to incubation mixtures containing either the 9000 *g* or microsomal fractions. Concentrations of $ZnSO_4$ of less than 5% resulted in poor recovery.

The formaldehyde produced by the enzymatic demethylation of ethylmorphine (2×10^{-3}M) using 9000 *g* or microsomal fractions was measured by using the optimal conditions for color development determined by our experiments. Formaldehyde production appeared to be equivalent when PCA and 5% or 20% $ZnSO_4$ were used, but values obtained with TCA were invariably lower (Table 1).

From these studies it appears that the choice of protein precipitant, the temperature at which the color is developed, and the duration of incubation are factors which must be considered in comparing the measurements of formaldehyde production from different laboratories. The procedure developed by Nash is a rapid and accurate one, but there should be an awareness of possible pitfalls in its application to biological systems.

The use of either a 5% or 20% solution of $ZnSO_4$ as a protein precipitant appears to offer the best combination of color stability, short interval of color development (30 min at 60°), and low blank values for the measurement of formaldehyde production by liver fractions.

Department of Pharmacology,
West Virginia University,
Morgantown, W. Va., U.S.A.

R. E. STITZEL
F. E. GREENE
R. FURNER
H. CONAWAY

REFERENCES

1. T. C. BUTLER, *J. Pharmac. exp. Ther.* **108**, 11 (1953).
2. J. AXELROD, *J. Pharmac. exp. Ther.* **114**, 430 (1955).
3. B. N. LADU, L. GAUDETTE, N. TRAUSOF and B. B. BRODIE, *J. biol. Chem.* **214**, 741 (1955).
4. S. SZARA and J. AXELROD, *Experienta* **15**, 216 (1959).
5. P. MAZEL, J. HENDERSON and J. AXELROD, *J. Pharmac. exp. Ther.* **143**, 1 (1964).
6. G. C. MUELLER and J. A. MILLER, *J. biol. Chem.* **202**, 579 (1953).
7. D. A. MACFAYDEN, *J. biol. Chem.* **158**, 107 (1945).
8. T. NASH, *Biochem. J.* **55**, 416 (1953).
9. L. LEADBEATER and D. DAVIES, *Biochem. Pharmac.* **13**, 1607 (1964).
10. A. RUBIN, T. TEPHLY and G. MANNERING, *Biochem. Pharmac.* **13**, 1007 (1964).
11. W. FRISELL and C. MACKENZIE, in *Meth. biochem. Anal.* **6**, 67 (1958).

Biochemical Pharmacology, 1966, Vol. 15, pp. 1003–1006. Pergamon Press Ltd., Printed in Great Britain.

A comparison of the isomers of warfarin*

(*Received* 12 *January* 1966; *accepted* 3 *February* 1966)

THE anticoagulant, *Rac*-warfarin [3-α-(acetonyl-benzyl)-4-hydroxycoumarin] has been widely used throughout the world as a rodenticide and clinically for the prevention of thrombosis.[1] The resolution of this important compound in this laboratory by West *et al.*[2] has made possible a study of the relative activity of its enantimorphs. The results of that study formed the basis for this report. Since the absolute configuration has also been determined by West *et al.*[2] the results are of special significance.

MATERIALS AND METHODS

Acute prolongation of prothrombin times. For 5 days preceding the experiment and throughout its duration, male Sprague-Dawley rats, 175 to 225 g, were housed 4 to 5 to a cage and maintained on a

* Published with the permission of the Director of Wisconsin Agricultural Experimental Station. Supported in part by the Research Committee of the Graduate School from funds of the Wisconsin Alumni Research Foundation.

semisynthetic low-K ration.[3] Single doses of the isomers of warfarin were given orally in corn oil via a calibrated dropper (0·0125–0·075 ml) either as solutions or homogenized suspensions. These small volumes were dropped directly into the rats' mouths which were held open by gentle pressure of the thumb.

Blood samples for the determination of prothrombin time were taken by heart puncture from rats lightly anesthetized with diethylether. Blood (0·9 ml) was drawn into a syringe containing 0·1 ml of 0·1 M sodium oxalate. After centrifugation, the plasma was further diluted with 7 parts of a 0·9% saline solution according to the Link modification (Campbell *et al.*[4]) of the Quick method[5] for the determination of prothrombin times. Commercial thromboplastin (Difco) was used. Prothrombin times beyond 250 sec were not determined, because of the poor reproducibility of the method in this range. These few animals (3) were arbitrarily excluded from the statistical analysis of the results.

