Twenty-four-hour urine specimens were extracted and fractionated according to the method described by Gallagher, Peterson, Dorfman, Kenyon, and Koch.[1] Samples were assayed for their estrogenic potency by the vaginal smear technic, using mice. Androgenic† assays were done by a modification of the Zimmerman method as described by Friedgood and Whidden.[2] We endeavored to correct for the inherent color of the samples by subtracting the equivalent of the reading obtained when an aliquot (equal in amount to that used for the actual determination) was treated with all the reagents except m-dinitrobenzene. This method gives values higher than those which would be obtained by biologic assay, but we feel that it is probably acceptable for use in a comparative study.

It will be seen that the male patients with mammary carcinoma did not differ significantly from the controls in regard to the amount of androgens and estrogens excreted. The low excretion of both androgens and estrogens in the aged, found by several previous investigators, and the daily variability in the excretion of androgens reported by Gallagher, *et al.*,[1] are confirmed.

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Comparative Activities of Certain Antihemorrhagic Compounds.

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Various quinones with vitamin K activity have been assayed in order to establish the comparative potencies of these compounds in relation to a common reference standard. The reference standard is an hexane extract of dried alfalfa equivalent in potency to 1 g of dried alfalfa per cc. This reference standard at 2 or more levels has been employed in all of our assays for the past 2 years. A secondary

1 Gallagher, T. F., Peterson, D. H., Dorfman, R. I., Kenyon, A. T., and Koch, F. C., *J. Clin. Invest.*, 1937, **16**, 695.

† Grateful acknowledgment is made to Ciba Pharmaceutical Products and to Schering Corporation for the crystalline androsterone; and to Parke-Davis for crystalline estrone.

2 Friedgood, H. B., and Whidden, H. L., *Endocrin.*, 1939, **25**, 919.

reference standard consisting of a sample of dried alfalfa has also been preserved. Neither of these standards has shown any loss of total or relative activity. In periodic assays the potency of the second reference standard has been obtained as 1.09, 1.03, 1.07, and 1.06 cc of the first standard per gram.

Some assays have been conducted as we have already described,[1] that is, by placing the supplement in the diet of depleted chicks and determining the prothrombin clotting time after 7 or more days. More recently,[2] after a depletion period of 10 to 14 days, we have given the supplement orally for 4 days and have determined prothrombin clotting time on the fifth day. Certain supplements have been assayed by both procedures and have given identical results. In a few cases, where evidence of loss of the supplement in the diet was encountered, the oral feeding was relied upon entirely. In all asays, the reference standard was employed at 2 or more levels in order to establish values for interpolation and calculation of potencies in terms of the standard. This may be done by means of the linear relation between the reciprocal of the prothrombin clotting time and the logarithm of the vitamin K dosage.[1] No "master curves," "response curves" or arbitrarily fixed values are required in our method. The results of the assays, which are in most cases average values of several determinations, are given in Table I . As a further illustration of the assay procedure, results of one recent assay are given in detail in Table II.

The activities listed in Table II agree substantially with those in Table I for the same compounds. The value for the sample of 2-methyl-4-amino-1-naphthol hydrochloride, corrected for the ethanol of crystallization, becomes 97,000, the same as that in Table I for this compound without ethanol of crystallization.

The activity of 2-methyl-1,4-naphthoquinone is the same whether administered orally in water or ethyl laurate solution (Table II). This has been our repeated experience. However, Dann[3] reported that 2-methyl-1,4-naphthoquinone is 3 times as potent when administered orally in oil as compared to water, while Ansbacher, Fernholz and Dolliver[4] state that it is twice as potent in water as in oil.

The tetra-sodium salt of 2-methyl-1,4-naphthohydroquinone di-

1 Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

2 Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, 1939, **130**, 787.

3 Dann, F. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 663.

4 Ansbacher, S., Fernholz, E., and Dolliver, M. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 652.

TABLE I.
Antihemorrhagic Activities of Certain Derivatives of 2-methyl-1,4-naphthoquinone.

Compound	Reference standard per g, cc	Source
2-methyl-1,4-naphthoquinone	205,000	Authors
2-methyl-1,4-naphthoquinone, dimerized	14,000	F. Giral
2-methyl-1,4-naphthohydroquinone	190,000	Authors
2-methyl-1,4-naphthohydroquinone, diacetate	92,000	"
2-methyl-3-hydroxy-1,4-naphthoquinone (Phthiocol)	500	R. J. Anderson
2-methyl-3-acetoxy-1,4-naphthoquinone	900	Authors
2-methyl-3-amino-1,4-naphthoquinone	3,000	"
2-methyl-3-nitro-1,4-naphthoquinone	800	"
2-methyl-3-palmityl-1,4-naphthoquinone	25,000	"
2,3-dimethyl-1,4-naphthoquinone	5,400	Merck & Co.
2-methyl-4-amino-1-naphthol hydrochloride	97,000	E. A. Doisy
2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (tetra sodium salt + 6 molecules water)	101,000	Hoffman-LaRoche
2-methyl-3-phytyl-1,4 naphthoquinone (vitamin K_1)		
natural	62,700	P. Karrer
"	61,600	E. A. Doisy
"	55,000	Authors
" , diacetate	20,000	E. A. Doisy
synthetic	57,000	"
"	56,000	Authors
"	59,000	Merck & Co.
2-methyl-3-(?)-1,4-naphthoquinone (vitamin K_2)	50,000	E. A. Doisy

TABLE II.
Comparative Assay of Antihemorrhagic Compounds by 4-day Oral Administration to Depleted Chicks.

