the malarial parasites must entail the consideration of the host cell. When considering a few recent developments, the conclusion is unavoidable that the biosynthesis of fatty acids in red cells is probably of a minor order, if it exists at all (*12*).

Recent studies from several laboratories have established that red blood cells are capable of incorporating fatty acids into their phosphoglycerides in vitro (*13–15*). Erythrocytes of various animal species appear to exhibit this feature, but intact red cells of oxen and sheep are less active in vitro than those of rat, rabbit, and man. Though no study on this subject is available for monkey erythrocytes, it is reasonable to assume that they will behave like the erythrocytes of man, rabbit, and rat rather than those of oxen, sheep, and other ruminants. Significant differences were observed in the rate of esterification among various fatty acids. In rabbit erythrocytes, a dominant activity was observed for linoleic acid, but oleic and palmitic acids also were more extensively incorporated than were stearic, myristic, and lauric acids (*15*). Though there is no information available on the red cell metabolism of stearic acid, the most striking feature of the liver metabolism of labeled stearic acid in vitro (*16*) and in vivo (*17*) is the very active desaturation of stearic acid to oleic acid. The actual mechanism of this action still remains unknown. Activation of stearic acid to stearoyl-CoA is essential for desaturation in this system; stearoyl-CoA is therefore presumably the true substrate, the immediate product presumably being oleoyl-CoA (*17*).

On the basis of the present data we cannot assign a specific role for stearic acid in the growth and development of *P. knowlesi*. Since erythrocytes apparently incorporate fatty acids (*15*) rather than synthesize them (*12*), the fate of labeled stearic acid should provide information on its ability to support the growth and multiplication of *P. knowlesi* and possibly other species of human and simian plasmodia. Recent in vitro studies with *Plasmodium fallax* demonstrated that stearic, palmitic, and oleic acids labeled with C^{14} are incorporated into phospholipids of infected turkey erythrocytes (*18*).

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28 March 1967

Potency Difference between the Zwitterion Form and the Cation Forms of Tetrodotoxin

Abstract. *Tetrodotoxin can exist as a zwitterion or two different cations in aqueous solutions. The cation forms are more potent in nerve block. Thus the lactone-hemilactal configuration of tetrodotoxin influences potency. Electrostatic attraction, hydrogen bonding, and intermolecular hemilactal formation are possible mechanisms of interaction with membrane receptors.*

After the chemical structure of tetrodotoxin was worked out, it was reported that it could exist in equilibrium with two other forms (see Fig. 1). One of the hemilactal forms ($B^{\pm}$ in Fig. 1) has an electronegative charge at the oxygen, as indicated, while the other (BH^+) has a proton added to the oxygen. Thus, form $B^{\pm}$ is a zwitterion, while forms AH^+ and BH^+ are cationic. The relative mole fractions of the three forms depend upon the equilibrium constant (k) and the pK_a of BH^+. According to Goto *et al.* (*1*), the pK_a value for the protolysis $BH^+ + H_2O \rightleftharpoons H_3O^+ + B^{\pm}$ is 8.76; Tsuda *et al.* (*2*) report the value is 8.84. The guanidinium group which gives an electropositive charge to the molecule should be associated with a pK_a value of about 11 or 12. We know that tetrodotoxin has potent blocking action on many electrical excitation phenomena (*3*). This block appears to be specific for the early permeability change, usually associated with Na^+ influx. By altering the *p*H of a physiological solution containing tetrodotoxin over a reasonable range such as 7 to 9, it should be possible to determine whether the zwitterion is more or less potent in blocking excitation than the cationic forms. This in turn might tell us something about a possible mechanism for interaction of tetrodotoxin with a receptor site.

The preparation consists of the isolated sciatic nerve of the frog *Rana pipiens* set up in a manner similar to that of others (*4*). Since the nerve sheath constitutes a barrier to blocking drugs, the tests were all carried out on a desheathed portion of the nerve. The kinetics of block can be

