogen, then pulverized and extracted with perchloric acid in a −20° room as described by Feinstein.[11] The extract was subsequently analyzed for inorganic phosphate (IP) and creatine phosphate (CP) according to the method of Furchgott and deGubareff[12] with the exception that the final volume was 1·0 ml. The analysis for CP was performed within 1 hr of the initial freezing, since samples stored overnight at −20° were found to have significantly less CP (ca. 0·5 μmole/g). The CP levels reported in this study are about 50 per cent of the amount reported in the left atrium of the guinea pig by Furchgott and deGubareff.[10] However, this difference may be due to variation between species.

Analysis of adenine nucleotides

The enzymatic, spectrophotometric method of Kalckar[13] was used in this study for the determination of adenine nucleotides. Adenylic acid deaminase (preparation A) and adenyl pyrophosphatase were prepared as described by Kalckar.[14] Myokinase was obtained from Boehringer & Soehne. The nucleotide content was calculated as described by Feinstein.[11] All values are expressed as micromoles per gram of auricle.

RESULTS

Anticoagulants (Dicumarol, Warfarin, Tromexan, Cumachlor, Sintrom, Phenindione)

Pharmacological effects. All the anticoagulants tested produced varying degrees of depression in amplitude as well as in rate of contraction in the rat double-auricle preparation. Dicumarol produced a negative inotropic effect in a concentration range that uncouples oxidative phosphorylation[2] and depresses the respiration of rat liver slices.[1] Figure 1 illustrates a typical time course of depression obtained when Dicumarol

was used and Fig. 2 illustrates the effect with Warfarin. In the present study the experiments were arbitrarily terminated when a 60 to 80 per cent depression of contractility occurred. The other anticoagulants produced similar depression, but there was some difference with respect to time of onset (Table 1).

TABLE 1. EFFECT OF ANTICOAGULANTS ON CONTRACTILITY AND CREATINE PHOSPHATE CONTENT OF RAT AURICLES*

	Number	CP	P	Amplitude	Average time to depression
		(μmole/g)		(% of control)	(min)
Control		1·89 ± 0·19			
Dicumarol	6	1·17 ± 0·18	0·01–0·02	36	6
Control		2·83 ± 0·23			
Warfarin	6	0·83 ± 0·02	0·001	37	19
Control		1·97 ± 0·11			
Tromexan	6	1·47 ± 0·17	0·02–0·05	35	22
Control		1·97 ± 0·13			
Cumachlor	5	0·74 ± 0·18	0·001	23	18
Control		2·12 ± 0·21			
Sintrom	7	1·54 ± 0·66	0·05	29	25
Control		1·71 ± 0·10			
Phenindione	6	0·90 ± 0·26	0·01–0·02	22	15

* Double-auricle preparations were incubated without and with anticoagulant at a concentration of 5×10^{-5} M except for Phenindione and Warfarin which were 5×10^{-4} M. Control and experimental samples were analyzed for high-energy phosphates as described in the text.

Effect on creatine phosphate and inorganic phosphate. Table 1 reveals that all the anticoagulants tested produced a significant decrease in the CP content of the rat auricles at this degree of depression of contractility. However, the inorganic phosphate content was not significantly altered.

Effect on adenine nucleotides. None of the anticoagulants tested produced significant changes in the adenine nucleotide content of the rat auricles at the time of depression. Inasmuch as little is known about the tissue content of these biochemical entities, the control values (μmole/g tissue) and standard error of the means for 35 double-auricle preparations are presented: AMP 0·21 ± 0·02; ADP 0·15 ± 0·03; ATP 1·48 ± 0·07; total adenine nucleotides 1·87 ± 0·08; IP 3·60 ± 0·13; CP 2·08 ± 0·09.

Effect of duration of action. In general the anticoagulants produced arrythmias, progressive declines, or complete cardiac standstill if the action was allowed to proceed beyond the arbitrarily chosen 60 to 80 per cent depression as the terminal point of this study. However, Warfarin more consistently produced relatively longer, steady-state levels of depression. In three experiments the CP levels at 40 min (0·87 ± 0·04) were significantly less than those of controls (2·08 ± 0·09) but no different from those observed at 20 min (0·83 ± 0·02), the degree of depression of amplitude being the same at both times.

