days have been made on rats restricted to a nitrogen-low basal diet, supplemented for various periods with casein, and an acid or an enzymic digest of casein. 2. The enzymic digest fed in comparison with casein, resulted in nitrogen retention of approximately the same magnitude as the unhydrolyzed protein. 3. The acid digest, supplemented with 0.2% tryptophane likewise gave positive nitrogen balances, but without the addition of the essential tryptophane, was refused by the animals. 4. When both the unsupplemented acid digest, and the enzymic digest were administered by stomach tube, the latter was well retained, but the former gave a slight negative balance with some indication that it was partially effective in supplying animal requirement for nitrogen.

11010

Quantitative Biological Assay of Vitamin K and Its Application to Several Quinone Compounds.*

FLEMINTINE PEIRCE DANN. (Introduced by Carl Nielsen.)

From the Abbott Laboratories, North Chicago, Illinois.

The Biological Assay Technic. When this work was started in 1937, the 2 methods used for the biological assay of the antihemorrhagic factor were the curative technic of Schønheyder[1] and the preventive method of Almquist and Stokstad.[2] The Schønheyder method which depends on a curative effect is theoretically more accurate than a preventive one. In the application of the method, the long and involved technic of taking blood samples and determining the blood clotting time is a definite disadvantage. Later, Dam and Glavind[3] revised the Schønheyder method without simplifying these steps.

In the meantime, some work[4] had been done in our laboratory on the Almquist and Stokstad preventive method. Finally, a method for the biological assay of the antihemorrhagic factor was developed,

* The author is indebted to Dr. H. Dam for his standard spinach tablets, to Dr. R. J. Anderson for the phthiocol compound, to Dr. Byron Riegel for a sample of the natural vitamin K_1, to Dr. E. A. Doisy for a specimen of his natural vitamin K_1, and to Dr. L. F. Fieser for a sample of the synthetic vitamin K_1.

1 Schønheyder, F., *Biochem. J.*, 1936, **30**, 890.

2 Almquist, H. J., and Stokstad, E. L. R., *J. Nutri.*, 1937, **14**, 235.

3 Dam, H., and Glavind, J., *Biochem. J.*, 1938, **32**, 1018.

4 Dann, F. P., *Am. J. Physiol.*, 1938, **123**, 48.

using the diet and blood-clotting technic of Almquist and Stokstad, the 3-day curative period of Dam, *et al.*, the oral administration of the vitamin K test dose to the chicks individually, and a vitamin K standard as a positive control for each group of chicks. This curative method which has been used with the vitamin K standard for a year and a half is quantitative within the limits of error for a biological assay, provided the procedure is carried out with certain precautions. Chicks are obtained from a source where the diet of the hens is standardized and is the same the year round. The fish meal and yeast in the deficient diet is continuously extracted with ether until the ether wash no longer contains any fat. The wire bottoms and cages are kept very clean throughout the assay period to prevent coprophagy. The incision of the main wing vein is made with a flexible razor blade, and not less than 0.5 cc of blood is withdrawn. The blood clotting time of 10 or more chicks is determined before the assay is started; if 70% or more of these negative controls do not show a clotting time of over 30 minutes, the chicks are continued on the deficient diet and the clotting time is checked every few days until the chicks are depleted of vitamin K. For a finished report, not less than 20 chicks are used to a level and the standard is run concurrently with the unknown. The potency is calculated in vitamin K units per gram by the formula, dose of standard/dose of unknown $\times$ units per gram in the standard. When the potency of 5000 vitamin K units per gram was assigned to our standard, a dose of 0.2 mg fed per chick per day gave a blood clotting time of approximately 2 minutes. Since then our method of assay and technic of taking blood clotting time have been modified. Essentially the same procedure has been followed with other standards for biological assay work, namely, arbitrarily assigning a definite potency to a standard and retaining the same factor although later the original assay method was modified. The standard selected in this case was a vitamin K oil concentrate prepared from alfalfa with the hexane and most of the sterols removed. For oral administration, it is diluted with sesame oil and 0.1 cc measured by a syringe into gelatin capsules, the unknown being fed in the same way. This standard has proved to be a workable and stable material. The data in Table I show no difference in potency of a sample of our standard kept in a screw-top bottle without nitrogen at 40°C for 14 months and one stored at 5°C for the same period.

Discussion of Assay Methods. During the last year, many assay technics have been suggested and later modified. The described 3-day curative method is the only one which has been consistently adhered to so that enough data have accumulated to check the ac-

TABLE I.
Correlation of Standard A and the Dam Standard with Data on Stability of Standard A.

