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Bioassay of Water-Soluble Antihemorrhagic Compounds by Intravenous Administration.*

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Since the natural vitamin K compounds are not soluble in water and in many cases superior therapy could be attained by intravenous administration, several different water-soluble antihemorrhagic compounds have been prepared. (Almquist and Klose;[1] Thayer, *et al.*,[2] Fieser and Fry;[3] Foster, *et al.*,[4] and Ansbacher, *et al.*[5]).

Supplementing our previous reports (Thayer, *et al.*),[6] we have assayed 2-methyl-1,4-naphthoquinone and several closely related water-soluble compounds by different methods in order to determine their respective potencies. Originally our products for assay were administered orally but recently the realization that the potencies by parenteral administration might be very different has led us to employ intravenous injection. Compounds which are active orally might be inactive parenterally due to their avoidance of the enzymes of the gastro-intestinal tract or to their too rapid excretion by the kidneys.

The potencies have been estimated from a comparison of the effects of the compounds under investigation and 2-methyl-1,4-naphthoquinone on the, (1) mean prothrombin time, (2) mean clotting time, and, (3) percentage of positive responses.[7] Each

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TABLE I.
Bioassay of Water Soluble Compounds (Intravenous Administration)

Compounds	Dosage, μg	Equivalent in μg of standard,‡ μg	Chicks used (21 days of age) No.	Response normal clotting time, %	Clotting time, mean; S.E., min	Prothrombin time, mean; S.E., sec
1,4-dihydroxy-2-methylnaphthalene monosuccinate	0.751†	0.68	12	58	17.8 ± 6.8	41.9 ± 4.9
	1.502	1.0	30	93	6.7 ± 0.5	29.1 ± 2.2
2-methyl-1,4-naphthoquinone (standard)	0.961		10	80	12.7 ± 4.9	35.7 ± 4.1
	1.432		20	100	6.0 ± 0.8	28.6 ± 1.4
Controls lot 1	—		10	0	129.7	58.0
" " 2	—		10	0	109.2	60.6
di-potassium-1,4-dihydroxy-2-methylnaphthalene-disulfate	2.503	1.0	14	64	29.1 ± 6.4	44.3 ± 8.6
	6.006	2.3	15	86	7.0 ± 0.82	33.5 ± 0.48
	6.005	2.3	14	71	8.9 ± 1.1	37.1 ± 1.50
2-methyl-1,4-naphthoquinone (standard)	0.963		10	60	9.9 ± 5.6	28.6 ± 1.2
	0.966		15	100	4.7 ± 0.7	28.6 ± 4.1
Controls lot 3	—		10	0	155.0	61.2
4-amino-2-methyl-1-naphthol*	1.002	0.74	29	89	6.7 ± 0.8	32.8 ± 1.5
	1.501	1.1	15	93	7.0 ± 1.4	27.8 ± 1.2
	1.504	1.0	15	93	7.1 ± 0.9	30.3 ± 3.6
2-methyl-1,4-naphthoquinone (standard)	1.434		20	100	6.1 ± 0.90	27.4 ± 1.6
Controls lot 4	—		10	0	104.0	60.6
4-amino-3-methyl-1-naphthol*	1.305	1.0	15	93	5.7 ± 0.65	32.5 ± 1.2
4-amino-2-methyl-1-naphthol*	1.305	1.0	15	73	8.4 ± 1.4	35.5 ± 1.30
Controls lot 5	—		10	0	296.6	67.8
Normal chicks	—		10	100	4.3 ± 0.1	26.5 ± 1.0

*As the crystalline hydrochloride containing 0.5 M ethanol.

†The superscript designates the lot of chicks used for that assay.

‡The values of column 3 were obtained by multiplying the values of column 2 by the ratio of the molecular weights of 2-methyl-1,4-naphthoquinone and the respective compound.

assay has been controlled by an accompanying assay of a standard substance (usually 2-methyl-1,4-naphthoquinone) on the same lot of deficient chicks to eliminate the variation in deficiency found in different groups of chicks.