Ten-day lethality. Male Sprague-Dawley rats, 175 to 200 g, were placed on a ration consisting solely of freshly ground whole corn (i.e., not degerminated) 2 days prior to the start of the experiment (when first exposed to cornmeal rats gorge.) They were then switched to cornmeal containing the anticoagulant isomers; this was maintained as their sole ration until death. These rations were prepared by mixing an acetone solution (ca. 0·5%) of the anticoagulant with the meal and then allowing it to air-dry. Ten animals were housed in each cage; (−)(S)-warfarin was fed at levels of 1·79, 2·51, and 3·54 mg/kg cornmeal; (+)(R)-warfarin was fed at levels of 14·1, 20·0, and 28·2 mg/kg cornmeal ration.

Statistics. A four-point assay was used to determine the relative potencies in the prothrombin-time test.[6] The dosage–mortality curve was determined by the method of Bliss,[7] and the calculation of this relative potency was done according to Miller *et al.*[8]

The rotation of the (R)-warfarin was $[\alpha]_D^{25}$ + 149·0 ± 0·5° (C2, 0·5 N sodium hydroxide). The rotation of the (S)-warfarin was $[\alpha]_D^{25}$ − 148° (C 1·2, 0·5 N sodium hydroxide).

RESULTS AND DISCUSSION

Prothrombin time four-point assay. It was found that a single oral dose of (−)(S)-warfarin was 5·5 times as active as a single oral dose of (+)(R)-warfarin in the rat prothrombin-time assay. Each point in Fig. 1 represents the mean response of 9 animals, as measured 24 hr after dosing.

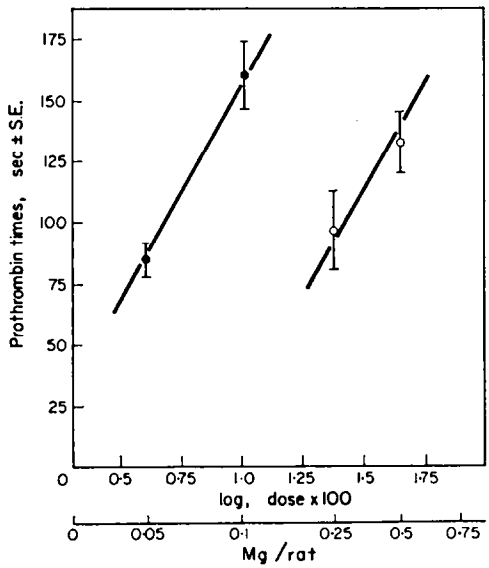

FIG. 1. Assay of the isomers of warfarin by their effect on the prothrombin time of rats 24 hr after oral administration. Each point in the figure represents 9 rats. Relative potency = 5·5. Fiducial limits at a probability of 0·95 are 4·1 and 7·5.
●—(−)(S)-warfarin. ○—(+)(R)-warfarin.

Prothrombin time response. Parallel time-response curves were found for equipotent doses of the isomers, as shown in Fig. 2. Each point on the curve represents the average response of 5 animals dosed at appropriate intervals so that all blood samples and all prothrombin-time determinations were made at the same time.

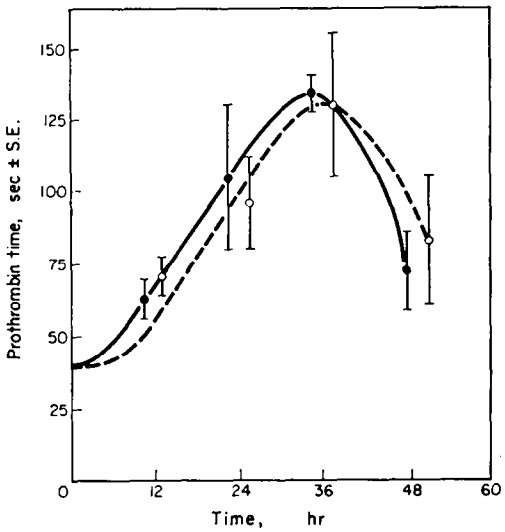

FIG. 2. The effect of oral doses of the enantimorphs of warfarin in a ratio of 5:1 [(+)(R):(−)(S)] on the prothrombin time of 200-g male rats. Each point in the figure represents 5 rats.
0·075 mg ●—(−)(S)-warfarin. 0·375 mg ○—(+)(R)-warfarin.