Reversibility of anticoagulant effect. After induction of the typical depressant action of Warfarin, the auricles were washed free from drug. Recovery of contractility occurred slowly but nearly completely (Fig. 2). Along with this restoration of contractility there was an increase in the CP content toward control levels. In the preparation illustrated in Fig. 2 the recovered aricles contained 2·42 μmoles of CP/g while the accompanying depressed auricle contained 0·73 μmole/g. The depression has been reproduced on the same auricle three times, which further illustrates the reversible nature of this effect.

Other reversibility studies were performed with Dicumarol. It appeared that removal of drug after cardiac depression, followed by washing with Krebs solution, did not consistently result in significant alterations in contractility or CP levels. This finding was not unexpected in view of the previously noted binding of Dicumarol to tissue components.[1]

The differences in ease of reversibility noted between reversal with Warfarin and Dicumarol may be explained in terms of differential binding affinities. However, further studies would be necessary to confirm this explanation.

A few attempts were made to reverse the depression with vitamin K_1, but these were unsuccessful. This is not unexpected in an experiment of this sort in view of the insolubility of vitamin K_1 in aqueous media, which would preclude its uniform distribution in the solution.

TABLE 2. EFFECT OF NITROPHENOL ANALOGUES ON CONTRACTILITY AND ON CREATINE PHOSPHATE CONTENT OF RAT AURICLES*

Agent	Number	CP	P	Amplitude	Time to depression
		(μmole/g)		(% of control)	(min)
None	35	2·08 ± 0·09			
2,4-Dinitrophenol	4	0·23 ± 0·05	<0·001	27	3·2
o-Nitrophenol	3	2·36 ± 0·34	0·4	89	30
p-Nitrophenol	6	0·92 ± 0·15	<0·001	25	17
2,4-Dinitroaniline	4	2·30 ± 0·10	0·4–0·5	23	3·1
2-Amino-4-nitrophenol	5	0·74 ± 0·11	<0·001	39	30
2,6-Dichloro-4-nitrophenol	4	1·29 ± 0·13	<0·001	38	2·8

* Auricles were incubated without and with the indicated agents at a concentration of 5×10^{-5} M, except for 2-amino-4-nitrophenol which was 5×10^{-4} M, until the indicated depression was attained. The auricles were then analyzed for high-energy phosphates.

Nitrophenols

Inasmuch as several nitrophenols have been shown to depress the respiration of liver slices and uncouple oxidative phosphorylation, it appeared of interest to study whether these properties, which they shared with anticoagulants, would also extend to the parameters investigated in the present study.

Figure 3 illustrates the depressant effect with 2,4-dinitrophenol. Table 2 reveals that most of the nitrophenols under investigation depressed the force of contraction of the auricles, except for *o*-nitrophenol. Since negligible depression was obtained with this agent, the experiments were terminated after 30 min of exposure. Although CP levels

