FACTORS AFFECTING EXFLAGELLATION IN CULTURED *PLASMODIUM FALCIPARUM* GAMETOCYTES

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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Dr. Samuel K. Martin

(Walter Reed Project,KEMRI).

DEDICATION

To my husband Mr. Wilson Odhiambo, children Franklin, Denis and more so to youngest Master Sam Martin Odhiambo.

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ACKNOWLEDGEMENTS

I am grateful to Dr. Aba Nwachukwu of Zoology Department, University of Nairobi for her supervision without which the whole study would not have been possible.

I am indebted to Dr. Samuel K. Martin, of Clinical Research Centre (CRC), Kenya Medical Research Institute (KEMRI) for introducing me to malaria research and his practical help, technical expertise and supervision which made the entire study a reality.

Sincere thanks are due to Dr. C. R. Roberts, the Director, Walter Reed Project (Kenya) for sponsoring my whole study at the University including funding of the thesis. This work was realised as a result of Walter Reed Project's generous financial and material support, including importation of a Phase Contrast Microscope with closed circuit television CA I kappa - S-VHS TV camera connected to a Carl Zeiss axioskop research teaching microscope and a CFM/15/2 high resolution colour 14" monitor with S-VHS input.

I wish to acknowledge the Director, KEMRI, for granting me permission to pursue the study to the end. Thanks to Dr. J.I. Githure, the Director Biomedical Sciences Research Centre (BSRC) for giving me permission to carry out the research project at the BSRC. I am grateful to Dr. Baldip Khan, the head of Malaria Laboratories in BSRC for advice and material support during the study. I am obliged to Dr. W. M. Watkins, the Director of Wellcome Trust laboratories, Nairobi for material support.

I am grateful for the technical expertise which was provided by Mr. Josephat Kabui Mwangi of Walter Reed Project (Kenya) and practical training provided by Dr. Sam Martin, Dr. Baldip Khan and Mr. A. V. O. Ofulla. Thanks for the good advice and encouragement from both the directors of CRC and WRP, Drs. J. B. O. Were and C. R. Roberts respectively. More technical advice was provided by Mr. Daniel Kariuki, Mr. Steve Kaniaru, Mr. Fredrick Kiarie, Mr. James Gitonga, Mr. Timothy Kamau, all of KEMRI and Mr. Joseph Koros from WRP. I am indebted to all of them.

Last but not least I wish to thank Dr. R. S. Copeland. Mr. Joseph Mulehi and Mr. Isaac Ouma Odero of WRP and Mr. Reuben Agwanda (Biostatistics of Medical Research Centre- KEMRI) for generous assistance with data analysis and final preparation of this thesis. Not to forget Mr. Francis Shukhuban and Joe Mwaura of ILRAD for the graphics.

ABSTRACT

Mature gametocytes transform into male and female gametes immediately after removal from the vertebrate bloodstream by a blood sucking insect or by venipuncture and exposure to air. Cultured gametocytes are routinely induced to undergo exflagellation by exposure to air in a petri dish. Of all the changes that take place in the environment of the gametocytes, temperature was found to be the over riding factor responsible for holding gametocytes in the inactivated state. At temperatures above 30^oC, mature cultured *P. falciparum* gametocytes remain quiescent in 10% serum even at low pCO_2 tension, alkaline pH (7.9) and 25 mM bicarbonate. When the temperature of the medium was allowed to drop below 30^0C, gametocytes emerged and microgametocytes consistently exflagellated at pH 7.4 even in the absence of bicarbonate ion.

Phosphoinositide (PI) hydrolysis has been reported in the induction of several biological functions like the activation of platelets, mast cells, leukocytes, and the secretion of hormones,but these reactions have not been shown to occur in parasitic protozoa. Both second messengers, Inositol trisphosphate {Ins (1,4,5)P3} and diacylglycerol (DAG), associated with PI hydrolysis have been identified in the activated gametocytes. To further investigate the role of PI hydrolysis products in malaria gametogenesis, compounds that have an effect on the metabolism and biological functions of these messengers were tested in an *in vitro* system. 2,3 Diphosphoglycerate (2,3 DPG) and magnesium (Mg^{2+}), inhibitors of Ins (1,4,5)P3 phosphatase; calcium ionophore, A-23187 and lipid soluble DAG analogue, 1-oleoyl-2-acetyl-glycerol (DAG) all stimulated exflagellation in suspended animation buffer, pH 7.4, at room temperature (23^0 C). In addition, alkaline pH; bicarbonate; methylxanthine inhibitors, caffeine and theobromine and the aminoglycoside, gentamycin also stimulated exflagellation.

