FIG. 3. " " " 2, ×5000.
FIG. 4. Single crystals in dried smears of gastric secretion (human), ×5000.
FIG. 5. A very thick deposit of dried gastric secretion (human) before electron bombardment, ×3000.
FIG. 6. The same field as Fig. 5 after electron bombardment, ×3000.

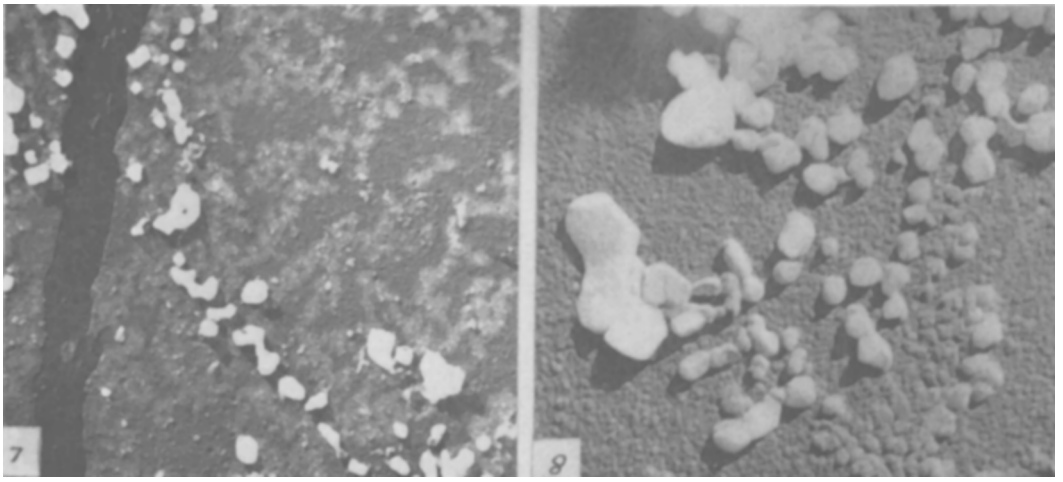

FIG. 7. A shadow cast preparation of gastric secretion (human) showing large NaCl crystals and amorphous background material, ×4200.
FIG. 8. A shadow cast preparation of NaCl itself. The field is part of a frond at high magnification to show the manner in which the fronds are formed of countless tiny crystals, ×25000.

NaCl identification with some weak unidentified lines also present.

1. Hollander, Franklin, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 749.

2. Watson, John H. L., and Preuss, Luther E., *J. App. Phys.*, 1950, v21, 904.

3. Henning, N., and Norpoth, L., *Z. f. Klin. Med.*, 1933, v126, 1.

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Diphenylacetyl-1,3-Indandione as a Potent Hypoprothrombinemic Agent. (19548)

JOHN T. CORRELL, L. L. COLEMAN, STUART LONG, AND RALPH F. WILLY.
(Introduced by M. H. Kuizenga.)
From the Research Laboratories, Upjohn Co., Kalamazoo, Mich.

As a result of the therapeutic importance of anticoagulant drugs, the literature reveals a continuing search for compounds which will inhibit blood coagulation. A recent review by Seegers(1) includes a summary of the published information on prothrombinopenic substances, citing studies on indandione derivatives as hypoprothrombinemic drugs. A number of 1,3-indandiones, including two previously studied by Kabat, Stohlman and Smith (2), were prepared in these laboratories and submitted to us for evaluation as to their potential prothrombinopenic properties. Out of the group studied, 2-diphenylacetyl-1,3-indandione (U-1363; Dipaxin*) was a particularly potent hypoprothrombinemic substance.

The data establishing the above observation,

* Trademark, Upjohn Co.

and the results obtained upon further biologic evaluation of U-1363 as an oral anticoagulant, constitute the basis of this report.