The basal diet used in all of our experiments is the one described by Almquist.

Assays—The compounds were dissolved in 0.85% NaCl solution. The chicks were slightly anesthetized with ether and the solution was injected directly into the jugular vein of the chick (0.1 cc of solution). Bleeding was minimized by the use of a fine needle (27 gauge) and by entering the vein through the subcutaneous tissues. Eighteen hours after the injection blood was drawn from the brachial vein into a small tube (micro test tube of Fischer)[8] and placed at once in a thermostat adjusted to 38.5-39.5°C and the time for coagulation determined. The chick's head was clipped off with scissors and 1.8 cc of blood collected in a vial containing 0.20 cc of 0.1 M sodium oxalate solution. The method of Almquist and Klose[9] was used for determining prothrombin time.

Early in our work on Vitamin K, we realized that the variability of the degree of deficiency of different lots of chicks (see values for the controls Table I) could produce gross inaccuracies in the bioassay. Originally we standardized the deficiency of each lot of chicks by determining the response to 2 different dosages of a stock solution which had been obtained from alfalfa. Later,[2] we suggested the adoption of pure crystalline 2-methyl-1,4-naphthoquinone as the basic standard. The variability of the response of these lots of chicks (1, 3 and 6) to the administration of 0.96 μg of 2-methyl-1, 4-naphthoquinone (Table I) emphasizes the necessity of ascertaining the response of each lot of chicks to a standard.

On a weight basis, 1,4-dihydroxy-2-methylnaphthalene monosuccinate, 4-amino-2-methyl-1-naphthol and 4-amino-3-methyl-1-naphthol administered intravenously are, perhaps, slightly less active than 2-methyl-1,4-naphthoquinone; on a molar basis all are fully as active. The potency of the disulfate on a weight basis is somewhat less than one-sixth and on a molecular basis approximately one-third that of 2-methyl-1,4-naphthoquinone.

Summary. Using intravenous administration it has been found that on a molecular basis the potencies of all the compounds with

[8] Fischer, A., *Pfluger's Arch. ges. Physiol.*, 1930, **225**, 737.

[9] Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

the exception of the disulfate are approximately equal to that of the standard, 2-methyl-1,4-naphthoquinone. The disulfate is about one-third as potent on a molecular basis, but owing to the much larger molecular weight its activity per milligram is somewhat less than one-sixth that of 2-methyl-1,4-naphthoquinone.

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Riboflavin Determinations on Normal Liver and Liver Tumor.

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It was decided to use a fluorometric method in making quantitative estimations of riboflavin from fresh liver tissue after several checks had been run against the Snell and Strong method.* The fluorometric method here used is first to determine the total fluorescence, then eliminate the riboflavin by raising the alkalinity to pH 11 and determining the "interfering" fluorescence. The difference between these two values gives the approximate riboflavin value. The fluorescence was measured with a photocell using suitable optical filters.

Liver tumors from Osborn-Mendel rats which had been fed 2-amino 5-azo toluene for a long period were used. These animals were kindly put at our disposal by Dr. E. Emmart. Samples of liver which had been perfused with saline were ground with n/10 HCl, autoclaved for 15 min. and clarified by precipitation at pH 5-6, followed by filtering through No. 42 paper. Recovery of added riboflavin was about 97% by this fluorometric method.

Some of the material was given a short low temperature drying by the lyophile process eliminating most of the water. The data in the tables indicate that the difference between normal and tumor liver is not due to water content.

Comparisons were made between normal livers from normal rats, nontumor bearing liver from dye-fed rats, liver tumors from dye-fed rats, residual liver from which the tumor had been excised, fetal liver, and leg muscle. In the case of a general riboflavin deficiency it would be expected that lower riboflavin values for the muscle would be ob-

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