In contrast, phospholipase inhibitors, quinine and chloroquine, inhibited all the above compounds tested.Neomycin, an aminoglycoside that inhibits

phospholipase-C activity also inhibited the above triggers as did heparin, an antagonist of the Ins (1,4,5) P3 receptor.None of these permissive conditions initiated exflagellation at 37^{0}C.

Our understanding of the biochemical and physiological mechanisms fo initiation of the sexual cycle of this parasite may lead to new strategies for controlling the transmission of malaria.

CHAPTER 1

1.0 INTRODUCTION

The aetiological agent for the disease malaria, is a protozoan of the genus *Plasmodium* which is transmitted by various species of *Anopheline* mosquitoes. Malaria has been reported to occur in several countries extending from 60°N to 40°N but its distribution is non-uniform owing to variations in climate, vector density, transmission efficiency of vectors, size of parasite reservoir and frequency of human/vector contact. The sexual cycle of the parasite occurs in the mosquito gut and commences immediately after gametocytes are ingested in the blood meal. Since the sexual cycle is responsible for disease transmission, there would be no transmission if gametocytes do not exflagellate or if exflagellation takes place in the vertebrate hosts. The sexual cycle is equally important to the parasite because it provides the opportunity for gene recombination.(Trager,1986).

Most work in malaria research has been on the asexual cycle which causes morbidity and mortality. Even though this aspect is important, more attention needs to be given to the gametocyte which is ultimately responsible for the transmission of this disease. Most of the work that has been done on gametocytes has been on near relatives of *Plasmodium* e.g. *Hemoproteus*, *Hepatocystis* or avian malaria such as *Plasmodium gallinaceum* and *Plasmodium elongatum*. and *P. berghei* (Bishop and

MacConnachie,1960 ; Martin *et al* , 1978; Nijhout,1979: Kawamoto *et al* 1991)

Following the establishment of techniques for the continuous culture of *P. falciparum* (Trager and Jensen, 1976), mature gametocytes have been successfully cultured *in vitro* (Carter and Beach,1977; Smalley and Sinden,1977; Carter and Miller,1979; Ifediba and Vanderberg,1981; Ponnudurai *et al*, 1982 a,b; Sinden *et al*, 1985; Vermeulen *et al*, 1986; Ponnudurai, *et al* 1989). It is now possible to study the biochemical and physiological aspects of gametocytes of the most malignant malaria parasite, *P. falciparum*.

The intracellular processes that trigger gametogenesis are yet to be completely understood (Martin *et. al*, 1978; Kawamoto *et. al*, 1990). Reports from other cell systems, (neutrophils, platelets, and nerve cells) implicate phosphoinositide hydrolysis as one of the mechanism through which the cells are stimulated, (Downes,1983; Rink *et al.*, 1983;Nishizuka,1984; Vergara, *et al*, 1985;Vicentini,*et al* 1985;Worley, *et al*. 1987). There is a possibility that like responsive mammalian cells, phosphoinositide hydrolysis products may be involved in the synchronization of gametogenesis. The similarity in the kinetics of responsive plasmodial and mammalian cells suggest that biochemical pathways for signal transduction may be highly conserved. In view of the overwhelming human disease capacity of *P. falciparum* the fact that its gametocytes can be routinely obtained by *in vitro* culture, an attempt has been made to determine the role played by chemical and physiologic factors in the activation of cultured gametocytes. The compounds known to have an effect in the phosphoinositide hydrolysis heve been tested in

exflagellation to elucidate the role of the crucial biologic trigger (IP3), a substance known to release bound calcium from biologic membranes (Downes,1983; Shute and Smith 1985; Vergara *et al* 1985;Supattapone *et al* , 1987; Goldschmidt-Clermont and Janmey 1991). Therefore, the more primitive malarial parasite could serve as a simpler model for investigating these important events and the knowledge gained could contribute to the development of methods to control malaria and could impact favorably on a globally relevant disease.