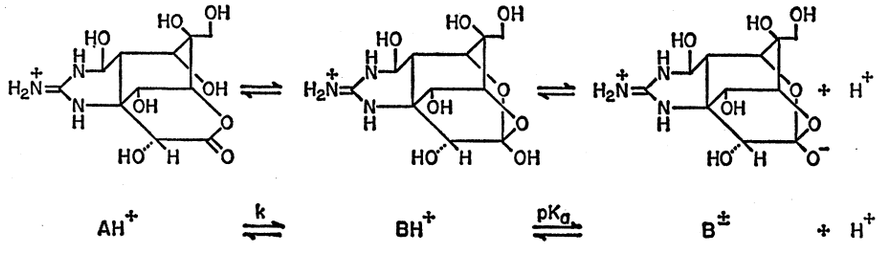

Fig. 1. Equilibria of tetrodotoxin in water solution, showing the distribution of the three molecular species. AH^+ and BH^+ are cations, while $B^{\pm}$ is a zwitterion. [Modified from Woodward (*10*)]

studied readily by observing the reduction in amplitude of the A spike of the compound action potential, as a function of time, on the cathode-ray oscilloscope. The tetrodotoxin was tested in three different bathing solutions. One solution contained 111.2 mmole of NaCl, 1.9 mmole of KCl, 1.1 mmole of $CaCl_2$, 2.4 mmole of $NaHCO_3$, 0.1 mmole of NaH_2PO_4, and 11.1 mmole of glucose. This solution we will call normal frog Ringer (NFR). The second solution contained 111.2 mmole of NaCl, 1.3 mmole of KCl, 1.1 mmole of $CaCl_2$, and 8 mmole of 2-amino-2-hydroxymethyl-1, 3-propanediol (tris) buffer. This we will call tris buffer Ringer (TBR). The third solution had 8 mmole of imidazole instead of tris and will be called IBR. The concentration of salts for TBR and IBR are from Mauro *et al.* (*4*) and are used routinely in this laboratory. The concentration of tris and imidazole buffers is the same as the tris used by Ritchie *et al.* (*5*), who did similar experiments by altering the *p*H of solutions containing conventional local anesthetics. The *p*H was changed over the range of approximately 7 to 9 by adding appropriate amounts of NaOH or HCl.

Table 1. Mole percent of the three molecular species of tetrodotoxin in aqueous solution at *p*H 7.05 and 8.90.

Molecule species	Mole percent		Ratio: conc. at *p*H 7.05 to conc. at *p*H 8.90
	*p*H = 7.05	*p*H = 8.90	
	k = 1		
AH^+	49.5	29.6	1.67
BH^+	49.5	29.6	1.67
$B^{\pm}$	0.965	40.8	0.02
	k = 9		
AH^+	9.83	4.46	2.20
BH^+	88.4	40.1	2.20
$B^{\pm}$	1.72	55.4	0.03

