Heretofore, no sera from persons convalescing from an infection with Japanese B virus have been available for study by means of complement-fixation tests, so that it was not known whether a similar cross antibody reaction occurs in the sera of human beings. Table I shows that sera from 3 convalescent persons which fixed complement in the presence of Japanese B virus failed to do so with St. Louis virus. Furthermore, 15 sera which contained complement-fixing antibody against Japanese B virus showed no antibody against the virus of Western equine encephalitis. Two sera which were positive against Japanese B virus were negative against lymphocytic choriomeningitis virus, and 2 other such sera were negative against Eastern equine encephalitis.

The identification of Japanese B virus as the causative agent of the Okinawan outbreak of encephalitis by means of the serologic evidence described above was confirmed by the isolation of a virus from the brain of a patient (No. 53) dying of encephalitis. This virus was isolated by a group of medical officers of the U. S. Army and Navy (two of the authors of the present communication, L.T. and J.P., were in this group). A sample of this agent was sent to our laboratory on Guam. An antigen suitable for complement-fixation tests was prepared from brains of mice dying of infection with the third passage of this virus. As shown in Table II, the Okinawa virus antigen fixed complement in the presence of sera of hamsters immunized against the Nakayama strain of Japanese B virus, but no significant complement-fixation occurred when it was tested against serum of guinea pigs immunized against Western equine encephalitis virus. The virus isolated on Okinawa was, therefore, shown to be Japanese B virus by means of its specific complement-fixation reaction.

Summary. 1. By complement-fixation tests carried out with human sera, an outbreak of encephalitis which began on Okinawa in July, 1945, was promptly shown to be due to Japanese B virus. 2. The tests showed that at least 11 of 20 patients studied had suffered an attack of encephalitis caused by this virus. 3. None of 15 human sera which were positive against Japanese B virus showed complement-fixing antibodies against Western equine encephalitis virus, and a few such sera were negative when tested against the viruses of Eastern equine encephalitis, St. Louis encephalitis, and lymphocytic choriomeningitis. 4. A virus isolated from a patient on Okinawa suffering from encephalitis was identified by complement-fixation reactions as Japanese B virus.

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Observations on Antimicrobial Action of 2, 3-Dichloro-1, 4-Naphthoquinone, and its Reversal by Vitamins K.

D. W. WOOLLEY.*

From the Rockefeller Institute for Medical Research, New York City.

Since Ter Horst and Felix[1] have found a marked antifungal action of 2,3-dichloro-1,4-naphthoquinone, the idea occurred that this effect might be linked with the structural similarity of the compound to vitamin K, much as that of the sulfonamides is related to their analogy with the metabolite *p*-aminobenzoic acid. This view seemed probable since Kuhn *et al.*[2] had shown previously that 6,7-dichloro-9-ribityl-isoalloxazine had antimicrobial action which was reversible by riboflavin. The change involved in passing from the vitamin riboflavin to the inhibitor 6,7-dichloro-9-ribityl-isoalloxazine was the replacement of 2 methyl groups attached to

* With the technical assistance of M. L. Collyer.

1 Ter Horst, W. P., and Felix, E. L., *Ind. Eng. Chem.*, 1943, **35**, 1255.

2 Kuhn, R., Weygand, F., and Möller, E. F., *Ber. deutsch. chem. Ges.*, 1943, **76**, 1044.

TABLE I.

Amounts of 2,3-dichloro-naphthoquinone and of 2-methyl-naphthoquinone Required to Cause Half-maximal Inhibition of Growth of Various Microörganisms.

Organism	Amount for half-maximal effect	
	2,3-dichloro-naphthoquinone μg per cc	2-methyl-naphthoquinone μg per cc
Saccharomyces cerevisiæ	0.0017	0.23
Endomyces vernalis	0.015	0.28
Lactobacillus casei	20	20
Staphylococcus aureus	10	10
*Escherichia coli**	35	15
Hemolytic streptococcus H69D	35	40

* This organism was tested in the synthetic medium described earlier[6] for its growth. In the highly purified, but more complex medium used for the other bacteria in this work the quinones were less active, *e.g.*, 2-methyl-naphthoquinone was just detectable at 50 μg per cc.

a benzenoid nucleus with Cl atoms. If a similar type of structural alteration were applied to vitamin K, *i.e.*, if the 2 alkyl side chains were exchanged for Cl atoms, one would arrive at 2,3-dichloro-1,4-naphthoquinone. Therefore, since we had been investigating the types of structural change which would convert metabolites into specific inhibitory competitive analogs,[3] it was decided to determine whether the action of dichloro-naphthoquinone bore relationship to that of vitamin K. If the effects of the compound could be negated by the vitamin the actions of the two might conceivably be connected.