In contrast to invertebrates, most vertebrates are homiothermic with a closed blood circulatory system. Therefore, when gametocytes make the transition from the vertebrate blood stream into the gut of an insect, the temperature and carbon dioxide (pCO_2) tension of their environment drop simultaneously. The role that each of these concomitant environmental factors play in activating gametocytes has been elucidated using primarily *Haemoproteus* and *P. gallinaceum* gametocytes (cited by Bishop and McConnachie, 1960) . It is currently believed that mature gametocytes remain inactive because of the low pH (7.4) maintained in the closed vertebrate circulation. When gametocytes are removed either by venipuncture or a blood sucking insect and exposed to air, the pCO_2 tension drops down to ambient levels and the concomitant rise in pH triggers the events associated with gametogenesis (Carter and Nijhout, 1977, Nijhout and Carter; 1978). A pivotal role has also been given to the bicarbonate ion in that gametocytes can be held inactive outside the vertebrate circulation in a buffered solution lacking this ion. It was reported that addition of bicarbonate to the solution lacking the ion adds to the environmental trigger, alkaline pH to the intracellular processes that result in gametogenesis (Bishop and McConnachie,1960). However, the

independent role of environmental factors in modulation of gametogenesis in *P. falciparum* have not been clearly delineated.

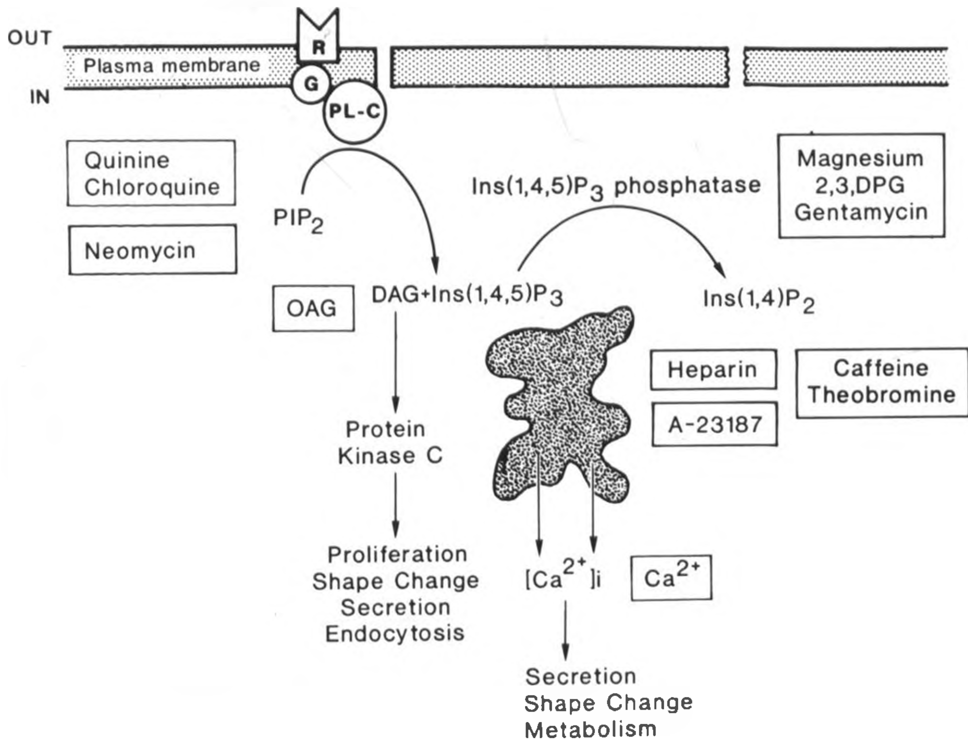
The role of temperature in maintaining suspended animation (SA) needs to be defined. Despite the fact that Carter and Nijhout (1977) used bicarbonate free buffer (pH 7.4) to maintain the viability of gametocytes and prevent the induction of gametogenesis several others in keeping with earlier studies of gametogenesis such as those of Danilewsky (1889), used high temperature (Micks *et al* 1948;Kawamoto *et al*.,1990;Kawamoto et al 1991). In addition, the latter investigators did not routinely remove serum and bicarbonate from their test solutions. Kawamoto *et al* , (1990) reported that *P. berghei* gametocytes, unlike *P. gallinaceum*, exflagellate at high pH in the absence of bicarbonate ions, indicating specificity for these triggers. Therefore, the independent role of pCO_2 tension, alkaline pH, bicarbonate ion, temperature and serum in triggering gametocyte activation need to be clearly defined for each plasmodial species.