Methods. Plasma prothrombin concentrations were estimated by a modified Quick(3) one-stage technic with Factor V (Ac-G) in the thromboplastic mixture. A standard thromboplastic mixture consisted of 12.5 ml of hog lung extract, 25 ml of hog serum (Ac-G) (4), 5 ml of 2.5% $CaCl_2$ solution, 0.5 ml of phenol, and distilled water q.s. to 100 ml. Aliquots were frozen and one was thawed daily during an investigation. Rabbits weighing approximately 3 kg were individually caged and fasted for 24 hours prior to the start of a prothrombinopenic study. From an ear vein was drawn 4 ml of blood into 0.4 ml of 1.34% sodium oxalate solution. The blood samples were centrifuged and the plasma assayed. Prothrombin times were determined on 100% plasma and after dilution with saline to 50%, 25%, and 12.5% plasma. Three determinations at each dilution were averaged. The values thus obtained for a given rabbit, before receiving any test compound, established the normal curve for that animal. Hence, each rabbit served as its own control. Subsequent prothrombin times were compared with the control values, and the existing prothrombin concentration calculated in terms of per cent of normal. Blood samples were obtained daily over the experimental period except in the chronic prothrombinopenic studies when determinations were made about every third day. Drug administrations were oral, either in capsules or by stomach tube. Food and water were consumed *ad lib.* after the initial bleeding. For the acute toxicity studies, U-1363 was administered by stomach tube in a 10% gum acacia suspension to rats and mice, and by capsule to rabbits. In the chronic toxicity evaluation, U-1363 was given by stomach tube daily for 14 days to rats and rabbits.

Results. Acute response. Table I presents the acute prothrombinopenic response of rabbits to single doses of 3,3′-methylenebis-4-hydroxycoumarin (Bishydroxycoumarin, U.S.P. XIV; Dicoumarol†), 3,3′-carboxymethylenebis-4-hydroxycoumarin ethyl ester (Ethyl biscoumacetate; Tromexan‡), and 17 analogues of indandione. Under the conditions of this test, U-1363 was the most potent. The data show that a pronounced and comparable prothrombin depletion resulted from 50 mg/kg of ethyl biscoumacetate, 10 mg/kg of bishydroxycoumarin, and only 0.05 mg/kg of U-1363. The entire acute response scatter curves for these 3 compounds at these dosages are presented in Fig. 1. With each of the drugs there was considerable individual variation in response. The average curves, however, demonstrate that after the single dose of ethyl biscoumacetate there was only a brief period of effectiveness before the prothrombin level returned to normal. In contrast, the small dose of U-1363 and relatively larger dose of bishydroxycoumarin resulted in

TABLE I. *Evaluation of Compounds as Oral Hypoprothrombinemic Anticoagulants. Response to a single dose.*

Compound (-1,3-indandione)	Dose, mg/kg	No. of rabbits	Lowest prothrombin values, (avg) % of normal
Ethyl biscoumacetate*	50	5	36
Bishydroxycoumarin*	10	10	38
2-diphenylacetyl-(U-1363)	.05	10	38
2-acetyl-	10	3	88
	50	2	60
2-phenylacetyl-	2	3	82
	50	2	50
2-triphenylacetyl-	5	3	86
2-pivalyl-	10	3	10
	5	3	18
	2	3	60
2-α,α-diphenylpropionyl-	50	2	50
2-α,β- "	50	2	50
2-β,β- "	50	2	45
2-isobutyryl-	50	3	40
2-isovaleryl-	50	3	40
2-α-phenyl-γ-methyl valeryl	50	2	50
2-benzoyl-	10	2	50
2-p-methoxybenzoyl-	10	3	100
	50	3	70
2-phenylazo-	10	3	88
2,2′-methylenebis-	10	3	90
2-(3′-keto-1′-indanylidene)	10	2	100
	50	2	70

* Not indandiones.

† Trademark, Wisconsin Alumni Research Foundation.

‡ Trademark, Geigy Co.

