dodecylammonium acetate (*8*). This he explained by postulating that, until a critical concentration of surface-active ion is reached, the concentration ratio of the two competing counterions in the surface phase is proportional to the concentration ratio in the bulk solution.

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References and Notes

1. D. A. Haydon, in *Recent Progress in Surface Science*, J. F. Danielli, K. G. A. Pankhurst, A. C. Riddiford, Eds. (Academic Press, New York, 1964), vol. 1; P. Sennett and J. P. Oliver, in *Chemistry and Physics of Interfaces* (American Chemical Society, Washington, D.C., 1965).
2. F. De Korosy, *Nature* **191**, 1363 (1961).
3. L. S. Hersh, *J. Phys. Chem.* **72**, 2195 (1968).
4. There is also the possibility of a different equilibrium distribution of the alkyl trimethylammonium chloride molecule at the two interfaces due to the different KCl concentrations. If this occurs, a membrane diffusion potential caused by the different mobilities of the quaternary and chloride ions would arise and would have an opposite sign to that of the original net Donnan potential.
5. They were given a heat treatment of 3 hours at 580°C followed by cooling at the rate of 80°C per hour. Leaching was performed in 1.0*N* HNO_3 at 95°C for 15 hours. Two washes were applied (i) 8 hours in 0.1*N* HNO_3 at at 95°C, and (ii) 60 hours in 0.05 molal KCl at 95°C. The porous glass had a pore diameter of 62 Å and a surface area of 183 m^2/g as determined by a BET (Brunauer, Emmett, and Teller) analysis with nitrogen. The fixed-charge concentration $\bar{X}$, as determined by the Meyer-Sievers method (*3*), was 0.024 molal.
6. The effect of the alkyl trimethylammonium chlorides on the reference electrodes was checked by the use of a positively charged membrane. The electrodes were checked at regular intervals for proper response without the membranes.
7. The alkyl trimethylammonium chlorides (Lachat Chemicals, Inc.) were 99.5 percent pure. Solution concentrations were checked by K. F. Sugawara, who used a spectrophotometric method coupled with a $CHCl_3$ extraction.
8. D. W. Fuerstenau, *J. Phys. Chem.* **60**, 981 (1956).
9. P. Somasundaran, T. W. Healy, D. W. Fuerstenau, *ibid.* **68**, 3562 (1964).
10. D. C. Grahame, *Chem. Rev.* **41**, 441 (1947).
11. I thank Mrs. M. C. Burke for preparing solutions and making potential measurements.

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Vitamin K and Coumarin Anticoagulants: Dependence of Anticoagulant Effect on Inhibition of Vitamin K Transport

Abstract. *Coumarin anticoagulants inhibit the release of plasma clotting factor VII by vitamin K in liver slices from vitamin K–deficient animals without inhibition of protein synthesis. When the ratio of vitamin K to coumarin anticoagulant is kept constant, but the concentrations are increased, the inhibition disappears. This suggests that the pharmacological action of coumarin anticoagulants depends on irreversible inhibition of normal vitamin K transport to its site of action. At higher concentrations of vitamin K the inhibition can be surmounted, because vitamin K can enter the cell by an alternate route that is not inhibited by coumarin anticoagulants.*

Coumarin anticoagulants act as vitamin K antagonists, since their pharmacological effect is limited to a decrease in the concentrations of the vitamin K–dependent clotting factors of plasma and can be reversed by vitamin K. Our previous work indicated that this antagonism is due to irreversible inhibition of the transport mechanism by which vitamin K normally, that is, at physiological concentrations, reaches its site of action. This inhibition can be bypassed or surmounted, because when larger doses are given vitamin K can reach its site of action by an alternate route, possibly simple diffusion, which is not susceptible to inhibition by coumarin anticoagulants. So far, this explanation has been adequate to interpret the results of many experiments that were designed to test its validity in intact animals (*1*, *2*).

The recent finding (*3*), that addition of vitamin K to liver slices from rats deficient in vitamin K as well as rats treated with a coumarin anticoagulant initiates the release of the vitamin K–dependent clotting factors of plasma into the medium, makes it possible to test the validity of the above explanation in an in vitro system. The results of such a study are reported here.