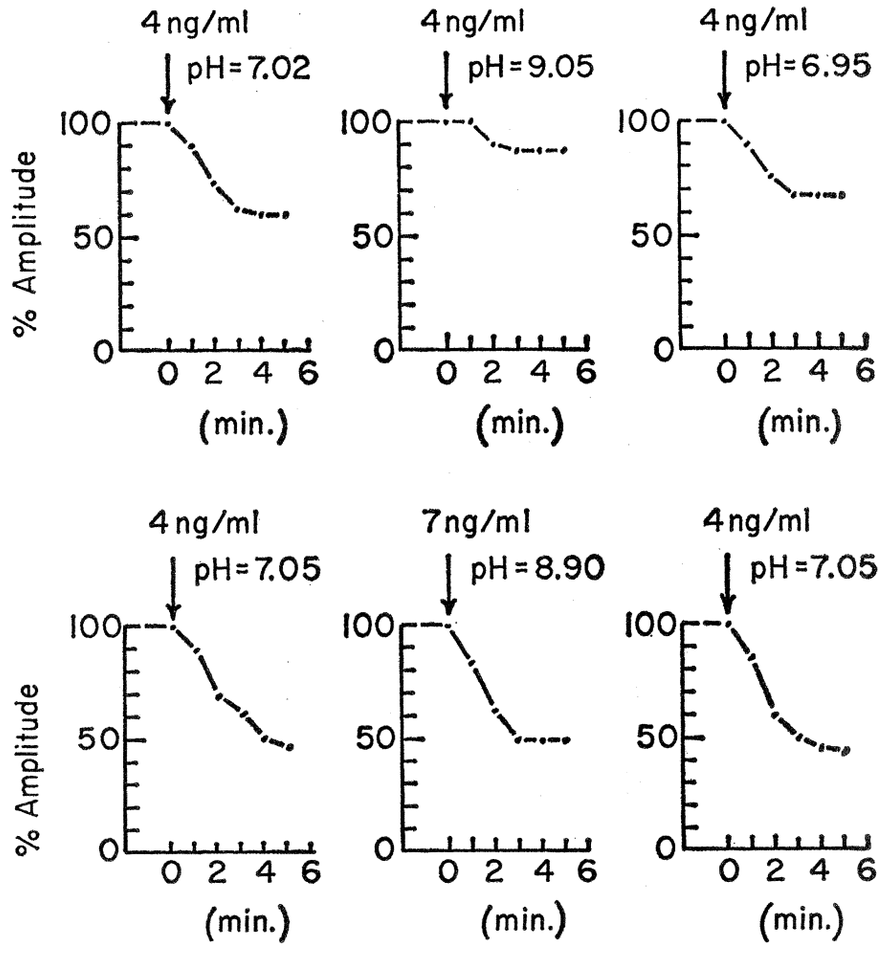

Fig. 2. Blocking curves for the compound action potential of the frog sciatic nerve. Tetrodotoxin in tris buffer Ringer was administered to a desheathed region of the nerve; the concentration and *p*H value of each application are indicated. In the upper series of three curves, the same blocking solution was used for all three blocks after adjusting the *p*H each time. In the lower series of curves, an attempt was made to find equi-effective concentrations. The duration for drug application was constant at 5 minutes; stimulus frequency was 30 per second.

The potency of block was greater at lower *p*H values with all three types of bathing solutions. The three successive blocking curves on the same nerve bathed in TBR shown in the upper part of Fig. 2 illustrate the results very clearly. Since tetrodotoxin is somewhat unstable chemically at alkaline *p*H, the obvious control of changing the *p*H of the same blocking sample back to *p*H 7 was carried out numerous times. Since identical results were seen with all three buffer systems, this would indicate that the buffering compounds themselves were not responsible for the change in excitation. However, to eliminate the possibility of any effects attributable to the *p*H change itself, electrophysiological control studies of nerves bathed in all three solutions at *p*H 7 and 9 were carried out. Curves of conduction velocity, spike amplitude, and strength duration were practically identical. Ritchie *et al.* (*5*) also report that *p*H changes in this range do not alter excitation to any degree. We are aware that desheathing the nerve can cause changes in the properties of the nerve fibers (*6*). However, any such changes should be constant for all experiments.

An attempt was made to determine the equi-effective molar concentrations of tetrodotoxin at the two *p*H values. An example is seen in Fig. 2. As the *p*H was changed from 7.05 to 8.90, the concentration had to be approximately doubled. Table 1 shows the calculated values of the relative concentrations of the three tetrodotoxin species in solution at *p*H 7.05 and 8.90, assuming a *k* value in the range 1 to 9 and pK_a equal to 8.76. In the almost equi-effective solutions of 4 ng of tetrodotoxin per milliliter at *p*H 7.05 and 7 ng/ml at *p*H 8.90, the ratio between the concentrations of the cationic species varies from 0.96 to 1.3 as we let *k* (see *7*) vary from 1 to 9. The one-to-one relationship between the biological responses to the two solutions is thus paralleled by a similar relationship for the concentrations of AH^+ and BH^+ at the two *p*H values.

Since the experimental results show that the concentration of tetrodotoxin had to be increased to maintain equal potency at *p*H 8.90, this indicates clearly that the cationic forms are more potent than the zwitterion. Were the zwitterion the active form, the potency of the solution should increase by a sizable factor as the *p*H is changed from 7.05 to 8.90. As can be seen in

Table 1, the relative amounts of both AH^+ and BH^+ increase by a factor of about 2 as the *p*H is changed from 8.90 to 7.05.

The electric charge of the guanidinium group should not change over the *p*H range used in this study. This implies an essential role for the hemilactal-lactone part of the molecule. Since there are two cationic forms in solution, it is difficult to determine which is the active form. There are a number of possible mechanisms to explain the interactions of both cations with receptor sites.

First, there is the possibility that the absence of the electronegative charge decreases the electrostatic repulsion by negative sites of the membrane surface (Fig. 3A). This mechanism applies equally to both cations.

Second, the protonic hydrogen of BH^+ may serve to form a hydrogen bond between the tetrodotoxin molecule and the receptor site (Fig. 3B). Since tetrodotoxin is now thought to act on the outside of the cell membrane (*8*), the receptor is most probably a protein. However, hydrogen bonding may also occur with phosphate groups. Since surfaces of proteins themselves are sensitive to *p*H change, we must not overlook the possibility that the potential binding sites are affected by the *p*H changes. This might happen without changes in any of the electrophysiological parameters that we measured.