Supplement In ethyl laurate solution	Daily dose	No. chicks	Avg prothrombin clotting time, sec	Reference standard per g, cc
Reference standard	0.15 cc	9	31.7	—
2-methyl-1,4-naphthoquinone	0.5 γ	10	37.5	191,000
" "	2.0 γ	10	22.7	
In water solution				
2-methyl-1,4-naphthoquinone	0.5 γ	10	39.9	187,000
" "	2.0 γ	10	22.9	
2-methyl-4-amino-1-naphthol hydrochloride (½ molecule ethanol)	3.0 γ	9	25.8	87,500
2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (tetra sodium salt + 6 molecules water)	3.0 γ	10	24.9	95,700

phosphoric acid ester is of particular interest because our results show, in agreement with the report of Foster, Lee and Solmssen,[5] that on a molecular basis this compound is more potent than the

[5] Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

methyl naphthoquinone. Potencies for the compound as administered (containing 6 molecules of water of crystallization) were found to be 101,000 and 95,700 in two assays. On the molecular basis, these values would be 310,000 and 294,000 respectively or 1.48 and 1.54 times the activity of the methyl naphthoquinone used in the same assay. There remains no doubt that this is the most potent antihemorrhagic compound yet prepared. The suggestion[5] that this phosphoric acid ester may represent a step in the metabolism of the methyl naphthoquinone seems to be logical. On the other hand, Ansbacher, *et al.*,[4] have claimed that this di-phosphoric acid ester is only 1/20 as potent as the methyl naphthoquinone.

The 2-methyl-1,4-naphthohydroquinone diacetate (92,000) calculated to the 2-methyl-1,4-naphthoquinone basis has a corrected potency (124,000) which is much less than that of the methyl naphthoquinone. Similarly the diacetate of dihydro-vitamin K_1 (20,000) has a corrected potency of 22,600 which is a little more than one-third the potency of the original preparation of vitamin K_1 (61,600). Our results agree qualitatively with those of Binkley, *et al.*,[6] in respect to this potency-lowering effect of reductive acetylation.

The activity of the water-soluble phenol, 2-methyl-1,4-naphthohydroquinone (190,000), which is spontaneously oxidized by air to the methyl naphthoquinone is not appreciably different from that of the latter, probably because the phenol is converted to the naphthoquinone before or soon after absorption. In this respect our results agree with those of Thayer, *et al.*[7]

Dimerization of the methyl naphthoquinone as catalysed by light involves 2,3 condensation. The condensed product which we obtained from Dr. F. Giral exhibited a potency (14,000) corresponding to only a few percent of unchanged methyl naphthoquinone. It was known to be not quite pure. The pure dimer is probably completely inactive. This result is of interest in relation to the proposed use of 2-methyl-1,4-naphthoquinone as a standard and to its stability as such.

In two joint assays of preparations of vitamins K_1 and K_2 obtained from Professor E. A. Doisy we found the same potency ratio, 1.25, which is almost exactly the inverse molecular ratio of these compounds or the direct ratio of their respective contents of methyl naphthoquinone. This observation is most easily explained by assuming

[6] Binkley, S. B., Cheney, L. C., Holcomb, W. F., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., 1939, 98th Meeting, Am. Chem. Soc., Boston.

[7] Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, A. R., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**, 2563.

that in each case the long side chain of these compounds is split off to the same extent, perhaps completely.

If this view of the metabolism of vitamins K_1 and K_2 is correct, the potencies of these compounds can not be greater than that of their corresponding content of the methyl naphthoquinone. Their potency is actually less since we obtain, after repeated assays, a potency for 1 milligram of vitamin K_1 equal to that of 300 μg of 2-methyl-1,4-naphthoquinone, whereas it actually contains 380 μg. Correspondingly, the potency of vitamin K_2 appears to be 240 μg out of a theoretical 307.

The phytyl side chain of vitamin K_1 and the larger side chain of vitamin K_2 seems nonspecific, as indicated by the fact that the methyl naphthoquinone itself is more active without these side chains. Furthermore, a side chain of an entirely different character, the palmityl group, also gives rise to a compound with fairly high activity (Table I).

The phthiocol used in our assays was in some instances submitted to a further purification by washing a solution of phthiocol in dilute sodium bicarbonate repeatedly with ethyl ether. The potency of the phthiocol was unchanged by this treatment. The ratio of its potency to that of the methyl naphthoquinone is 1 to 400 according to our assay values. Flynn and Warner[8] reported a potency ratio 1 to 500 for these compounds. Fernholz and Ansbacher[9] have claimed, however, that the potency ratio may be as low as 1 to 4000.

The antihemorrhagic activity of 1 cc of our reference standard is equivalent to that of 5 μg of 2-methyl-1,4-naphthoquinone. All of our values are convertible on this basis since the reference standard has been used in all assays pending the time when a universal standard might be adopted.

[8] Flynn, J. E., and Warner, E. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 190.

[9] Fernholz, E., and Ansbacher, S., *Science*, 1939, **90**, 315.