Effect of Selected Antibiotics on Prothrombin Time of Rats and the Relationship to Coumarin Anticoagulants[1] (34346)

JAMES B. POLSON AND WALTER D. WOSILAIT

Department of Pharmacology, University of Missouri, School of Medicine, Columbia, Missouri 65201

Although the coumarin anticoagulants reportedly block the biosynthesis of clotting factors in the liver, the mechanism of action of these drugs is not yet known. In recent years the steps involved in protein biosynthesis have been elucidated; however, it is not yet known whether inhibition of protein synthesis at any of the steps results in a decrease in clotting factor synthesis comparable to the effects produced by the coumarin anticoagulants. Various antibiotics are known which inhibit protein synthesis. For example, Nogalamycin (1) and actinomycin D (2) inhibit at the level of the DNA-dependent synthesis of RNA; and puromycin (3) and cycloheximide (4) inhibit at the level of the ribosomes. In the present studies, an analysis of the effects of these antibiotics was carried out for purposes of comparison with one of the coumarin anticoagulants (warfarin). Preliminary reports have been presented elsewhere (5–7). The purpose of these studies was to determine which of the antibiotics, if any, would simulate the action of the coumarin anticoagulants, thus providing information which might clarify the mechanism of action of the anticoagulants.

Materials and Methods. Drugs and animals. Puromycin and cycloheximide were obtained from Nutritional Biochemicals Corp. Actinomycin D was generously provided by Merck, Sharp and Dohme and Nogalamycin by the Upjohn Company. Warfarin was obtained from Endo Laboratories, Inc. Vitamin K_1 (Aquamephyton) was obtained from Merck, Sharp and Dohme. The drugs were dissolved in 0.9% NaCl unless specified otherwise.

Female rats of the Sprague-Dawley strain, weighing 250–375 g, were used for most of the studies. The hamsters were of the golden Syrian strain.

Administration of drugs and blood sampling technique. Drugs were usually administered by the intraperitoneal route. Animals in the control groups received injections of the solvent in which the experimental drug was dissolved and the injections were administered in the same volume and on the same schedule as for the experimental animals. Oxalated blood samples were obtained by heart puncture under light ether anesthesia (8), centrifuged, and stored at about 0° until assayed.

The prothrombin time. Usually the prothrombin time was measured within 2 hr after collection; however, some samples were frozen and stored at —18° until assayed. Separate studies showed there was no change in the prothrombin time when the plasma was stored in the frozen state (—18°).

The prothrombin time was measured on 0.1 ml of 12.5% plasma by the one-stage procedure described by Campbell *et al.* (9). Simplastin, (0.2 ml) (Warner-Chilcott) was used to start the reactions. Using this procedure, the prothrombin time for untreated rats ranged from 23 to 25 sec.

Factor VII assay. Factor VII content of 0.01 ml of plasma was measured by two different procedures. The first was described by Owren and Aas (10). The second was an adaptation of the procedure of Gaston and Spivack (11) to rats. Rats were injected with

[1] This research was supported in part by Grant AM-10425 from the U. S. Public Health Service. It represents part of the work carried out by J. B. P. in fulfilling the requirements for the Ph.D. degree at the Department of Pharmacology, University of Missouri, School of Medicine, Columbia, Missouri.

warfarin (1 mg/kg). After 18 hr, the plasma was obtained and used for assay purposes. The main effect on the clotting factors at this time is a marked depression of factor VII while the others are affected slightly (12). In both procedures the amount of factor VII was estimated from a standard curve made with various dilutions of normal plasma added to the deficient plasma. One unit of factor VII was defined as the amount present in 0.01 ml of normal plasma.

Statistical analysis. Preliminary studies showed the prothrombin time data obtained for animals treated with the agents used were not necessarily normally distributed. Therefore statistical analyses were carried out using the Wilcoxon Rank Sum Test (13) which is a nonparametric test that may be applied to data of that type.

Results. Prolongation of the prothrombin time. Nogalamycin and actinomycin D were selected for study because of their inhibitory effects on RNA biosynthesis, first to determine whether these agents would prolong the prothrombin time *in vivo*, and to what extent they would simulate the action of the coumarin anticoagulants. These results of representative experiments using Nogalamycin are shown in Fig. 1 and actinomycin D are shown in Table I. Both drugs produced a prolongation of the prothrombin time, however, a lag period of several hours elapsed before measurable effects were observed. The amount required to prolong the prothrombin time measurably was lethal and the animals died within 2 or 3 days. Lower doses were used in an attempt to produce effects with sublethal amounts, however, these did not prolong the prothrombin time.