Material	Daily dose, mg	No. of chicks	Avg clotting time, min
Vitamin K Std. A—stored at 5°C	0.2	22	7.96 ± 1.05*
" " " " " " 40°C	0.2	21	7.86 ± 1.08
Std. Spinach Tablets—Dam	50.0	10	7.27 ± 1.23
" " " "	75.0	22	4.65 ± 0.39†
Negative controls		20	More than 25.66

* On this dose level, the standard deviation is great. The most sensitive level for accurate interpretation of results is the 75 mg dose on the Dam standard with a percentage error of approximately 10%,† or a dose level of 0.30 mg on Standard A and 0.45 mg on Standard B which is ⅔ as active as A.

curacy of the technic. In the meantime, other methods were tried without any increase in accuracy. Almquist, Mecchi and Klose[5] modified their original preventive method and suggested from 1 to 2 weeks' curative period. This method in which the chicks are placed on test after only 1 week on the vitamin K-free diet and therefore do not reach a state of avitaminosis, is not precisely a curative technic. It is based on the assumption, as is the preventive method, that the vitamin K reserve is the same in each group of chicks, which is not the case. Recently, Almquist and Klose[6] modified the technic of taking the simple blood clotting time by determining the whole blood prothrombin time. In checking this method in our laboratory negative controls which had a blood clotting time of over 30 minutes showed a prothrombin time of from 47 to 93 seconds, while the normal chick's prothrombin time was 25 to 37. Such a close relationship between the deficient and the normal chicks produces slight differences in dose responses, decreasing the assay's sensitivity.

Thayer, *et al.*,[7] have reported on the 3-day curative method using a dose response curve for interpretation of results and a unit defined as that quantity of vitamin which produces a clotting time of 10 minutes or less in 50% of a group of 10 or more chicks. Such a dose level is very close to a borderline level where the individual variation is great. On the other hand, the calculated standard deviation will not give an accurate picture of this variation where the clotting time is not observed after 30 minutes. Later, Thayer, *et al.*,[8] modified their method to an 18-hour test, while Ansbacher[9] re-

5 Almquist, H. J., Mecchi, E., and Klose, A. A., *Biochem. J.*, 1938, **32**, 1897.

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

7 Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 478.

8 Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 194.

9 Ansbacher, S., *J. Nutri.*, 1939, **17**, 304.

duced the test period to 6 hours. In our hands, these short procedures work very nicely for preliminary research work where one is only interested in approximate potencies but are not accurate enough for quantitative work.

The Vitamin K Activity of Several Quinone Compounds. The earliest chemical studies on vitamins K_1 and K_2 indicated that they contained the quinone structure.[10] The vitamin K activity of the pure substances and the related quinone compounds was reported by different workers.[11] These reports were conflicting, partly due to the assays being preliminary and partly due to the different methods and units used. Data collected in this laboratory on the vitamin K activity of these compounds in terms of 1 unit may help to clarify the confusion in this field.

The two compounds which first created the most interest in the recent literature were the 2-methyl-3-hydroxyl-1,4-naphthoquinone known as phthiocol and the 2-methyl-1,4-naphthoquinone. Preliminary assays were first run on these compounds and later were reassayed by the 3-day curative method with a greater number of chicks to a level. The activity of the phthiocol in an oil medium is on the borderline of 15 units per mg; the sodium salt in a saline solution is definitely not as active as the original compound in oil, being less than 10 units per mg. The 2-methyl-1,4-naphthoquinone contains 2500 units per mg when administered orally in an oil solution. (Table III.) The first sample of this compound with a m.p. of 100-101°, assayed 2250 units; later a slightly purer sample, m.p. 104-106°, assayed 2500. The activity is significantly less with other solvents and different methods of administration (Table II). The reason for this is not clearly understood at present.

Dr. Marjorie B. Moore of our research staff first suggested the

[10] Dam, H., Geiger, A., Glavind, J., Karrer, D., Karrer, W., Rothschild, E., and Soloman, H., *Helv. chim. Acta*, 1939, **22**, 310; McKee, R. W., Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1295; Binkley, S. B., MacCorquodale, D. W., Cheney, L. C., Thayer, S. A., McKee, R. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1612.

[11] Thayer, S. A., Cheney, L. C., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1932; Karrer, P., and Geiger, A., *Helv. chim. Acta*, 1939, **22**, 945; Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1611; Fieser, L. F., Bowen, D. M., Campbell, W. P., Frey, E. M., and Tates, M. D., *J. Am. Chem. Soc.*, 1939, **61**, 1926; Ansbacher, S., and Fernholz, E. J., *J. Am. Chem. Soc.*, 1939, **61**, 1924; Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, R. A., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**, 2563; Tishley, M., and Sampson, W. L., *J. Am. Chem. Soc.*, 1939, **61**, 2563; MacCorquodale, D. W., McKee, R. W., Binkley, S. B., Cheney, L. C., Holcomb, W. F., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, 1939, **130**, 433; Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, 1939, **130**, 787.