Liver slices were prepared from vitamin K–deficient rats as well as rats that had been treated with a coumarin anticoagulant (warfarin) whose plasma concentrations of factor VII were less than 5 percent of normal. One gram of slices in 10 ml of bicarbonate buffer containing uniformly labeled ^{14}C-L-leucine (2.6×10^6 disintegrations per minute) was incubated in a Dubnoff shaker at 37°C under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide. After 4 hours, 0.5 ml of the medium was removed and mixed with 0.1 ml of 2 percent (by weight and volume) ethylenediaminetetraacetate (*p*H 7.4), and factor VII was determined by the method of Koller (*4*). The concentration of factor VII in the medium is expressed as the percent of factor VII, on the basis of its concentration in normal rat plasma being 100 percent. In the presence of vitamin K, the appearance of factor VII in the medium increased with time and reached a maximum between the 3rd and 4th hours of incubation.

Protein was isolated from the combined medium and slices by the method of Manchester and Young (*5*) and was assayed for the incorporation of ^{14}C-L-leucine by the liquid scintillation technique after solubilization with NCS (*6*). The efficiency of counting was between 55 and 60 percent.

Figure 1 shows the results obtained with slices from vitamin K–deficient rats. In the absence of vitamin K_1 little factor VII was found in the medium after 4 hours of incubation. Addition of vitamin K_1 at a concentration of $10^{-6}M$, the concentration required for saturation of the specific transport mechanism, increased the amount of factor VII from 1.6 to 12.0 percent. Simultaneous addition of warfarin, at concentrations of 10^{-9} to $10^{-6}M$, partially or completely inhibited this response. There were no significant differences in the incorporation of ^{14}C-L-leucine in the presence and absence of vitamin K_1 or warfarin. Thus, in slices from vitamin K–deficient animals, warfarin inhibited the response to vitamin K at concentrations that have no effect on protein synthesis.

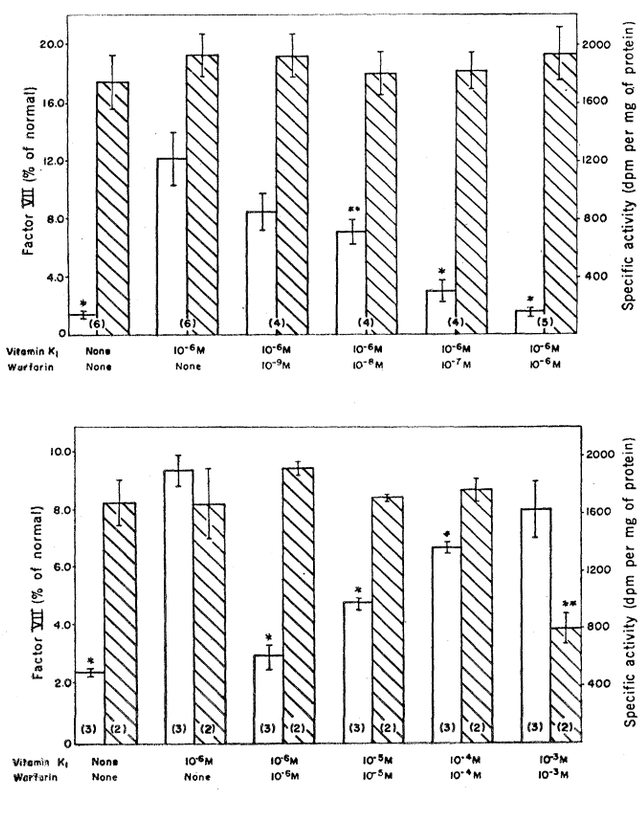
When the experiment was carried out with slices from animals pretreated with warfarin (Fig. 2), vitamin K_1 again increased the amount of factor VII in the medium, but the concentration required to produce an equivalent response was $4 \times 10^{-4}M$ compared to $10^{-6}M$ for slices from vitamin K–deficient animals. A similar, approximately 100-fold difference has been found between the potency of vitamin K_1 in vitamin K–deficient rats compared to those treated with a coumarin anticoagulant (*7*). The concentrations of warfarin required to inhibit the release of factor VII were also higher than in the previous experiment, 10^{-5} to $10^{-3}M$ compared to 10^{-9} to $10^{-6}M$, and at these higher concentrations of warfarin protein synthesis was inhibited. Thus, in slices from animals pretreated with warfarin, the warfarin inhibited response to vitamin K_1 only at concentrations that also inhibited protein synthesis, as measured by incorporation of ^{14}C-L-leucine.