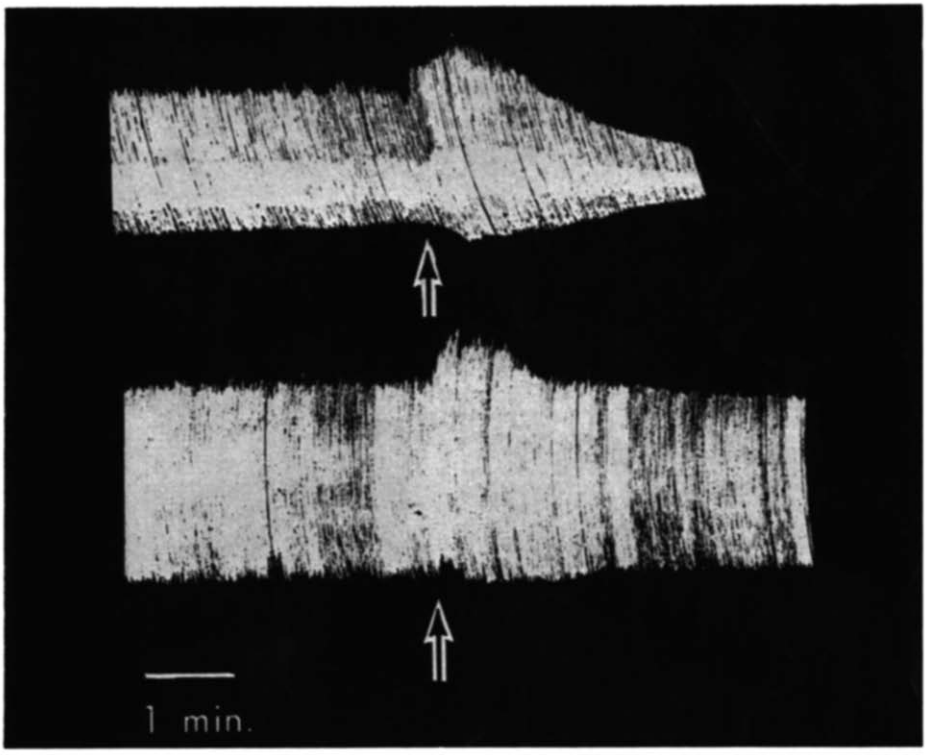

FIG. 1. Effect of Dicumarol (5×10^{-5} M) on rat auricles. After equilibration, Dicumarol was introduced into the experimental (upper) and fresh media into the control (lower) at the times indicated by the arrows. After termination of the experiment the auricles were analyzed for high-energy phosphates as described in the text.

facing page 806

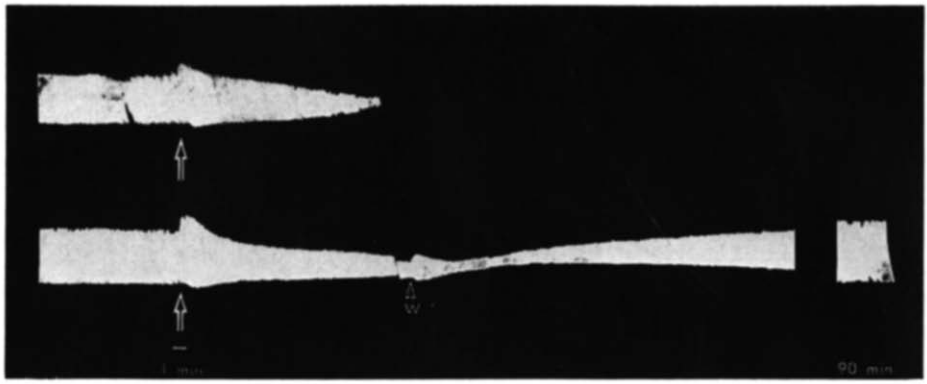

FIG 2. Reversal of Warfarin (5×10^{-4} M) depression. Warfarin was added to the muscle chambers at the time indicated by the first arrow and washed out at W. After termination of the experiment the auricles were analyzed for high-energy phosphates.

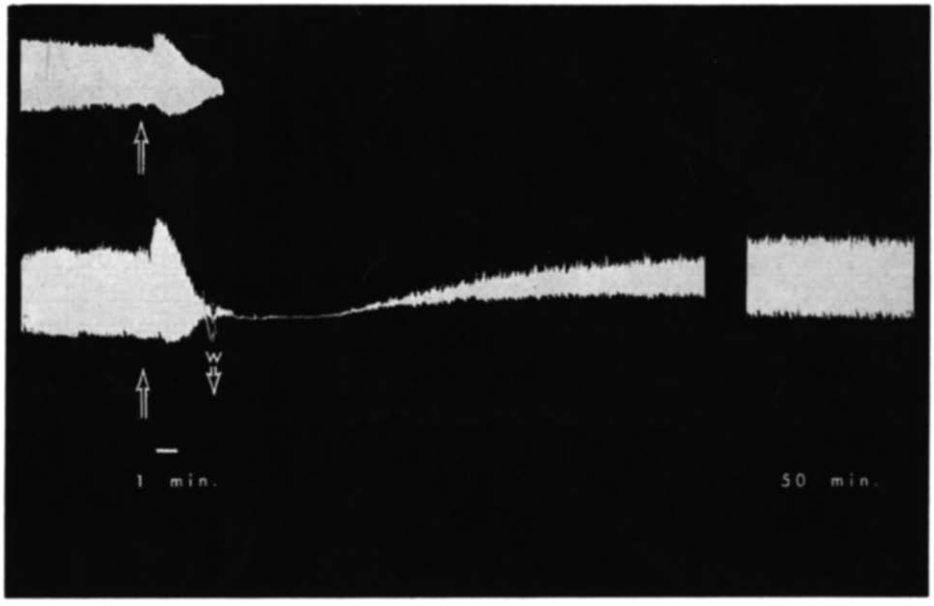

FIG. 3. Effect of 2,4-dinitrophenol (5×10^{-5} M) on rat auricles. Dinitrophenol was introduced into the muscle chambers at the time indicated by the first arrows, and washed out at W.