Both second messengers produced by PI hydrolysis, inositol (1,4,5) trisphosphate (Ins 1,4,5)P3 and diacylglycerol (DAG) have been isolated in activated mature malaria gametocyte of *P. falciparum* (Martin *et al*., 1990). In the present study, various chemicals which affect Ins (1, 4, 5)P3 dependent pathways were examined for the induction of exflagellation of cultured *P. falciparum* gametocytes. Two compounds, magnesium (Mg^{2+}) and 2,3 diphosphoglycerate (2,3 DPG)which are known inhibitors of the enzyme phosphatase which breaks down Ins (1, 4, 5)P3 and hence prevent its accumulation as it is produced in other cell systems (Brass and Joseph, 1985; Shute and Smith,1985; Joseph and Williamson,1986) were studied. Work by Foster *et. al*, (1989) suggested that the

dephosphorylation of Ins (1,4,5)P3 to inositol 1,4, biphosphate is magnesium dependent. Previous experiments with caffeine, a substance known to release bound calcium, showed that xanthine oxidase inhibitors can bypass the obligate requirement for bicarbonate and trigger exflagellation of *P.gallinaceum* gametocytes (Martin et al 1978). In sarcoplasmic reticulum (SR), using 5mM of caffeine, a compound known to release bound calcium from internal stores, the results showed that SR still retained sufficient amounts to elicit a large contracture after removal of neomycin which is an inhibitor of contraction (Nishizuka,1984;Shute and Smith,1985).

Kawamoto *et al*, (1990), reported that exflagellation of *P.berghei* and was strongly inhibited at pH 7.3 by caffeine at 5mM. Tanabe and Dois (1989)also reported that the same compound which is also a potent inhibitor of cyclic adenosine monophosphate-phosphodiesterase (CAMP-PDE) is also a strong inhibitor of exflagellation of *P. berghei* and *P. falciparum*. Earlier work, (Martin, *et. al*, 1978) reported caffeine as a strong trigger of exflagellation of *P. gallinaceum* at pH 8.0 and a weak trigger at pH 7.4. The aminoglycoside antibiotic, neomycin, has been shown to block neuromuscular transmission and also inhibit the enzyme -calcium dependent phosphatidyl inositol phosphodiesterase-phospholipase C (Shute and Smith,1985). However, low concentrations of the antibiotics, stimulated the enzyme activity and the inhibiting effect observed at the higher concentration of the drugs was more marked at the lower calcium concentration. Neomycin was reportedly a constant inhibitor of contractions of swine muscles at 2 mM (Foster *et al* , 1989) while at lower concentrations (0.5 mM) its inhibitory effect was only observed after equilibrium time was increased from 20 to 40 minutes.

Figure 1: Schematic diagram of a receptor activated phosphoinositide-linked signal transduction system.



1.1 LITERATURE REVIEW

1.1.1 Exflagellation

The process of exflagellation is the earliest known phenomenon associated with the life cycle of the malaria parasite. It was observed by Laveran in 1881 at the time of his discovery of the human malaria parasite. (edited by Kean *et al* , 1982) Laveran , saw male gamete formation or "exflagellation" in the blood of an infected patient and thereby confirmed the vital nature of the malaria agent. Later Danilewsky (1885) and Marchoux (1899), described this process in *Haemoproteus*. (reviewed by Micks *et al* ., 1948, edited by Kean *et al*, 1982). However, it was MacCallum (1897) who realised that this dramatic event represented the production of the male gametes, and who subsequently observed the process of fertilization of the large female gamete (macrogamete) by the diminutive male (microgamete). He also observed exflagellation and fertilization in *Plasmodium falciparum*. (MacCallum,1897). It was not, however, until Ross in 1898 noted flagellated organisms in the stomach of the mosquito that the significance of these previous observations was understood (Micks, *et al*., 1948). From then, numerous elegant studies have been done upon the events of gamete formation and dispersal by Anderson and Cowfry, 1928 (reviewed by Sinden in 1983;Aikawa *et al*, 1984; Mons *et al*, 1985)

In natural transmission of malaria, insect vectors become infected by ingesting vertebrate red cells containing gametocytes, the sexual stages of the parasite. The mature macrogametocytes (female) and microgametocytes (male) are quiescent in the vertebrate blood stream, but

upon entering the insect midgut, they emerge from their host cells and develop into gametes. Microgametogenesis is particularly striking with the development and release of motile flagellated microgametes by the process of exflagellation. In a matter of minutes, the male gametes fertilize the female gametes and the sporogonic cycle is initiated in the vector (Hawking *et al*, 1971; Carter and Nijhout 1977, 1978; Martin, *et al*, 1978). The gametocytes also emerge from the corpuscles and exflagellation occurs in blood withdrawn artificially from the vertebrate host, provided that the blood is exposed to air (Micks, *et al*, 1948; Bishop and McConnachie, 1960 ; Nijhout and Carter, 1978).