2,3-dichloro-naphthoquinone† was inhibitory to the growth of all microbial species tested. The amounts of the compound required to produce half-maximal effect are shown in Table I. It can be seen that there was very great variation in the susceptibility of various species to the agent. Bacterial forms examined were relatively resistant, while the yeasts were exceedingly sensitive to the quinone. The minute concentration needed to affect *Saccharomyces cerevisiæ* made this compound one of the most toxic known for this organism. The growth tests with bacteria were conducted in the highly-purified medium of Landy and Dicken,[4] and those with the yeasts in the synthetic medium previously described.[5] Growth was estimated turbidometrically in the usual manner,[6] and in the case of those species which produced acid, the turbidometric results were checked by estimating the final pH of the cultures. The quantity of agent which caused half-maximal inhibition of growth was determined by interpolation on a curve relating dose to response. It was necessary that the dichloroquinone be added after the various media were autoclaved, for when it was heated in the medium or even in water, its potency was reduced about 100 times, probably due to the conversion of the substance to 2-chloro-1,4-naphthoquinone.[7] To avoid heat-sterilization, a 2% solution of the dichloro-naphthoquinone in acetone was diluted with sterile water.

The toxicity of vitamin K in the form of 2-methyl-1,4-naphthoquinone was determined for each species of microörganism, for it is known that this compound, like many other quinones, is rather harmful to microörganisms. The data in Table I indicate the concentrations of this form of the vitamin which were necessary to cause half-maximal inhibition of growth under the same conditions as were used in the trials with the dichloroquinone. However, synthetic vitamin K_1 in contrast to 2-methyl-naphthoquinone was not inhibitory

[3] Woolley, D. W., *Science*, 1944, **100**, 579.

† The 2,3-dichloro-naphthoquinone was kindly supplied by Dr. G. L. McNew of the United States Rubber Company.

[4] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1943, **52**, 106.

[5] Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

[6] Woolley, D. W., *J. Biol. Chem.*, 1941, **140**, 453.

[7] Beilstein, F., *Handbuch der Organischen Chem.*, 1897, **3**, 372.

TABLE II.
Effect of 2,3-dichloro-naphthoquinone and of 2-methyl-naphthoquinone Singly and Together on the Growth of *Saccharomyces cerevisiæ*.

2,3-dichloro-naphthoquinone	2-methyl-naphthoquinone	Turbidity* of culture
μg per cc	μg per cc	
0	0	39
0.002	0	78
0.005	0	93
0.01	0	99
0.005	0.04	60
0.005	0.02	68
0.005	0.01	77
0.005	0.005	85
0.002	0.02	48
0.002	0.01	66
0	0.05	46
0	0.20	65

*Turbidity is expressed as per cent of the incident light transmitted by the culture when the uninoculated basal medium is considered to have 100% transmission.

to the growth of either *Lactobacillus casei* or *S. cerevisiæ*.

With *S. cerevisiæ* and with *Endomyces vernalis* the harmful effects of 2,3-dichloro-naphthoquinone were antagonized by vitamin K. Over a limited range of concentration this antagonism was competitive as can be seen from the data in Table II. However, the inhibition index[5] (the ratio between concentration of inhibitor and that of vitamin required to negate the action of the former) was 0.1, and therefore, a point was soon reached, as the concentration of dichloro-naphthoquinone was raised, beyond which it was not possible to reverse the inhibitory effect with the vitamin, for the concentration of the vitamin was then in the toxic range. With *E. vernalis*, it was barely possible to achieve complete counteraction of the effect of the analog for, since the dichloro-naphthoquinone was less poisonous than with *S. cerevisiæ*, the dosage of the vitamin K required for reversal was approaching the toxic level.