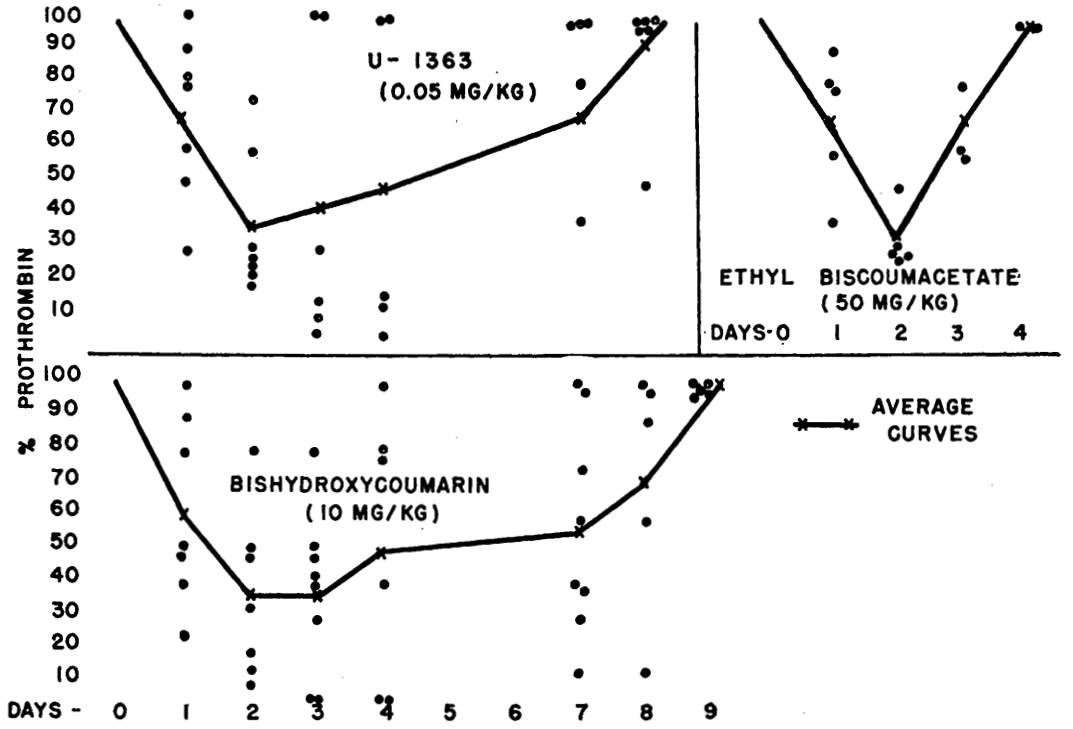

FIG. 1. Response of rabbits to a single oral dose of prothrombinopenic drugs.

more prolonged periods of prothrombin depletion.

Chronic response. Because U-1363 had demonstrated in single doses an acute hypoprothrombinemic potency, repeated doses were administered orally to 12 rabbits to determine the chronic regime which would lower the prothrombin level to around 30% of normal and maintain it at approximately that level for several weeks. Prothrombin determinations were made intermittently, usually every third day. Typical response curves are presented in Fig. 2 for four of the rabbits. Again, there was significant variation in the dose requirements of individual animals. Rabbits 397 and 394 maintained reasonably uniform low prothrombin concentrations on doses of 25 μg/kg administered, usually, every 1 or 2 days. Rabbit 390 was slightly more resistant, requiring several 50 μg/kg doses which were occasionally reduced to 25 μg/kg. Rabbit 389 responded to doses of 100 μg/kg, with a few days interspersed when only 50 μg/kg were needed.

Histopathologic observations. From this group of 12 rabbits, an animal was sacrificed at each of 4, 8, 10, 12, and 13 weeks for pathologic study. A gross examination was made, and sections of heart, kidney, liver, adrenal, lung, spleen, stomach and duodenum were taken for microscopic evidence of systemic toxicity. No such evidence was found, except for pulmonary petechia in moderate numbers in some of the rabbits, indicating a possible increase in capillary fragility. Hematologic studies revealed no effect on hemoglobin concentration or red and white cell count.

Acute and chronic toxicity. The acute oral LD_{50} for U-1363 was found to be 3 mg/kg for rats, 340 mg/kg for mice, and 35 mg/kg for rabbits. The number of animals used in these tests were 38, 75, and 21 respectively. The chronic oral LD_{50} for U-1363 was indicated as greater than 0.1 mg/kg/day with 12 rats. With 12 rabbits, a value of 0.25 mg/kg/day was obtained.