1.2.0 External stimuli of Exflagellation

Reseachers on this process incriminated temperature, carbon dioxide tension, pH of the medium, and the presence of bicarbonate as the physiologic factors triggering exflagellation.(Micks *et al*, 1948; Bishop and MacConnachie,1960 ; Roller and Desser, 1973; Carter and Nijhout,1977)

1.2.1 Effect of temperature

Exflagellation was earlier reported to be completed within a rather wide range of temperatures (8.5° C to 43° C)[cited by Micks *et al*, 1948]. Kligler and Mer (1937), observed exflagellation of *Plasmodium vivax* , *Plasmodium falciparum* and *Plasmodium malariae* at 21°C and beyond, depending upon the species while temperatures of 15 to 16°C retarded the

process. (reviewed by Micks *et al* ., 1948, Roller and Desser 1973). Marchoux and Chorine(1932) worked on *Haemoproteus* and found that these changes took place over a wide range of temperature (13-43°C). For *Plasmodium gallinaceum*, *Haemoproteus columbae* and *Leucocytozoon simondi* exflagellation was reportedly not affected by temperature (Bishop and McConnachie,1956). Kawamoto *et al* (1990), suggested that induction of microgametogenesis may be composed of two distinct mechanisms, one a temperature-dependent DNA synthesis and the other a pH dependent control of development of events leading to microgamete assembly and exflagellation. Cultivation at pH 8.0 and 37°C did not induce development, and gametocytes remained in the non-activated form, having the DNA contents of 1.5C (Kawamoto *et al*, 1990). The above reports indicated contradicting temperature conditions at which exflagellation occurs in various malaria species. In the present work, the role of temperature in the induction of exflagellation in cultivated *Plasmodium falciparum* gametocytes is defined.

1.2.2 Effect of carbon dioxide tension and pH

Using *Plasmodium gallinaceum*, *Haemoproteus columbae* and *Leucocytozoon simondi* , it was demonstrated that exflagellation in whole blood was suppressed in atmospheres containing 5% carbon dioxide (Bishop and McConnachie, 1960). In addition, Marcheaux and Chorine (1932) demonstrated that blood pH values below 7.6 suppressed exflagellation. The two factors called attention to the carbon dioxide of the blood as the agent which restrained gametogenesis in *Haemoproteus* as Chorine(1933) noted that the process in *P. falciparum* occurred in the

presence of saline-citrate but that hydrochloric acid and carbonic acid gas arrested it. They concluded that it was hydrogen ion concentration which was important in exflagellation (both reviewed by Bishop and McConnachie, 1956).

Marcheaux and Chorine (1932), working on *Haemoproteus*, found that the exposure of the gametocytes to carbon dioxide prevented their emergence from the corpuscles; this inhibition could be reversed by subsequently exposing the blood to air, provided that the exposure to carbon dioxide had not been too prolonged. Similar results were obtained by Chorine(1933)with *P. praecox (P. falciparum).* This work was repeated using *P. gallinaceum* by Bishop and McConnachie,1956, who obtained satisfactory recovery from the inhibitory effect of the gas only after exposure to 5% carbon dioxide in 95% oxygen or nitrogen, recovery from 100% carbon dioxide being more variable. Marchoux and Chorine believed that the actual factor controlling the emergence of gametocytes from the corpuscles was a change in the pH of the blood due to the loss of carbon dioxide. However, Chorine in 1933 pointed out that this probably only triggered off a series of reactions in the blood which brought about the changes in the erythrocytes (Bishop and McConnachie,1960). They recorded a rise in the pH of fresh drawn, normal and infected chick blood, exposed to air, but, in experiments with infected blood, in which the rise in pH was inhibited, they were unable to confirm that pH is the critical factor controlling the development of the gametocyte. Bishop and McConnachie, (1956) reported that the pH of whole blood could affect gametocytes in *P. gallinaceum* but they concluded that pH is not the sole regulatory agent since no exflagellation occured when infected cells were suspended in buffered sodium chloride solutions regardless of pH. In a

Bishop and McConnachie (1960) reported that gamete development progressed in saline solutions containing bicarbonate but they neglected to investigate the effect of pH under these new conditions.

