obstructive jaundice of over 6 months' duration, the secondary liver damage robs the test of its value in differential diagnosis. If obstructive jaundice develops in patients with pre-existing parenchymatous disease of the liver, the test shows a confusingly high blood level of galactose. This occurred in 2 cases (identified on the chart by asterisk) in which obstructive jaundice was associated with impaired clearance of galactose. Application of this test to other forms of liver disease is now in progress.

Conclusion. The intravenous galactose tolerance test offers valuable assistance in the differentiation of the parenchymatous or "medical" type from the obstructive or "surgical" type of jaundice.

11699

Antihemorrhagic Activity of Tetra Sodium 2-Methyl-1, 4-Naphthohydroquinone Diphosphoric Acid Ester and Other Naphthoquinone Derivatives.

J. LEE, U. V. SOLMSSEN, AL STEYERMARK AND R. H. K. FOSTER.
(Introduced by E. Chargaff.)
From the Laboratories of Hoffmann-La Roche, Inc.

In a recent communication[1] we reported preliminary data on the antihemorrhagic activity of the tetra sodium salt of 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester (for brevity, referred to hereafter as substance N-123). At this time preliminary tests indicated that doses of 0.6-0.8 γ administered subcutaneously to chicks on a vitamin K-free diet reduced the clotting time to below 10 minutes. In this report we are presenting more complete observations.

Ansbacher *et al.*[2] recently reported the potency of this phosphoric acid ester to be much less than we indicated. Further work has shown that while occasional chicks require only 0.6-0.8 γ of substance N-123 to reduce the clotting time to 10 minutes or less the average is somewhat higher. This average is still far below that reported by Ansbacher *et al.* Furthermore we find the average dose

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

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

of methyl naphthoquinone to be higher than reported by the latter authors.[2]

Fieser and Fry[3] did not consider the phosphoric acid ester a very potent preparation. They stated it was active at 25 γ but not at 10 γ. The conflict between the results of Ansbacher *et al.* and those of Fieser and Fry on the one hand and ours on the other raises the question of purity of the preparations with which those authors worked. In our experience pure preparations of the tetra sodium salt are only obtained after isolation and purification of the intermediate diphosphoryl chloride and the free diphosphoric acid.

*2-Methyl-*1,4-*naphthohydroquinone-diphosphoryl chloride* is a viscous yellow oil with an odor characteristic for an acid chloride. It cannot be distilled without decomposition.

Analysis:

Found	P 14.46%
Calc. for $C_{11}H_8O_4Cl_4P_2$	P 15.23%

2-Methyl-1,4-naphthohydroquinone diphosphoric acid ester is a pink-tinted, crystalline substance, m.p. 212° (uncorr.) under decomposition.

Analysis:

Found	C 39.75, 39.83%, H 3.65, 3.75%, P 17.87, 17.79%
Calc. for $C_{11}H_{12}O_8P_2$	C 39.52%, H 3.59%, P 18.56%

*Tetrasodium 2-methyl-*1,4-*naphthohydroquinone diphosphoric acid ester* is a colorless crystalline hygroscopic substance containing various amounts of moisture of crystallization depending upon the drying treatment. A typical sample showed after drying to constant weight at 160°C 20.40, 20.60% of moisture (calc. for 5 H_2O, 18.6, calc. for 6 H_2O, 20.3%).‡ The sodium content of the moisture-containing substance was found as 18.02%, 17.02% (calc. 17.95%) and the phosphorus content 12.07%, 11.99% (calc. 12.11%).

For the anhydrous materials the figures correspond fairly closely to the theoretical data.

Calc. for $C_{11}H_8O_8P_2Na_4$	Na = 21.79%, P = 14.69%
Found	Na = 22.10, 20.98%, P = 14.8, 14.7%

Immediately before preparing test solutions the water content is controlled or the solutions themselves are assayed for phosphorus.

In carrying out the test we have adopted an 18 hour curative

---

[3] Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, 1940, **62**, 228.

‡ The moisture calculated as water. Crystallized from water, methanol and acetone, the moisture probably consists of 2 molecules of methanol and 3 molecules water (theoretical moisture content 21.8%). The material used in these studies was prepared in this manner. Pending definite determinations of the constitution, the substance was referred to as containing the equivalent of 6 molecules of water.

assay period, a modification of Ansbacher's[4] original method. Day-old white leghorn cockerels were placed on the Almquist[5] diet for 2 weeks. Many chicks developed the characteristic hemorrhages and control samples did not clot in 2 hours. Substances tested were administered subcutaneously or by stomach tube.