According to the proposed explana-

tion, it can be predicted that in slices from vitamin K–deficient animals the inhibition should disappear if the ratio of vitamin K to warfarin is kept constant, but the concentrations are increased, because at higher concentrations vitamin K would be expected to reach its receptor site by the alternate pathway. This prediction has been shown to hold in vitamin K–deficient animals (*1*). The results of the experiment described in Fig. 3 show that it also holds for liver slices from vitamin K–deficient animals. At a concentration of $10^{-6}M$, warfarin inhibited nearly completely the response to $10^{-6}M$ vitamin K_1. When this ratio of vitamin K_1 to warfarin (1 : 1) was kept constant, a 10- and 100-fold increase in the concentrations caused a partial, and a 1000-fold increase a complete reversal of the inhibition. In agreement with the proposed scheme, the concentrations of vitamin K_1 (10^{-5} to $10^{-3}M$) at which the inhibition was overcome are of the same magnitude as those that led to the production of factor VII in slices from animals pretreated with warfarin, in which only the alternate pathway is initially available.

Before attempting an explanation of the above findings, it will be helpful to compare the effect of warfarin on the in vitro system with its effect in intact animals. In vitamin K–deficient animals and in liver slices from vitamin K–deficient animals, simultaneous administration of warfarin inhibits the response to vitamin K. In contrast, simultaneous administration of warfarin has no effect on the response to vitamin K in animals that had been treated with a coumarin anticoagulant (*1*), but in slices from these animals warfarin inhibits the response to vitamin K. However, this inhibition occurs at concentrations of warfarin (5×10^{-4} to $10^{-3}M$) that also inhibit incorporation of ^{14}C-L-leucine, while the inhibition in slices from vitamin K–deficient animals is produced without concurrent inhibition of incorporation. Furthermore, in the experiment with increasing concentrations of a constant ratio of vitamin K_1 and warfarin (Fig. 3), inhibition of incorporation of ^{14}C-L-leucine is also observed only when the concentration of warfarin is $10^{-3}M$. Thus it appears that the difference between the effects of warfarin on the response to vitamin K in animals pretreated with a coumarin anticoagulant and in liver slices from these animals can be attributed to the high concentrations of warfarin (5×10^{-4} to $10^{-3}M$) that can be tested in the in vitro system. Such high concentrations of warfarin cannot be attained in intact animals, because they produce acute toxic effects. Therefore, the inhibition by warfarin of the vitamin K–initiated release of plasma clotting factors in slices from animals pretreated with a coumarin anticoagulant is not related to its pharmacological action in intact animals. This conclusion also offers an explanation for the observations of Pool and co-workers (*8*), who found that slices from animals pretreated with a coumarin anticoagulant show normal protein synthesis but no formation of vitamin K–dependent clotting factors, and that concentrations of warfarin that inhibit formation of plasma clotting factors in slices from normal animals also inhibit protein synthesis.

At present, the pharmacological effect of warfarin in intact animals, reduction of concentrations of vitamin K–dependent clotting factors of plasma, can be produced in vitro only when vitamin K and warfarin are added simultaneously to slices from vitamin K–deficient animals, because under these conditions warfarin inhibits the uptake of vitamin K. Therapeutic doses of warfarin reduce the concentration of the vitamin K–dependent clotting factors of plasma in normal animals, while analogous



