modifications would probably be without effect. Fifty-percent reductions in the amounts of most proteins would scarcely be lethal unless the modified protein chains themselves were the lethal agent (a dominant effect).

There are three known genes (*14*) in RNA phages, and it was expected that several types of *azure* mutants would be found. However, all mutants tested so far appear to be similar. Either there is a mutational "hot spot," which is especially sensitive to nitrous acid, or mutants in the other functions are so "leaky" as to be undetectable.

The *azure* mutants that we isolated are restricted early in their growth, but when they escape this early restriction, they proceed to grow normally. Putting together what is known about RNA phage physiology (*18*) with the properties of these mutants, we offer the following interpretation of the results. The f2 *azure* mutations are at the codon that normally results in termination of the chains of the phage RNA polymerase. The polymerase itself is a multimeric protein. Each monomer that is synthesized has a probability of being properly terminated equal to the frequency of chain-termination in the different host bacteria. However, the improperly terminated monomers might still participate in forming the multimeric enzyme. Should any of the monomers be abnormal, an irreversible block in replication of the input-RNA occurs. Alternatively, the proper number of monomers must assemble by a fixed time or the RNA is inactivated. These kinds of models provide the necessary amplification of suppression. Were the probability of proper chain-termination 0.5 each time a protein chain was synthesized, then in only 6 percent of the bacteria would there be a tetramer, for example, with all four chains properly terminated. Such a hypothesis also aids in explaining why efficiency of the formation of infective centers is a function of the number of input-phage particles and why the *azure* mutants cannot be rescued. It is the protein molecules synthesized directly from the input-RNA that are involved.

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Stearic Acid as Plasma Replacement for Intracellular in vitro Culture of Plasmodium knowlesi

Abstract. *A chloroform extract of Cohn's fraction IV-4 of human plasma successfully replaced whole fraction IV-4 for the intracellular in vitro culture of* Plasmodium knowlesi. *We are now able to report the successful replacement of monkey plasma by stearic acid.*

Recently we reported (*1*) the successful replacement of monkey (*Macaca mulatta*) plasma by Cohn's fraction IV-4 (*2*) of human plasma for the intracellular in vitro cultivation of *Plasmodium knowlesi*. While this was significant progress toward our goal of attaining a truly chemically defined growth medium, it was emphasized that fraction IV-4 is a complex mixture of known and unknown materials (*3*). Experiments were conducted to identify the substance(s) which might replace fraction IV-4. By fractionation of fraction IV-4 it was found that a chloroform extract successfully replaced fraction IV-4, suggesting that the active material might be a lipid. Proceeding from this result we are now able to report the successful replacement of monkey plasma by stearic acid for the intracellular in vitro cultivation of *P. knowlesi*.

The techniques for maintaining, culturing, counting, and evaluating the malarial parasites were based on those previously described (*1*, *4*). The synthetic medium originally described by Anfinsen *et al.* (*5*) was modified as described previously (*1*). The technique for the preparation of plasma-free host cells has also been reported previously (*1*).

In order to ascertain the nature of the active materials in fraction IV-4, a simple fractionation was employed. Two and one-quarter grams of Cohn's fraction IV-4 of human plasma (Nutritional Biochemicals Corp.) were suspended in glass-distilled water and made up to a final volume of 15 ml. The suspension was cooled to 4°C and, over a period of 2 hours, 10.6 g of "enzyme grade" ammonium sulfate (Nutritional Biochemicals Corp.) were added in small portions with continuous stirring. After the salt ceased to dissolve, the stirring was continued for an additional half hour. Undissolved crystals were removed by centrifugation at 2000 rev/min for 10 minutes. The suspension was drawn off and filtered through a microfiber glass disc (type AP20, Millipore Corp.) with suction. The filtrate was labeled fraction A and was dialyzed against glass-distilled water until a test for SO^{--}_4 in the external liquid was negative. The precipitate (fraction B) was suspended in 15 ml of water and dialyzed in the same manner as was fraction A.

Half of fraction A was extracted three times with equal volumes of chloroform. The interface material and the aqueous layer were drawn off and air was passed through the liquid until there was no longer an odor of chloroform. This was labeled fraction C. The combined chloroform layers were evaporated to dryness by warming to 60° to 70°C. The residue was dissolved in water (volume equal to that of fraction A originally extracted). Air was passed through to remove any residual chloroform. This solution was labeled fraction D. For addition to the cultures

Table 1. Effect of fractions of Cohn's fraction IV-4 of human plasma on in vitro cultivation of *Plasmodium knowlesi* in the absence of plasma.

Additions to culture medium	Parasites per 100 red blood cells		Ratio 24/0 hours	Degenerate and extracellular forms (%)
	At 0 hour	At 24 hours		
Fraction IV-4	3.2	4.1	1.3	4
Fraction A of IV-4	3.2	2.6	0.8	12
Fraction B of IV-4	3.2	2.9	.9	6
Fraction C of IV-4	3.2	2.2	.7	6
Fraction D of IV-4	3.2	3.6	1.13	2

summarized in Table 1 corrections were made for dilution that occurred upon dialysis, so that the amount of the individual fraction added to the culture corresponded to that which would have been present in the amount of fraction IV-4 previously found to be effective (*1*).