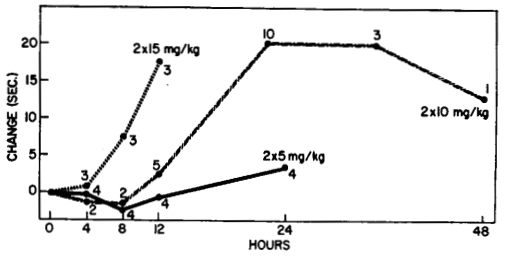

FIG. 1. The effect of nogalamycin on the prothrombin time of rats. Nogalamycin was administered at 0 and 3 hr at the indicated dose. Average values are presented along with the sample size at each point. No significant increase was observed for the control group (4).

TABLE I. The effect of Actinomycin D (1 mg/kg, ip) on the Prothrombin Time of Rats.[a]

Time (hours)	n	Prothrombin time (sec)	p value
0	9	23.9 ± 0.5	NS[b]
12	9	26.4 ± 2.1	NS
24	8	38.8 ± 3.3	0.01
36	5	53.1 ± 15.5	0.02

[a] The changes are presented as mean ± the standard error and the p value is the probability of no difference from the control group (3) receiving 0.9% NaCl.

[b] NS = not significant at the 0.05 probability level.

In order to take into consideration the possibility that the prolongations observed were due to an interaction of the drugs with the clotting factors in the circulation, amounts of the drugs calculated to be present *in vivo* were added to plasma samples *in vitro*. However, no prolongation resulted, indicating the results *in vivo* were not due to the effects of the drugs on the clotting factors in the circulation.

In view of the fact that puromycin and cycloheximide inhibit protein biosynthesis at the level of the ribosome, their effects on the prothrombin time were also studied (Fig. 2). The effect was reversible and most animals returned to normal by 48 hr. Furthermore, the dose used was nonlethal and the animals were alive 2 weeks later. Several different dose levels and schedules were tried, however the most optimal regimen was six hourly injections of 25 mg/kg.

The effects produced by cycloheximide (1 mg/kg) were similar to those produced by puromycin. Larger amounts of cycloheximide in divided doses increased the prothrombin time measured; however, the higher dose levels were not pursued further due to toxicity. Studies were carried out to determine whether the effects of puromycin and cycloheximide could be reversed by vitamin K_1, however, thus far a reversal by the vitamin has not been observed.

Puromycin and cycloheximide did not pro-

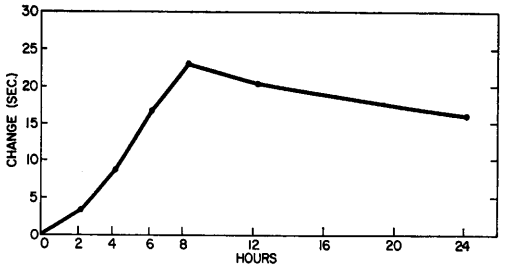

FIG. 2. The effect of puromycin on the prothrombin time of rats. Puromycin was administered in 6 hourly injections of 25 mg/kg each. The time of the first injection was designeated zero time. Average values ($n = 6$ to 8) are presented. $p \leq 0.02$ for 4, 6, 8, 12, and 24 hr.

duce prolongations when separately mixed with plasma *in vitro*.

Experiments with puromycin were carried out using hamsters in order to determine whether prolongations could also be observed in other species besides rats. The effects were similar to those observed in rats and comparable dose levels were effective.