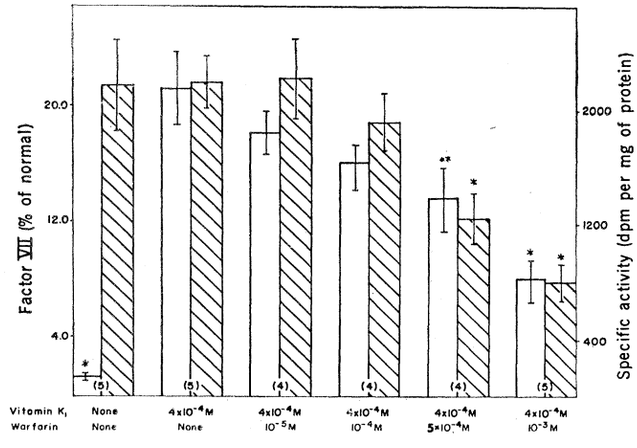


Fig. 1 (upper left). Inhibition by warfarin of the response to vitamin K_1 in liver slices from vitamin K–deficient rats. White columns, concentration of factor VII present (expressed as percentage of normal) in the medium after 4 hours of incubation. Hatched columns, incorporation of ^{14}C-L-leucine into protein. Vertical bars represent standard error of the mean; $*P < .05$ and $**P < .10$ compared to the response of vitamin K_1 alone. Numbers in parentheses are the number of animals used. Fig. 2 (upper right). Inhibition by warfarin of the action of vitamin K_1 in liver slices from rats pretreated with warfarin. See Fig. 1 for explanation of graph. Fig. 3 (lower left). Reversal of the vitamin K antagonism of coumarin anticoagulants by an increasing concentration of a constant ratio (1 : 1) of vitamin K_1 and warfarin in liver slices from vitamin K–deficient rats. In this experiment the incubation period was 3 hours. See Fig. 1 for explanation of graph.

concentrations (10^{-9} to $10^{-6}M$) of warfarin have no effect on slices from normal animals, because slices are not viable for a period sufficient to deplete them of the vitamin K already at the site of action. The results reported here, therefore, indicate the need for a reinterpretation of the significance of previous studies of the effects of warfarin and other indirect anticoagulants on isolated tissue preparations.

Finally, it has not escaped our notice that in slices from vitamin K–deficient animals, warfarin appears to inhibit the release of factor VII without inhibiting protein synthesis. However, this does not necessarily indicate that the appearance of factor VII requires no *de-novo* protein synthesis. Since the synthesis of factor VII and other vitamin K–dependent clotting factors probably is only a very small fraction of the total protein synthesis carried out by the liver, specific inhibition of factor VII synthesis might not have been detected by the method used to measure total protein synthesis. Other workers (*10–13*), studying the effects of specific inhibitors of protein synthesis in intact animals and in in vitro systems on the response to vitamin K, have failed to show conclusively whether the response depends on *de novo* protein synthesis or on the transformation and release of precursors of clotting factors.

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References and Notes

1. J. Lowenthal and J. A. MacFarlane, *J. Pharmacol. Exp. Therap.* **143**, 273 (1964).
2. ———, *ibid.* **157**, 672 (1967).
3. J. Lowenthal and E. L. Simmons, *Experientia* **23**, 421 (1967).
4. F. Koller, A. Loeliger, F. Duckert, *Acta Haematol.* **6**, 1 (1951).
5. K. L. Manchester and F. G. Young, *Biochem. J.* **70**, 353 (1958).
6. D. L. Hansen and E. T. Bush, *Anal. Biochem.* **18**, 320 (1967). NCS is the trade name for the protein solubilizer obtained from Nuclear-Chicago Corp., Des Plaines, Ill.
7. J. Lowenthal and J. D. Taylor, *Brit. J. Pharmacol.* **14**, 14 (1959).
8. J. G. Pool and C. F. Borchgrevink, *Amer. J. Physiol.* **206**, 229 (1964).
9. A. M. Paolucci, P. B. Rama Rao, B. C. Johnson, *J. Nutr.* **81**, 17 (1963).
10. B. M. Barbior, *Biochim. Biophys. Acta* **123**, 606 (1966).
11. J. P. Olson, L. I. Miller, S. B. Troup, *J. Clin. Invest.* **45**, 690 (1966).
12. J. W. Suttie, *Arch. Biochem. Biophys.* **118**, 166 (1967).
13. R. B. Hill, S. Gaetani, A. M. Paolucci, P. B. Rama Rao, D. V. Shah, V. K. Shah, B. C. Johnson, *J. Biol. Chem.* **243**, 3930 (1968).
14. Supported by research grants from the Medical Research Council of Canada and the Ontario Heart Foundation. This study constitutes part of H.B.'s Ph.D. thesis to be submitted to the department of pharmacology.