The effect of fractions of Cohn's fraction IV-4 of human plasma on the in vitro cultivation of *P. knowlesi* in the absence of plasma is shown in Table 1. Results indicate that growth and multiplication of parasites in tubes containing fraction D (chloroform extract) of IV-4 were nearly as good as in control tubes with fraction IV-4. In this experiment 90 percent of the parasites were in the early ring stage when the experiment was started. Differential counts made at the end of 24 hours indicated that in tubes with fractions A, B, and C, separately, 80 to 90 percent of the parasites developed only to the mature trophic stage, whereas in tubes with fraction D, 40 to 50 percent developed to schizont and segmenter stages similar to those in tubes with the whole fraction IV-4. Such results suggest that a substance or substances in the chloroform extract of fraction IV-4 are required for intracellular asexual development of this strain of *P. knowlesi*. On the basis of these results and the reports in the literature that certain lipids have shown beneficial effects for in vitro cultivation of other organisms (*6–8*), stearic, oleic, linoleic, and palmitic acids were tested in our system. Stearic acid was the most effective.

In a series of four experiments conducted to test the effect of stearic acid on the growth and multiplication of *P. knowlesi* in a plasma-free medium, two sets of controls were used, one without and the other with plasma. Tubes in all experiments were run in duplicate. The results in Table 2 indicate that the growth and multiplication of parasites in tubes containing synthetic medium without plasma were very poor in comparison with the growth and multiplication of parasites in tubes containing plasma and synthetic medium. However, in tubes in which synthetic medium was supplemented with stearic acid, growth and multiplication of the parasites approached the results in control tubes with plasma. The percentage of degenerate and extracellular forms of parasites was higher in tubes containing only synthetic medium than in the control tubes with plasma. When the synthetic medium was supplemented with stearic acid, the percentage of these forms was approximately as low as in the control tubes with plasma.

Table 2. Effects of stearic acid and of plasma for in vitro cultivation of *Plasmodium knowlesi*.

Additions to culture medium	Parasites per 100 red blood cells		Ratio 24/0 hours	Degenerate and extracellular forms (%)	Glucose utilized (μmole/ml of culture)
	At 0 hour	At 24 hours			
		Experiment No. 66			
None	1.4	1.7	1.2	40	3.6
Stearic acid	1.4	7.4	5.3	6	4.8
Plasma	1.9	10.35	5.4	2	6.35
		Experiment No. 68			
None	1.8	2.2	1.2	28	1.1
Stearic acid	1.8	7.1	4.0	5	3.65
Plasma	2.5	8.8	3.5	3	3.75
		Experiment No. 75			
None	2.4	4.15	1.7	26	2.9
Stearic acid	2.4	7.6	3.2	8	5.35
Plasma	2.5	10.0	4.0	5	7.6
		Experiment No. 76			
None	2.55	4.8	1.9	27	3.5
Stearic acid	2.55	9.7	3.8	7	5.2
Plasma	2.8	12.0	4.3	3	6.35

Oleic, palmitic, and linoleic acids were also tested individually in the same manner as stearic acid, but the growth and multiplication of *P. knowlesi* in the presence of these lipids were as poor as in medium without plasma. These fatty acids were not pure, and thus comparison with purified stearic acid may not be justified.

Analysis of glucose in the cultures has been a routine procedure in our laboratory, and the extent of multiplication in such experiments can be fairly accurately predicted from the magnitude of glucose utilization throughout the culture period. It is evident from the data presented in Table 2 that the utilization of glucose was 50 to 75 percent less in tubes containing only synthetic medium than in the control tubes with plasma. When the synthetic medium was supplemented with stearic acid, the amount of glucose utilization approached that observed in the control tubes with plasma. From the results presented here, we can conclude that stearic acid can be substituted for plasma for the intracellular in vitro cultivation of *P. knowlesi*.

The ability of fraction IV-4 to replace plasma for growth of *P. knowlesi* was found to be dependent upon the concentration of IV-4 (*1*). A similar concentration dependence is observed for stearic acid. Lower concentrations of stearic acid (0.013 m*M* and 0.026 m*M*) did show adequate growth, but higher concentrations (0.080 m*M* and 0.106 m*M*) were definitely inhibitory. This result may be related to the effect observed by Dubos (*9*), who reported that low concentrations of certain organic acids stimulated growth of tubercle bacilli, but higher concentrations caused complete inhibition of growth.

Growth requirements for long-chain fatty acids have been repeatedly demonstrated for bacteria (*10*). More recently, the demonstration of the use of stearic acid as a replacement for serum was made for trypanosomes (*6*) and *Paramecium* (*7*). Pathogenic trichomonads required both stearic and oleic acids to replace plasma (*8*), and *Histomonas meleagridis* was shown to require cholesteryl stearate to replace cream (*11*). Though these lipids have been shown to be growth factors for the protozoa mentioned, the role of these materials is unknown.

Any discussion on the lipid requirement of an intracellular organism like

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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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

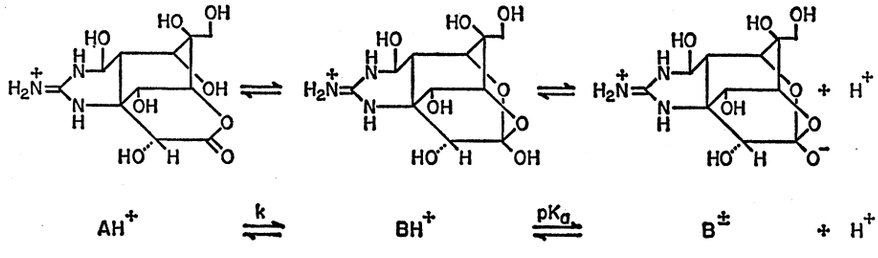

Fig. 1. Equilibria of tetrodotoxin in water solution, showing the distribution of the three molecular species. AH^+ and BH^+ are cations, while $B^{\pm}$ is a zwitterion. [Modified from Woodward (*10*)]