To minimize contamination of the sample by tissue juices, blood samples were taken by brachial venipuncture using a 1 cc tuberculin syringe and a half inch No. 25 needle. 0.25 cc of blood was drawn into the syringe and of this the first 0.20 cc was expressed slowly into a small vaccine tube. The tube was immediately stoppered with a paraffined cork and placed into an automatic tilting rack immersed in a water bath at 38.5°. Coagulation time was determined to the nearest minute, the end-point being taken when the sample ceased to flow.

Because chicks obtained at different times appear to vary somewhat in reactivity we employ a standard of comparison whenever accurate assays are desired. Originally we used a hexane extract of alfalfa as a standard but now employ only methyl naphthoquinone.

As a criterion of potency we employ a modified Thayer-Doisy[6] unit. This unit is defined as the amount of antihemorrhagic substance which will reduce the clotting time of 50% of vitamin K-free chicks to ten minutes or less under our assay conditions. Since it is a curative unit it may be represented by the expression CD50. A 10 minute clotting time, although not normal, is more satisfactory than the normal because it is on a curved portion of the dose-effect curve rather than on the horizontal position where there is little or no change in clotting time with change in dosage.

The method of determining the CD50 has been to inject 2 groups of chicks subcutaneously at different dose levels and plot the doses against the per cents of samples clotting at 10 minutes or less on logarithmic probability paper. The CD50 is that point where a straight line drawn between the two data crosses the 50% probability coördinate.

In Table 1 are given 3 sets of assays on substance N-123. The ratios of potency between N-123 and methyl naphthoquinone are 0.52, 0.44 and 0.47. Similar values have been observed in many other assays. The average CD50 for methyl naphthoquinone in 7 different assays was 0.64 γ with extremes of 0.61 and 0.75 γ. This dose only brings the average clotting time to 10 *minutes*

[4] Ansbacher, S., *Science*, 1938, **88**, 221.

[5] Almquist, H. J., *J. Biol. Chem.*, 1936, **114**, 241.

[6] Thayer, S. A., MacCorquodale, D. W., McKee, R. W., and Doisy, E. A., *J. Biol. Chem.*, 1938, **123**, cxx.

TABLE I.
Bioassay of Sodium 2-methyl-1,4-naphthohydroquinone Diphosphoric Acid Ester (MeNHQ Phos · $6H_2O$) and 2-methyl-1,4-naphthoquinone (MeNQ). Injections made subcutaneously using 0.2 cc of aqueous solutions.

Chick Group No.	Substance	N*	CD50	Ratio of Potencies
			γ	
505-506	MeNHQ Phos · $6H_2O$	24	1.31	.47
507-508	MeNQ	21	0.61	
542-543	MeNHQ Phos · $6H_2O$†	28	1.25	.52
540-541	MeNQ†	27	0.65	
544-545	MeNHQ Phos · $6H_2O$	37	1.65	.44
546-547	MeNQ	40	0.72	

*Divided about equally between the two dose levels.
†The actual doses injected were 0.5 and 1.0 γ per chick for MeNQ and 1.0 and 2.0 γ per chick for MeNHQ Phos · $6H_2O$.

whereas Ansbacher reported that 0.25 γ brings the average clotting time to *normal*. In his earlier reports he stated the activity to be 0.5 γ, which is more nearly in line with our figures. The average of 8 assays on substance N-123 is 1.34 γ per CD50 and the potency relative to methyl naphthoquinone as determined by these averages is 0.48.

In Table II are listed assays comparing oral and subcutaneous potencies of the 2 substances. Fewer animals were used in these tests so obviously the standard errors must be greater. Approximations of CD50's were obtained by drawing the best fitting lines on logarithmic probability paper, parallel to the average slope of many other assays. The oral CD50's were found to be 1.9 γ for substance N-123 and 1.5 γ for methyl naphthoquinone. The relative

TABLE II.
Comparison of Potencies After Oral and Subcutaneous Administration. (Assays conducted simultaneously.)

Chick group No.	Substance	Dose per chick	N	Route	Avg clotting time	% of samples clotting at 10′ or less
		γ			min	
553	MeNHQ Phos · $6H_2O$[4]	2	8	P.O.	14.6*	63
554	”	4	8	”	8.0	88
556	MeNQ	1	8	”	18.1*	12
557	”	2	6	”	6.5	100
552	MeNHQ Phos · $6H_2O$[4]	2	7	S.C.	12.8	43
555	MeNQ	1	7	”	10.3	57

*In both instances 2 samples had not clotted at 30 minutes. For purpose of averages, these samples were treated as though they had clotted at 30 minutes.