A third possibility would be that the tetrodotoxin first binds to a negative receptor site by the guanidinium group. If then a sterically suitably situated hydroxyl group on the receptor could react to form an intermolecular hemilactal configuration preferentially to the intramolecular reaction leading to BH^+, a secondary anchoring would take place between AH^+ and the receptor (Fig. 3C).

In conclusion, whatever the mechanism, this study supports the statements of Narahashi *et al.* (*9*) that more than the guanidinium group is involved in determining the potency of tetrodotoxin. More specifically, we have shown that the lactone-hemilactal configuration of the tetrodotoxin molecule is also important.

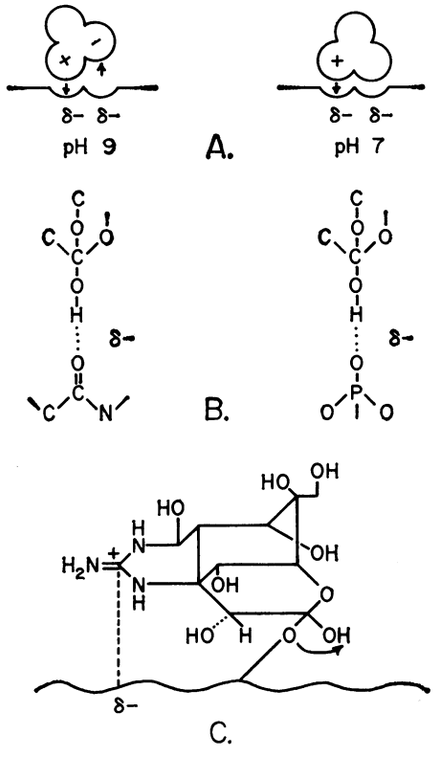

Fig. 3. (A) Models of the molecular interaction of tetrodotoxin with a receptor site on the outer layer of an excitable membrane. The zwitterion is partially repelled by electronegative regions of the cell membrane. The cationic form has less repulsion, thus resulting in a higher probability of proper steric interaction with the receptor site. (B) The structural formulas show possible interactions of the hemilactal part of BH^+ with an electronegative oxygen of a peptide chain, or an electronegative oxygen of a phosphate group by hydrogen bonding. (C) Postulated interaction of tetrodotoxin with a receptor site by electrostatic attraction and secondary intermolecular hemilactal formation.

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8 March 1967

Crystallization of a Sulfate-Binding Protein (Permease) from Salmonella typhimurium

Abstract. *Crystallization is reported of a protein that is a component of an active transport system for sulfate into* Salmonella typhimurium. *This appears to be the component that is specific for binding the substrate.*

Isolation of a protein with high specific affinity for a single sulfate anion has been reported (*1*). This protein was pure by the usual physical-chemical criteria. Its crystallization is reported here.

Purified protein (4 mg lyophilized from distilled water) was taken up in 0.55 ml of 1.8 m*M* potassium phosphate buffer, *p*H 7.0, at 24°C, and then 1.0 ml of 2-methyl-2,4-pentanediol (*2*) was added to produce a turbid suspension. Distilled water was added (0.3 ml) until the turbidity nearly disappeared. A few seed crystals, which were obtained by a similar procedure after 50 days at 4°C, were added and the tube was stoppered and kept at 4°C. Crystals were observed after 7 days (Fig. 1); the largest were more than 0.1 mm long. They were quite birefringent in the polarizing microscope.

The crystals were collected by centrifugation, washed with cold 80 percent

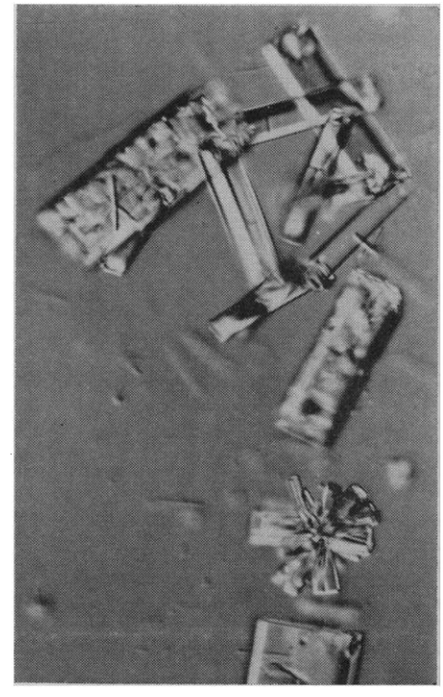

Fig. 1. Crystals of sulfate-binding protein. Nomarski optics were used. The largest crystal shown was 0.18 mm long.