Antidote studies. U-1363 appeared to have potentialities as an oral hypoprothrombinemic anticoagulant. Experiments were therefore designed to ascertain if there might be an

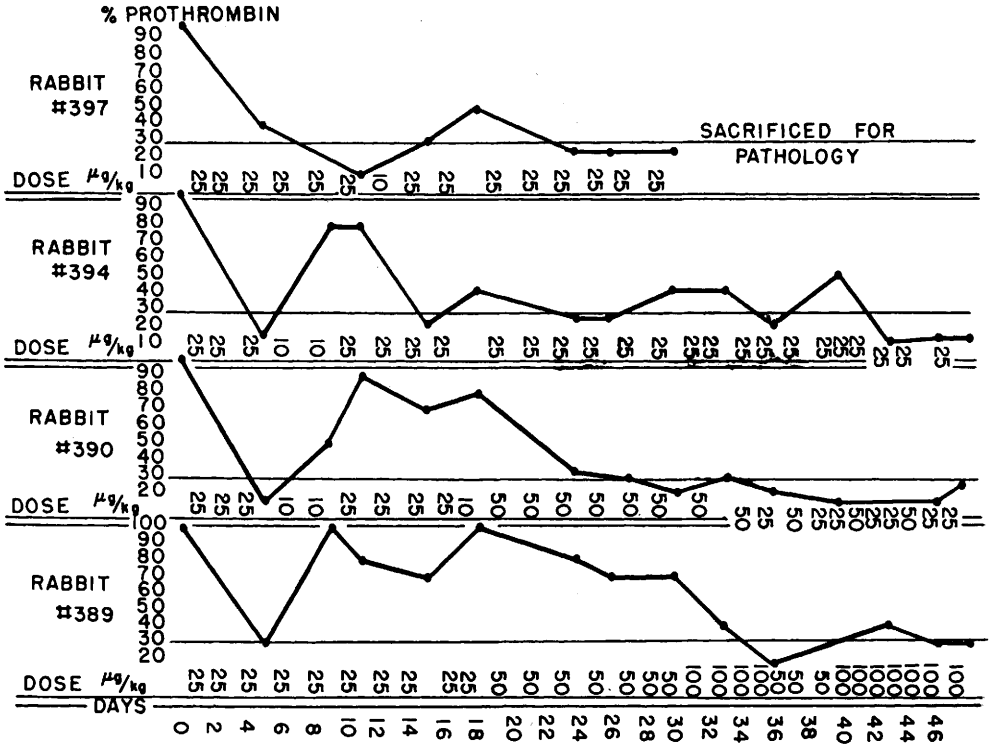

FIG. 2. Prothrombin response of rabbits to repeated oral administration of U-1363.

antidote for the hypoprothrombinemia produced by this drug. Thirty rabbits were given repeated doses of U-1363. After the last dose, prothrombin concentrations ranged from less than 10% (11 rabbits) to slightly above 30% (3 rabbits). Once hypoprothrombinemia was established, the administration of U-1363 was discontinued. Twelve of the animals, representing all degrees of depletion, served as controls receiving only food and water during the following 24 hours. At the end of this period, 6 controls showed either further prothrombin decreases or no improvement, 5 had slight to moderate increases. Only one of the group had returned to 100% of normal prothrombin concentration. The remaining 18 severely prothrombinopenic rabbits were administered amounts of vit. K_1 ranging from 20 to 400 mg per rabbit in 3 or 4 divided doses over the 24-hour period; 15 received the drug orally in capsules, 3 were injected intravenously with a fat emulsion of vit. K_1. At the end of the 24 hours, 13 had returned to 100% of normal prothrombin values, the remaining 5 had recovered to the relatively safe concentrations of 40 to 90% of normal. Thus vit. K_1, administered orally or intravenously in these doses, restored the prothrombin to normal within 24 hours after a U-1363 produced hypoprothrombinemia. In similar experiments, not reported in detail, it was found that a water-soluble vit. K analogue (tetrasodium 2-methyl-1, 4-naphtho-hydroquinone, diphosphate) had no influence on the hypoprothrombinemia produced in rabbits by U-1363.