TABLE III.
Results on Other Naphthoquinone Derivatives.
Oral administration of 0.2 in sesame oil. Eighteen-hour assay method.

Substance	Dose γ/chick	N	Clotting time
			min
2-n-butyl-3-hydroxy-1,4-naphthoquinone	1000	3	>30
2-allyl-3-hydroxy-1,4-naphthoquinone	1000	3	>30
3,4-dihydroxy-1,2-naphthoquinone (isonaphthazarine)	200	3	>30
γ,γ-dimethyldihydropyrano-1,4-naphthoquinone (α-lapachone)	200	3	>30
β,β-dimethyldihydrofurano-1,4-naphthoquinone	200	3	>30

strength of the former is thus 0.79. The subcutaneous assays in this series gave 2.2 γ and 0.9 γ respectively with a potency ratio of 0.41.

Ansbacher found the activity ratio by oral or intravenous administration between the phosphoric acid ester and methyl naphthoquinone to be about 1:20. Our data show this ratio to be slightly less than 1:2 by subcutaneous administration and our oral tests, although limited in number, indicate that the potency ratio must be far closer to 1:2 than 1:20.

In Table III are listed the bioassay results of 5 naphthoquinone derivatives not heretofore reported in the literature. These samples were prepared by one of us (A.S.) in the laboratory of the late Dr. Samuel Hooker.[7] We made no attempt to determine what the active dose was, if any, all substances being found inactive at the comparatively high doses studied. Neither the furano- nor the pyrano-naphthoquinones tested showed any activity, possibly due to the presence of the oxygen linkage in position 3. Isonaphthazarine (3,4-dihydroxy-1,2-naphthoquinone) was also tested in order to determine the effect of o-quinones on the activity, although it was known that vitamin K must be a p-quinone since it is yellow.[7]

Discussion. Substance N-123 used in these experiments contained the equivalent of 6 molecules of water of crystallization* which amounts to 20.2% of its weight, the total molecular weight being 530. The molecular weight of methyl naphthoquinone is 172 and the ratio 172:530 is 0.325. The average ratio of potency was found to be 0.48, however, so evidently the phosphoric acid ester is more active, molecule for molecule, than methyl naphthoquinone. This higher molecular activity is in the proportion of 0.48 to 0.325

[7] Hooker, S. C., and Steyermark, Al, *J. Am. Chem. Soc.*, 1936, **58**, 1163.

* The substance is hygroscopic and may even hquefy if the humidity is high. See also footnote ‡.

or 1.48, that is, substance N-123 is about 50% more active than methyl naphthoquinone on a molecular basis.

Summary. 1. The CD50 (Thayer-Doisy unit) by subcutaneous administration of tetra sodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester ($C_{11}H_8O_8P_2Na_4 \cdot 6H_2O$)[4] is 1.34 γ. 2. The subcutaneous CD50 of 2-methyl-1,4-naphthoquinone ($C_{11}H_8O_2$) is 0.64 γ.

3. The ratio of these potencies is 0.48 but on a molecular basis allowing for 6 molecules of water of crystallization the phosphoric ester is 50% more potent than methyl naphthoquinone.

4. Five naphthoquinone derivatives not hitherto reported are shown to be inactive in doses from 200 to 1000 γ.

11700

Pharmacological Observations on Tetra-Sodium-2-Methyl-1, 4-Naphthohydroquinone Diphosphoric Acid Ester.

R. H. K. FOSTER. (Introduced by E. Chargaff.)
(With the technical assistance of H. H. Clark.)
From the Pharmacology Laboratory of Hoffmann-La Roche, Inc.

The antihemorrhagic activity of tetra-sodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester ($C_{11}H_8O_8P_2Na_4 \cdot 6H_2O$)* referred to as N-123 hereafter) has been reported elsewhere.[1, 2] Molecule for molecule it was found to be about 50% more potent than methyl naphthoquinone. In this report we wish to give the general pharmacological properties.

Fatal Dose. Molitor and Robinson[3] reported the LD50 of 2-methyl-1,4-naphthoquinone as 500 mg/kg orally in mice and 75 mg/kg intraperitoneally. In 19-day chicks they found the intraperitoneal dose to be < 100 mg/kg. If their figures of 70% mortality for 100 mg and 90% mortality for 150 mg are plotted on logarithmic probability paper the LD 50 is calculated to be 76 mg/kg.

Although our routes of administration were such that a direct

* See footnote ‡ of previous paper.

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

2 Lee, J., Solmssen, U. V., Steyermark, Al, and Foster, R. H. K., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 407.

3 Molitor, H., and Robinson, H. J., *ibid.*, 1940, **43**, 125.