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Crystal and Molecular Structure of a Thymine-Thymine Adduct

Abstract. *Thymine-thymine adduct is a product isolated from thymine irradiated with ultraviolet light in frozen aqueous solution. This compound is presumably formed through the rearrangement of an initial photoproduct. Single crystal x-ray diffraction analysis has confirmed the molecular formula of the adduct, 5-hydroxy-6-4′-(5′-methylpyrimid-2′-one)-dihydrothymine, except for the possibility of a hydrogen atom on the 3′ nitrogren rather than the 1′ nitrogen, and has established the stereoconfiguration of the molecule.*

Ultraviolet irradiation of frozen aqueous solutions of thymine and of DNA has yielded cyclobutane-type dimers of thymine (*1*). Recently, a different kind of thymine-thymine product has been isolated from the ultraviolet irradiation of frozen aqueous solutions of thymine (*2*). From spectroscopic evidence the product was deduced to be 5-hydroxy-6-4′-(5′-methylpyrimid-2′-one)-dihydrothymine (*2*). For proof of the structure, a crystal of the adduct was subjected to an x-ray diffraction analysis.

X-ray diffraction data for a single crystal of the thymine-thymine adduct were collected photographically about two axes by the multiple film, equi-inclination Weissenberg technique for a total of 1844 independent reflections. The material crystallizes in the centrosymmetric triclinic space group of $P\bar{1}$ with two molecules per unit cell. The cell parameters are as follows: a, 9.44± .02 Å; b, 8.29 ± .02 Å; c, 7.57 ± .02 Å; α, 99.0°; β, 91.5°; and γ, 89.8°.

The crystal structure was solved directly by the symbolic addition procedure (*3*), and all the atoms were located on the first E-map. The coordinates and thermal parameters were refined by least-squares methods and the hydrogen atoms were found in a difference map. The agreement between observed and calculated structure factors is 10.2 percent. The molecular structure (Fig. 1) shows that the methyl group on carbon-5 of ring I and ring II on carbon-6 of ring I are both axial to ring I. The structure analysis indicates that an H atom is located on the 3′ nitrogen rather than the 1′ nitrogen.

Since the material crystallizes in a centrosymmetric system, the two molecules in the unit cell are a racemic pair. Ring II is planar to within ± .02 Å, while ring I is in the half-chair conformation with carbon-5 0.4 Å below and carbon-6 0.2 Å above the plane of the other four atoms. The angle of inclination between the two rings is approximately 96° (Fig. 2). Bond lengths in the dihydrothymine moiety are quite similar to those found for the molecules of dihydrothymine (*4*) and dihydrothymidine (*5*). A water of hydration crystallizes with the T-T adduct and is included in an extensive system of hydrogen bonding in the crystal.

The complete elucidation of the stereoconfiguration of T-T adduct proves its molecular structure and may

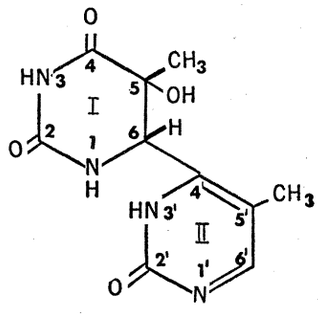

Fig. 1

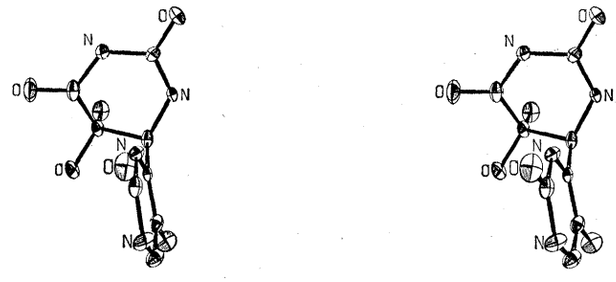

Fig. 2. Stereodiagrams of the configuration of thymine-thymine adduct as determined by x-ray analysis. The picture should be seen with a three-dimensional viewer for printed stereophotographs (*7*).