It was reported that gametogenesis in *P. gallinaceum* involves a bicarbonate dependent process and requires a continuous supply of glucose, presumably as an energy source (Nijhout and Carter, 1978). Emergence and exflagellation of gametocytes *in vitro*, occured independently of the carbon dioxide tension but were closely correlated with the pH of the external medium. In bicarbonate-saline, gametogenesis was initiated only if the pH exceeded 7.7. Their results suggested that gamete development of malaria parasites is stimulated when infected blood is exposed to air because the decrease in the carbon dioxide tension of the blood caused its pH to rise (Green,1978). In an attempt to examine the role of pH and carbon dioxide tension (pCO_2) in controlling the development of gametes, Nijhout and Carter (1978) used the equation described by Henderson -Hasselbalch, in which the pH of a solution containing bicarbonate is determined by the concentration of bicarbonate in solution and the pCO_2 of the atmosphere with which the solution is in equilibrium:

$$pH = pK' + \log \ HCO^{-}_3 / pCO_2 \times \alpha$$

where α (0.044) is the solubility coefficient of carbon dioxide in water and pK' equals 6.193 for the bicarbonate-containing solutions of ionic strength 170 at 21^0C (Sigaard- Anderson, 1964). Although they studied the effect of pH on exflagellation in relation to bicarbonate (HCO_3^-) and pCO_2 tension, they did not study the effect of temperature under these conditions. Kawamoto *et al*; in 1990, using *P. berghei* , suggested that an

increase in intracellular pH induced by the Na^+/H^+ exchange plays an important role in the induction of gametogenesis. Their results suggested that exflagellation of *P. berghei* differs from *P. gallinaceum* in that it occurs in the absence of sodium bicarbonate. From the above reference it is evident that exflagellation has been studied over a century but no conclusion has been reached as to the effect of each physiologic factors on exflagellation.

1.2.3 Effect of serum on exflagellation

Studies have shown that serum is also one of the physiological factors that affect exflagellation. Bishop and MacConnachie (1960), working with *P. gallinaceun* reported that gametocytes did not emerge from the corpuscles if they were washed and suspended in isotonic saline buffered to pH 7.78, a pH at which the emergence of gametocytes readily takes place in blood. The gametocytes subsequently emerged in large numbers and the male exflagellated when the saline was replaced by chicken plasma, thus suggesting that some factor (s) present in chicken plasma was necessary for the development of the gametocytes. Martin and co-workers (1978), used undiluted chicken serum, at pH 8.0 for the production of maximum number of centres of exflagellation and this whole serum was used as a standard in each experiment against which all other solutions were compared(Martin *et al* 1978; Nijhout 1979). It was reported that gametocytes developed inside the mosquito in the absence of ingested bicarbonate, a factor essential at a maximum concentration of 15 to 30mM for optimum *in vitro* exflagellation in saline (Nijhout, 1979). It was also observed that the male gametocytes exflagellated normally *in*

vitro in normal or inactivated horse serum, or in rabbit plasma. It was further noted that gametocytes washed and suspended in normal horse serum, completed their sexual development and produced oocysts in mosquitoes. In addition, hyper-immune chick plasma, as compared with normal chick plasma, had no significant effect on the time of onset or the frequency of exflagellation.

To date, conflicting reports have been published on the stimulatory effects of human serum, on exflagellation (Carter and Beach, 1977; Ponnudurai, *et al* .1982b; Ponnudurai *et al* 1986; Meuwissen and Ponnudurai, 1988). While earlier reports suggested possible substances in normal human serum which inhibited exflagellation to a variable degree and reduced mosquito infectivity, (Bishop and McConnachie, 1960), others did not observe any difference in the number of microgametocytes undergoing exflagellation either in foetal calf serum or human serum, (Carter and Beach 1977). However Nijhout and Carter, (1978), reported that decarbonated serum could not support exflagellation in *P. gallinaceum*. The role of serum in exflagellation of *P. falciparum* gametocytes needs clarification.