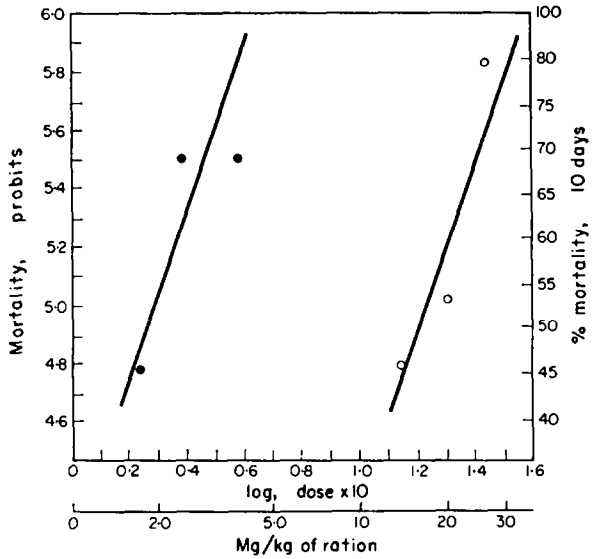

FIG. 3. Assay of the isomers of warfarin by their lethal effect in rats after 10 days when incorporated into the diet. Ten 200-g male rats at each dose level. Dose in terms of mg isomer/kg ration. Relative potency = 8·5. Fiducial limits at a probability of 0·95 are 5·2 and 13·9.
●—(−)(S)-warfarin. ○—(+)(R)-warfarin.

Lethality tests. In this test, (−)(S)-warfarin was 8·5 times as active as (+)(R)-warfarin, as shown in Fig. 3. The LD_{50} concentration determined at 10 days for (−)(S)-warfarin was found to be 2 mg/kg cornmeal ration. The LD_{50} concentration for (+)(R)-warfarin was found to be 17·7 mg/kg cornmeal ration.

The results found here are in general agreement with those of earlier studies in this laboratory.[9,10] The isomeric ratio of 5–8:1 found here and the average human maintenance dose of the racemic mixture of this compound (5–10 mg/day) puts this compound in the predicted range given by Pfeiffer[11] in his generalized scheme for the relationship between isomeric ratios and drug potency.

Department of Biochemistry,
University of Wisconsin,
Madison, Wis., U.S.A.

JOHN NELSON EBLE*
BRUCE D. WEST†
KARL PAUL LINK

* Pitman-Moore Div. of The Dow Chemical Co., P.O. Box 10, Zionsville, Ind., U.S.A. 46077.
† University of New Mexico, Albuquerque, N. M.

REFERENCES

1. K. P. LINK, *Circulation* **29**, 97 (1959).
2. B. D. WEST, S. PREIS, C. H. SCHROEDER and K. P. LINK, *J. Am. chem. Soc.* **83**, 2676 (1961).
3. K. P. LINK, R. S. OVERMAN, W. R. SULLIVAN, C. F. HUEBNER and L. D. SCHEEL, *J. biol. Chem.* **147**, 463 (1943).
4. H. A. CAMPBELL, W. K. SMITH, W. L. ROBERTS and K. P. LINK, *J. biol. Chem.* **138**, 21 (1941).
5. A. J. QUICK, M. STANLEY-BROWN and F. W. BANCROFT, *Am. J. med. Sci.* **190**, 501 (1935).
6. D. J. FINNEY, *Statistical Method in Biological Assay.* Hafner, N.Y. (1952).
7. C. I. BLISS, *Quart. J. Pharm.* **11**, 192 (1938).
8. L. C. MILLER, C. I. BLISS and H. A. BRAUN, *J. Am. pharm. Ass.* **28**, 644 (1939).
9. J. N. EBLE, Ph.D. Thesis, University of Wisconsin (1953).
10. T. H. LIN and W. F. BLATT. Unpublished observations.
11. C. PFEIFFER, *Science* **124**, 29 (1956).

Biochemical Pharmacology, 1966, Vol. 15, pp. 1006–1008. Pergamon Press Ltd., Printed in Great Britain.

Studies of the relationship between chemical structure and porphyria-inducing activity—II

(*Received* 26 *October* 1965; *accepted* 19 *January* 1966)

THE overproduction of porphyrins and porphyrin precursors in the livers of animals fed porphyria-inducing drugs results from an enhanced synthesis of the first enzyme in the porphyrin biosynthetic pathway, viz. δ-aminolaevulic acid synthetase.[1,2] Recent studies of the structure–activity relationships of porphyria-inducing compounds in chick embryo liver cells lead to the conclusion that steric rather than chemical factors are important for activity.[3] The most active compounds appear to have a planar portion with a side chain out of the plane.[3,4] This conclusion is difficult to reconcile with the emphasis placed by previous workers[5–7] on the importance of a free allyl group for porphyria-inducing activity in intact animals and chick embryos. For this reason a group of compounds previously tested in whole animals has been reinvestigated in chick embryo liver cells in order to evaluate the importance of the free allyl group for activity.