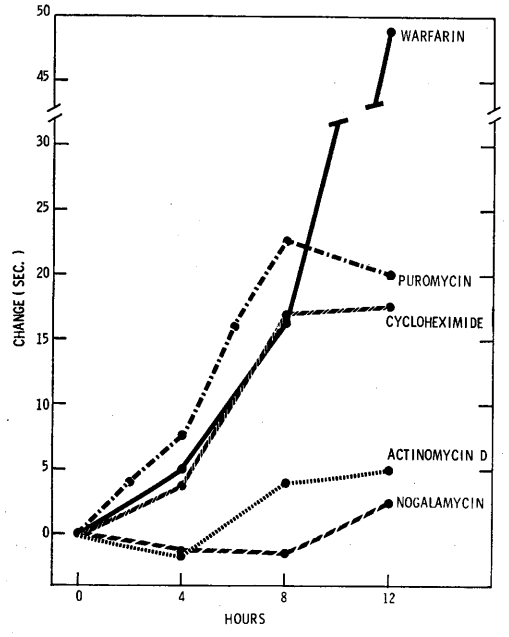

FIG. 3. A comparison of the onset of action of various antibiotics with warfarin. The following dosage regimens were employed: warfarin (1 mg/kg, ip); actinomycin (1 mg/kg, iv); Nogalamycin (2 × 10 mg/kg, ip); puromycin (6 × 25 mg/kg, ip); cycloheximide (1 mg/kg, ip). Each point represents the mean of 2–8 values.

Comparison with warfarin. One of the major differences between the effects of the various drugs was the lag period which occurred before prolongations were observed. In view of this, a more thorough analysis of the time period immediately following the administration of the drugs was carried out and compared with warfarin. During the 8-hr period following the (first) injection of the drugs, the effects produced by puromycin and cycloheximide paralleled the effects produced by warfarin (Fig. 3). The similarity between the time courses suggests that warfarin may also act at a site near the ribosomal level for the biosynthesis of clotting factors. The greater effect produced by warfarin after 8 hr may be due to the greater persistence of the drug at its site of action in the liver. Comparable effects on the prothrombin time due to Nogalamycin and actinomycin D were not observed in the early time period.

The effect of puromycin on the circulating levels of factor VII. The coumarin anticoagulants were reported to decrease the plasma levels of factor VII in Sprague-Dawley rats more rapidly than the other clotting factors (12). Therefore, it was deemed pertinent to determine whether puromycin also decreased the factor VII levels. The time course of the effect of puromycin on the factor VII content of the plasma is presented in Fig. 4. The circulating level of factor VII decreased measurably as early as 2 hr after the first injection of the drug. The decline continued until 8 hr which also was the time of the peak effect on the prothrombin time. In this experiment, the animals died shortly thereafter apparently because of the large number of heart punctures required to get a time course.

Since repeated heart puncture posed a problem, an experiment was carried out in which puromycin was administered to a group of rats (4) and the factor VII content was assayed on plasma drawn 8 hr after the first injection. The mean level (0.08 ± 0.02 units) was statistically different ($p = 0.02$) from the mean for an equal number of rats injected with saline on the same schedule. Thus, the administration of puromycin, like the coumarin anticoagulants, resulted in a

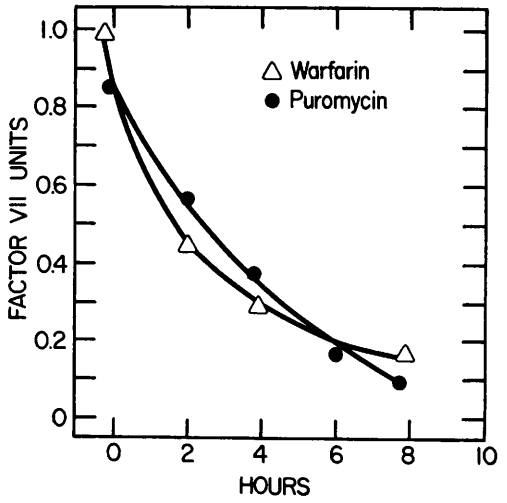

FIG. 4. Comparison of the effect of puromycin with warfarin on the plasma level of factor VII. Puromycin was administered to a pair of rats in 6 hourly injections (25 mg/kg, ip). The warfarin dose was 0.75 mg/kg. The time of the first injection was designated zero time.

rapid decrease of the circulation levels of factor VII.

Discussion. Nogalamycin and actinomycin D, administered in the amounts used in the present studies, reportedly inhibit most of the DNA-dependent synthesis of RNA (14, 15). One of the differences between the effects of these drugs on the prothrombin time and the effects of warfarin was the lag period of several hours which elapsed before measurable effects were observed. Furthermore, the lag period was not eliminated when actinomycin D was given by the intravenous route rather than the intraperitoneal route. Assuming the prolongations eventually observed were due to depletion of messenger RNA, the lag period might represent the time required for degradation of the messenger already present when the drugs were administered. An alternate explanation is that Nogalamycin and actinomycin D have no effect on the prothrombin time other than nonspecific toxic effects of the drugs. This possibility should be considered in view of the fact that only lethal amounts of the drugs were effective.