1.2.4 Effect of Mosquito gut factor

Measurement of mosquito gut content showed that there is a pH rise to as high as 7.9 within 5 minutes of feeding indicating a possibility that mosquitoes possess vector-specific synergistic or inhibitory substances capable of controlling gametogenesis in malaria parasites independent of the bicarbonate dependent pH control (Micks *et al.*, 1948;

Bishop and McConnachie, 1956;Wigglesworth,1974). Using avian plasmodia, Micks and co-workers (1948), found no correlation between exflagellation and pH but instead incriminated a chemical factor present in stomach cavity of *Culex pipiens* ,which greatly activated the gametocytes of *P. elongatum* to exflagellate and complete fertilization; another factor in *Aedes aegypti* and *A. quadrianulatus* inhibits these processes in the same parasite. This factor appeared to be associated with secretions produced by ingestion of blood, since unfed (non-blooded) stomach contents from the same species did not induce exflagellation upon their addition to infected blood from the bird (Micks *et al*, 1948). A later investigation reported a factor present in the mosquito midgut that also stimulated exflagellation in *P. gallinaceum* (Nijhout, 1979). The presence of this factor was demonstrated at pH values from 7.4 to 8.0 and over a wide range of concentrations. This factor, termed, mosquito exflagellation factor (MEF) was implicated as the physiological trigger during the transfer of malarial infection from man to the mosquito. Later attempts to reproduce this factor - MEF in different laboratories, have not been published.

1.3.0 Stimulus response coupling:

1.3.1 Incrimination of cyclic adenosine monophosphate (cAMP)

Work on exflagellation of plasmodium species has been done mainly on *P. gallinaceum* , *P. yoelii* and *P. elongatum* but very little has been done on *P. falciparum*, the most virulent species of human malaria. Martin *et al*. (1978), using *P. gallinacium*, induced male gametocyte

exflagellation by using certain phosphodiesterase inhibitors like caffeine. This finding implicates cyclic nucleotides in the initiation of this process. Previous experiments with caffeine, a substance known to release bound calcium, showed that xanthine oxidase inhibitors could bypass the obligate requirement for bicarbonate and trigger exflagellation of *P. gallinaceum* gametocytes (Martin *et al*, 1978). This work incriminated cyclic adenosine monophosphate (cAMP) in the exflagellation of *P. gallinaceum* gametocytes. Later work (Read and Ross,1990) have characterized cyclic AMP and Ca^{2+}- dependent protein kinases in *Plasmodium falciparum* by using fluorescence imaging. It is now possible to observe the dynamic intracellular biochemistry of single living cells, more so by using a fluorescent indicator for the adenosine 3',5'-cyclicmonophosphate (cAMP) signalling pathway (Adams *et al* 1991). Effect of cyclic AMP on the asexual maturation and gametocyte formation of *P. falciparum* grown *in vitro* have been examined and the compound was reported to inhibit both processes in a stage-specific manner (Inselburg,1983; Maswoswe *et al* 1985).

1.3. 2 Effect of drugs on exflagellation:

Investigation of the action of metabolic inhibitors on microgametogenesis in *P yoelii nigeriensis*, showed that colchicine was particularly effective in inhibiting exflagellation but pyrimethamine, which inhibits the enzyme dihydrofolate reductase thereby restricting nucleotide and DNA synthesis, did not inhibit exflagellation in *P. cynomolgi* (Sinden and Canning, 1977). Later studies of the inhibitory effects of anticytoskeletal agents on gametogenesis in *P. yoelii*, showed that, the colchicine, vinblastine

sulphate and cytochalasin B. inhibited gamete formation in a dose and time dependent manner (Sinden *et al* . 1985). Clinical observations have suggested that the gametocytes of *Plasmodium* are unusually resistant to a variety of antimalarials including quinine. the 4-aminoquinolines, pyrimethamine and sulphonamides (Sinden and Smalley 1979; Sinden, 1983). Earlier findings on this matter, showed that mature gametocytes were not affected by chloroquine at concentrations up to 1 mg/ml, but in contrast, the immature cells were readily destroyed at this concentration (Smalley, 1977; Smalley and Sinden, 1977; Sinden, 1982; Sinden, 1985). Chariya (1982), reporting on conditions favouring gametogenesis in continuous culture of *P. falciparum*, showed that of the compounds that inhibited cyclic adenosine monophosphate (cAMP) - phosphodiesterase enzyme activity, only caffeine, at appropriate concentrations could be used to enhance gametogenesis without exerting toxic effects on the culture. Other workers, working on the various effects of inhibitors of exflagellation concluded that the event was insensitive to Mitomycin C, but highly sensitive to inhibitors of protein synthesis and microtubule assembly (Sinden and Canning, 1977). Following the observation of a specific protein present on the male and female gametes and on the zygote, research was directed towards the production of a monoclonal antibody against the protein for blocking transmission (Rener *et al*, 1983; Vermeulen *et al* , 1985).