were decreased in most instances, there were two exceptions among the six agents studied in which the CP content was not lowered concomitant with a depression of cardiac activity. The adenine nucleotide content was not altered by any of these agents, except in the case of 2,4-dinitrophenol. In this case, there was a slight decrease in ATP (0·95 ± 0·11) and a slight increase in AMP (0·45 ± 0·19).

The reversible nature of the effect of 2,4-dinitrophenol is illustrated in Fig. 3. After removal of the drug, and allowing the auricles to incubate in Krebs solution, a return to normal contraction took place in 35 to 70 min. The CP content in the restored auricles was within control range or slightly above (3·0 μmole/g), whereas in the companion auricle preparation in which depression was not reversed by washing out, the CP level was 1·21 μmole/g. In a series of four reversal studies of this type, the CP and adenine nucleotide content was no different from that of control auricles.

DISCUSSION

It has been demonstrated that various anticoagulants lowered the CP content of rat auricles when the amplitude of contraction was depressed 60 to 80 per cent. A degree of specificity appears to be inherent, inasmuch as cardiac depression by ryanodine or acetylcholine was not accompanied by changes in the CP content.[10] Furthermore, the depressant effects produced by Warfarin and 2,4-dinitrophenol are reversed by washing out the agent. On the other hand, the effect produced by Dicumarol was not readily reversible, which may be due to tight binding of the drug by tissue proteins.

In general the agents used did not affect the adenine nucleotide content. A possible explanation for this finding is that the equilibrium constant for the ATP–creatine transphosphorylase reaction is toward ATP.[15] As long as this reaction is not rate limiting, any ADP produced as a result of uncoupling could be phosphorylated by CP. Consequently it is possible that, although uncoupling occurred, the effect would be manifest in a decreased CP content rather than in a decreased ATP content. It was also noted that the inorganic phosphate content did not increase as the CP level decreased. The additional phosphate may have diffused into the media or it may have been utilized by glycogen phosphorylase, thus forming various intermediates of carbohydrate metabolism, some of which serve as phosphate donors to ADP. This would also tend to keep the ATP levels elevated. It is also possible that a significant amount of the ATP is present in an intracellular compartment which is not readily available for the contractile process. The experimental approach employed provides only a measure of the amounts of high-energy phosphates present at the termination of the experiment and does not reveal the source or the rate of turnover of these substances.

It is tempting to ascribe a common role for the various effects that have been attributed to these anticoagulants. However, before this can be envisaged it will be necessary to determine whether the synthesis of the clotting factors, the increased coronary flow, the uricosuric effects, and the antibacterial action also are accompanied by changes in the high-energy phosphate content of the respective tissues. Experiments are in progress in this laboratory to determine whether inhibition of the synthesis of the clotting factors is related to any alterations in the high-energy phosphate content of liver.

Any unitarian hypothesis is subject to pitfalls, and this is evident from the findings relative to nitrophenols. In general the nitrophenols uncouple oxidative phosphorylation and also depress the respiration of rat liver slices. Thus, the present findings

that certain of the nitrophenols produced lowering of CP levels which paralleled their depressant effects on cardiac muscle is compatible with the results obtained with anticoagulants. However, 2,4-dinitroaniline and *o*-nitrophenol did not alter the CP levels with their depressant action on the heart. We may perhaps explain the dinitroaniline exception in light of the finding that this substance apparently does not uncouple oxidative phosphorylation,[16] hence another process may be responsible for this negative inotropic action.

In the case of *o*-nitrophenol we are confronted with an agent that uncouples oxidative phosphorylation in isolated mitochondria, yet it lacks an effect on respiration of intact liver slices. It would thus appear that problems of cell membrane permeability may be associated with this substance.

It is evident that any future studies of the present problem which attempts application to the situation *in vivo* will have to take into consideration the classical problems of specificity, protein binding, tissue concentration, and permeability as well as relative functional significance of the mitochondria within a particular tissue.

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