TABLE II.
Vitamin K Activity of 2-methyl-1,4-naphthoquinone.

Daily dose, micrograms	Solvent	Method of admin.	Avg clotting time	Dose of Std. B, micrograms	Avg clotting time	Potency in units per mg
0.6	Oil	Oral	4.44	450	4.87	2500
0.6	"	Intramus.	4.78	450	4.87	2500
2.0	Aqueous	Oral	5.38	450	5.40	750
1.5	"	Intramus.	4.93	450	5.40	1000
1.0	Propylene Glycol	"	5.51	450	5.62	1500

TABLE III.
Vitamin K Activity of Vitamin K_1 and Other Quinone Compounds.

Material*	Daily dose, micrograms	Avg clotting time	Dose of Std. B, micrograms	Avg clotting time	Potency in units per mg
Synthetic K_1	2.00	4.86	450	4.69	750
Natural K_1	1.50	4.18	450	4.26	1000
2-methyl-1,4-naphthoquinone	0.60	4.44	450	4.87	2500
2-methyl-1,4-naphthoquinhydrone	0.60	5.12	450	4.87	2500
2-methyl-1,4-naphthohydroquinone	0.75	7.16	450	4.35	<2000

* All these materials were dissolved in sesame oil and 0.1 cc fed orally per chick per day.

possibility of 2-methyl-1,4-naphthoquinhydrone having vitamin K activity. Later she found that this compound had been synthesized once before by the Spanish workers, Madináveitia and de Buruaga.[12] A sample of this compound with a m.p. of 106-109° (this was not a sharp m.p.) assayed 2500 units per mg (Table III). The 2-methyl-1,4-naphthohydroquinone, m.p. 158-159° with decomposition, contains slightly less than 2000 units per mg. In comparison with the vitamin K activity of these synthetic compounds, the natural vitamin K_1, $C_{31}H_{46}O_2$ (found: C, 82.42 H, 10.41) showed a potency of 1000 units per mg (Table III). The question arises whether this sample of natural vitamin K_1, which is very sensitive to light, was slightly inactivated. Later, a second specimen of this material carefully protected from light throughout the procedure, was assayed and checked the first results nicely. The sample of synthetic vitamin K_1 (found: C, 82.40 H, 10.44) was slightly less active than the natural K_1, being 750 units per mg. The molecular weight of vitamin K_1 is 2.6 times that of 2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinhydrone, hence the compounds are approximately equally active on a molar basis.

[12] Madináveitia, A., and de Buruaga, J. S., *An. Soc. Espanola Fisica Quim.*, 1929, **27**, 647; *Chem. Zent.*, 1930, **101**, pt. 1, 684.

Conclusions. (1) A 3-day curative technic for the biological assay of the antihemorrhagic factor has been developed. If a vitamin K standard is run with each group of chicks and certain precautions in the procedure are observed, the assay is quantitative within the limits of error for a biological assay. (2) The vitamin K activity of a compound varies significantly with the solvent and the method of administration. (3) The vitamin K activity of 2-methyl-1,4-naphthoquinone and of 2-methyl-1,4-naphthoquinhydrone is approximately the same as the natural vitamin K_1 on a molar basis.

11011

Action of Sulfanilamide on Hemolytic Enterococcus (*Streptococcus fecalis hemolyticus*).

ERWIN NETER.

From the Laboratories of the Children's Hospital, and the Department of Pathology and Bacteriology, University of Buffalo School of Medicine, Buffalo, N. Y.

It is generally agreed that sulfanilamide, sulfapyridine, and related compounds are effective in the treatment of infections due to a limited number of microörganisms only. *In vitro,* these chemotherapeutic substances may be bacteriostatic or bactericidal toward susceptible strains. There is, however, no absolute parallelism between the effectiveness of sulfanilamide *in vivo* and *in vitro;* nor between susceptibility or resistance of certain species or groups of microörganisms and their respective position in the system of bacteria. The susceptibility toward the action of sulfanilamide may be quite different even among relatively closely related groups. Thus, sulfanilamide may inhibit the growth of Lancefield Group A hemolytic streptococci in broth, while strains of hemolytic streptococci Group D (hemolytic enterococci) are quite resistant toward this drug (Bliss and Long[1]). Recently, it was reported[2, 3] that sulfanilamide in a concentration of 800 mg per 100 ml may continuously inhibit the growth of fibrinolytic hemolytic streptococci even when relatively large numbers were used for inoculation; in contrast, strains of hemolytic enterococci, characterized by their growth on 40% bile agar, by their capacity to reduce methylene blue and litmus in

1 Bliss, S. A., and Long, P. H., *New England J. Med.,* 1937, **217**, 18.

2 Neter, E., *J. Bact.,* 1938, **36**, 669.

3 Neter, E., *J. Lab. and Clin. Med.,* 1939, **24**, 650.