On the other hand, puromycin and cycloheximide which inhibit the biosynthesis of protein at the level of the ribosomes, produced effects which paralleled the effects of warfarin for the first several hours. This result suggests, but does not prove, that warfarin may also act at the ribosomal level or at a subsequent step in the synthesis of clotting factors. For example, warfarin might inhibit the addition of a nonprotein moiety to the newly formed protein portion of the molecule.

The effects of puromycin were of relatively short duration and multiple injections of the drug were required. This was probably due to the rapid metabolism of puromycin by the liver microsomes as reported by Mazel *et al.* (16).

The administration of of puromycin resulted in the rapid decline of the plasma levels of factor VII. Assuming the decline followed first order kinetics, a half-life in the range of 3 hr can be estimated from the data. Pyörälä (12) reported that the half-life of factor VII was in the range of 1.8–2.6 hr as determined in warfarin-treated rats whereas the half-lives of the other clotting factors were considerably longer. The close agreement for the half-lives of factor VII measured after injection of puromycin and warfarin further suggests that the sites of action for the two drugs are relatively close in the sequence of reactions involved in synthesis of the clotting factors. Although the results do not prove unequivocally that the coumarin anticoagulants act at the level of the ribosome, it appears that the site of action is probably not far removed from the ribosomal level in the biosynthesis of clotting factors.

Summary. Antibiotics with known intracellular sites of action, viz. Nogalamycin, actinomycin D, puromycin, and cycloheximide, were given to rats *in vivo* to determine which of them produce effects comparable to the coumarin anticoagulants. All of these agents prolonged the prothrombin time, however, in the case of Nogalamycin and actinomycin such effects could not be produced unless lethal amounts were used. A comparison of the onset of effect of these agents with warfarin was made. Nogalamycin and actinomycin showed a marked delay in onset while the effect of puromycin and cycloheximide coin-

cided closely with warfarin which suggests that warfarin does not act at the same site as Nogalamycin and actinomycin D. Since puromycin and cycloheximide simulated the action of warfarin during the early part of the time course, it seems likely that warfarin may act at or close to the site of action of these agents.

1. Bhuyan, B. K. and Smith, C. G., Proc. Natl. Acad. Sci. U.S. **54**, 566 (1965).

2. Golderg, I. H. and Rabinowitz, M., Science **136**, 315 (1962).

3. Yarmolinsky, M. G. and De la Haba, G. L., Proc. Natl. Acad. Sci. U.S. **45**, 1721 (1959).

4. Ennis, H. L. and Lubin, M., Science **146**, 1474 (1964).

5. Polson, J. B. and Wosilait, W. D., Biochem. Pharmacol. **16**, 1113 (1967).

6. Polson, J. B. and Wosilait, W. D., Pharmacologist **9**, 229 (1967).

7. Polson, J. B. and Wosilait, W. D., Pharmacologist **10**, 193 (1968).

8. Burhoe, S. O., J. Heredity **31**, 445 (1940).

9. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., J. Biol. Chem. **138**, 1 (1941).

10. Owren, P. A. and Aas, K., Scand. J. Clin. Lab. Invest. **3**, 201 (1951).

11. Gaston, L. W. and Spivack, A. R., J. Lab. Clin. Med. **72**, 548 (1968).

12. Pyörälä, K., Ann. Med. Exptl. Biol. Fenniae (Helsinki) Suppl. **3**, 43 (1965).

13. Wilcoxon, F., Biometrics **1**, 80 (1945).

14. Gray, G. D., Camiener, G. W., and Bhuyan, B. K., Cancer Res. **26**, 2419 (1966).

15. Schwartz, H. S., Sodergren, J. E., Garofaol, M., and Sternberg, S. S., Cancer Res. **25**, 307 (1965).

16. Mazel, P., Kerza-Kwiatecki, A., and Simanis, J., Biochim. Biophys. Acata **114**, 72 (1966).

Received June 9, 1969. P.S.E.B.M., 1969, Vol. 132.