With the other species examined, no reversal of the action of dichloro-naphthoquinone was accomplished by additions of vitamin K either as 2-methyl-naphthoquinone or as vitamin K_1. This was not surprising, however, for the fact that the inhibition index was less than 1 for this pair of structural analogs indicated that the amount of vitamin needed to exert a reversing effect was beyond the toxic level. Since 20 μg of dichloro-naphthoquinone were needed for half-maximal inhibition of growth of *L. casei*, 200 μg of 2-methyl-naphthoquinone would be necessary to counteract the effect. However, 2-methyl-naphthoquinone was toxic at levels lower than this. If 2-methyl-naphthoquinone were not such a potent inhibitor of microbial growth it might be possible to reverse the action of dichloro-naphthoquinone in more species. Perhaps the failure to reverse the anti-microbial action of 3,3′-methylenebis-(4-hydroxycoumarin)[8] may have a similar explanation, for it is known that the inhibition index for this compound and vitamin K in rats is less than 1 just as in the case of the present example.[9]

With *S. cerevisiæ*, vitamin K_1 was about equal (on a molecular basis) in activity to 2-methyl-naphthoquinone in causing reversal of the effects of dichloro-naphthoquinone, and phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) had 1/30 the potency. This is about the same relative potency of these 3 compounds when tested for their ability to cure vitamin K deficiency in chicks.

If dichloro-naphthoquinone acts in microbes by production of vitamin K deficiency, one would expect that it would cause signs of vitamin K deficiency in animals. Experiments were performed in which graded amounts of the substance were fed to weanling mice maintained on a highly purified adequate basal ration[10] minus vitamin K. The rate of

8 Goth, A., *Science*, 1945, **101**, 383.

9 Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, 1942, **23**, 589.

10 Woolley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 565.

TABLE III.
Effect of 2,3-dichloro-naphthoquinone on Growth and Survival of Weanling Mice Fed a Highly Purified Ration Free of Vitamin K.

Amt in diet, %	No. of animals	Deaths	Avg change in wt, g per wk
0	8	0	+5.0
0.1	4	0	+3.3
0.2	4	0	+1.4
0.5	14	7	—1.0

growth was diminished detectably when 0.1% of the diet consisted of the quinone, and when this was increased to 0.5%, half the animals died within 3 weeks (Table III). The mice which died did not, however, show signs of hemorrhage macroscopically as do mice fed 3,3'-methylenebis-(4-hydroxycoumarin). Furthermore, the prothrombin time of the diluted plasma of mice fed 0.5% of the quinone was not significantly greater than that of the controls or of normal mice. Finally, simultaneous administration of 2-methyl-naphthoquinone or of vitamin K_1 did not erase the toxic manifestations of 2,3-dichloro-naphthoquinone.

Since vitamin K has not been demonstrated in yeast, it may be that dichloro-naphthoquinone and the vitamin are just 2 foreign substances contending non-specifically in the yeast cells, which fall prey to either agent or to both, or to neither, depending on relative and absolute concentrations. However, vitamin K is known to exist in some microörganisms, and to be an essential for growth of the Johne's bacillus.[11] The concentration of the vitamin in yeast may be so small as to be undetectable by present methods, and yet sufficient to supply each cell with many molecules.

From the examples now at hand it would seem that the replacement of methyl groups attached to benzenoid nuclei in metabolites with Cl atoms can lead to inhibitory structural analogs which compete with these metabolites. More examples would be desirable to determine whether it is a type of change which is generally applicable.

Summary. 2,3-dichloro-1,4-naphthoquinone, an antifungal agent now in practical use, has been recognized as an analog of vitamin K. This substance has been found to be exceedingly toxic to yeasts and moderately harmful to the growth of bacteria. Its effect on yeast was reversed competitively by vitamins K over a limited range of concentration. While the effect on the growth of bacteria was not influenced by vitamin K in the form of 2-methyl-naphthoquinone, it was probable that this was due to the toxicity of the vitamin for these species.

11 Woolley, D. W., and McCarter, J. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 357.

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The Porphyrin of Harder's Gland.

EUGENE J. TOWBIN, PAUL E. FANTA, AND HAROLD C. HODGE.
From the Departments of Physiology, Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, N.Y.

The fact that choline poisoning stimulates the flow of brick red tears, designated as chromodacryorrhea,[1] has been reported and confirmed; however, the nature of the pigment, protoporphyrin[2] or blood,[3] is in dispute. The presence of a porphyrin in Harder's

1 Tashiro, S., and Stix, H., *Biol. Bull.*, 1935, **64**, 327.

2 Barnard, R. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 254.

3 Hodge, H. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 26.