Summary. Out of a group of compounds, including bishydroxycoumarin, ethyl biscoumacetate, and 17 analogues of indandione, tested in rabbits, 2-diphenylacetyl 1,3-indandione (U-1363) demonstrated the greatest hypoprothrombinemic potency. The prothrombinopenic response of rabbits to acute and chronic doses of U-1363 are reported. Histopathologic observations are reported for rabbits that were given U-1363 daily for several weeks. Acute and chronic toxicity data are reported for U-1363. Vit. K_1, but not a water-soluble vit. K analogue, is an

antidote to the hypoprothrombinemia produced by U-1363.

The authors wish to acknowledge the generous cooperation of the following associates from these laboratories: Drs. M. E. Speeter, D. G. Thomas and Mr. W. C. Anthony for preparing the indandione compounds; Dr. E. S. Feenstra for the histologic examinations; Mr. O. F. Swoap for the toxicity data; and Mrs. Russell Borst for technical assistance. The vit. K_1 emulsion was kindly furnished by Dr. F. J. Stare, Harvard University.

1. Seegers, W. H., *Pharmacol. Rev.*, 1951, v1, 278.
2. Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharmacol. Exp. Therap.*, 1944, v80, 160.
3. Quick, A. J., *J.A.M.A.*, 1938, v110, 1658.
4. Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, v152, 567.

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Two Dimensional Paper Chromatography of Proteins. II. Application to Blood Plasma Fractions.* (19549)

HENRY TAUBER AND EDWARD L. PETIT.

From the Venereal Disease Experimental Laboratory, U. S. Public Health Service, University of N. Carolina, School of Public Health, Chapel Hill, N. C.

Franklin and Quastel(1) have reported that proteins may be separated by means of 2-dimensional filter paper chromatography; aqueous buffers and the ascent principle were used. These investigators converted the proteins into protein-hemin complexes prior to chromatography and identified the position of each protein-hemin complex by treating the paper with benzidine and hydrogen peroxide. All proteins, however, do not combine with hemin. The method is also limited by the intensely colored background which obscures the color given by the protein-hemin complex. In order to overcome this interference the patterns must be immediately photographed. While Hall and Wewalka(2) have criticized the method of Franklin and Quastel, the former authors concluded that proteins of dissimilar nature could be separated by chromatography. Feigl(3), as early as 1937, showed that dyes may be used for the detection of proteins on filter paper. Based on this principle, we have developed a convenient staining reagent containing the fluorescent dye, eosin, and methyl orange and presented other improvements concerning the 2-dimensional procedure(4).

*A preliminary report was presented before the American Society of Biological Chemists at the meeting of the Federation of Amer. Soc. for Exp. Biol., New York City, April 15, 1952.

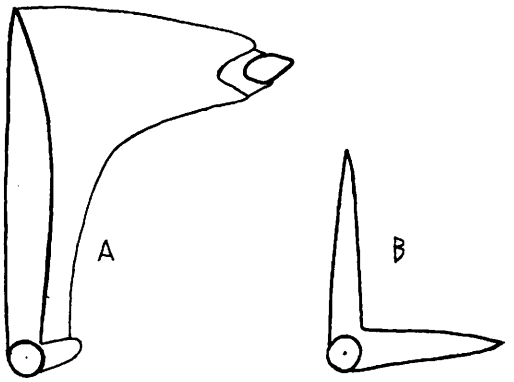

FIG. 1. Tracings of 2-dimensional filter paper chromatograms. A. = albumins, 120; B. = γ-globulins, 120. Quantities are in μg.

In the present paper we wish to describe the results obtained when our technic of 2-dimensional filter paper chromatography and staining was applied to human blood plasma albumins and γ-globulins fractions.

Experimental. The total protein applied in each experiment was 120 μg. The following solution of pH 6.0 was used as the developing agent in the first dimension: trisodium citrate (0.02 M), 9.25 ml (2 N) hydrochloric acid, and 50 g of sodium chloride per 5 l. The same buffer, after adjustment to pH 4.0, was used for dissolving the plasma proteins. Tartaric acid (2%) was employed in the second dimension.

In Fig. 1, "A" represents the tracing of the