1.3.3 Implication of cyclic guanine monophosphate

Kawamoto, *et al*, (1990), using *P. berghei* indicated that mobilization of the parasite's internal resources of calcium ions (Ca^{2+}) was a prerequisite in exflagellation, and they concluded that induction of

exflagellation requires both Ca^{2+} mobilization and an increase in cyclic guanine monophosphate (cGMP) levels by alkaline media They also reported that caffeine, a potent inhibitor of cAMP-phosphodiesterase enzymes (cAMP-PDE) strongly inhibited the exflagellation of *P. berghei* and *P. falciparum*.. Scheibel and co-workers (1987), reported that addition of ionophore- A-23187 which is a Ca^{2+} mobilising agent inhibits the asexual growth of *P. falciparum* while Kawamoto *et al* (1990) reported that treatment with Ca^{2+} mobilization agents, inhibited exflagellation in both *P. berghei* and *P. falciparum*. These reports on the effect of Ca^{2+} mobilising agents reveal contradicting effects of these compounds on exflagellation. Kawamoto *et al* (1990) elucidated compounds which are the possible producers of cyclic GMP and which are mediators of exflagellation.

1.3.4 Phosphoinositidase (PI) hydrolysis and exflagellation

Stimulation of cell-surface receptors initiates hydrolysis of a membrane bound inositol lipid, which produces at least two second messengers - diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (Ins (1,4,5)P3.[Muallem,et al 1985; Shears,1989; Goldschmidt-Clermont and Jamney,1991]. These messengers are generated by a membrane transduction process comprising three main compounds; a receptor, a coupling G-protein and phosphoinositidase C (Bleasdale,*et al* 1985). DAG acts by stimulating protein kinase C, whereas Ins (1,4,5)P3 releases calcium from internal stores (Beaven,*et al* , 1984; Supattapone *et al* , 1987; Berridge and Irvine, 1989; Lewin,1990; Goldschmidt-Clermont *et al* 1991). These pathways form the cornerstone of a ubiquitous transduction

mechanism now known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation. (Abdel-Latif,1986; Goldschmidt-Clermont,1990). Both second messengers produced by PI hydrolysis, Ins (1,4,5)P3 and DAG, have been isolated in inactivated mature malaria gametocytes of *P. falciparum* (Martin *et al*, 1990 in press). The DAG analogue,the synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol, which is a potent activator *in vitro* of Ca^{2+} activated,phospholipid-dependent protein kinase,was reported to intercalate into the membrane and directly activate protein kinaseC without interaction with cell surface receptors(Kaibuchi, *et al* , 1983).

Despite the recognition of important extracellular inducers of exflagellation, it is clear that the true nature of the secondary messenger systems involved in signal transduction remain inadequately characterized. Bishop, at her death had began investigations into the trigger for gametogenesis in malaria parasites, and 25 years later the question is still unanswered(Cox,1990). The intracellular processes that trigger gametogenesis are yet to be completely understood (Martin *et al*, 1978; Kawamoto *et al*, 1990;Contie,1991). From recent studies, (Martin *et al*, 1990; Kawamoto *et al*, 1990), there is growing evidence that exflagellation in cultured *P. falciparum* gametocytes is a complicated process, consisting of phosphoinositide hydrolysis, as several inositol polyphosphates have been obtained which exert concerted effects on calcium mobilization produced in regulatory sequence upon initiation of gametogenesis. The role of these second messengers in exflagellation have been studied in the present work.

Since the process of exflagellation is such a dynamic and critical process and it is not clear whether it is only influenced by environmental factors or if there is a contribution from the gametocytes' internal environment, there is a need to dissect out the role of environmental factors from the internal response coupling in *P. falciparum* gametocytes activation. A study of factors such as bicarbonate, alkaline pH, pCO_2 tension, temperature and serum is not conclusive enough for exflagellation to be fully understood. Compounds involved in signal transduction in other cell systems also need to be investigated in an attempt to find out whether phosphoinositide hydrolysis is involved in the stimulation/ inhibition process in *P. falciparum* gametocytogenesis. This study looks into the critical factors which prevent or trigger exflagellation in cultured *P. falciparum* gametocytes.

1.4 OBJECTIVES OF THE PRESENT STUDY.

The main aim of the present study was to examine factors that affect exflagellation of cultured *Plasmodium falciparum* gametocytes. The specific objectives of the study were:

1. To select a routine technique for *in vitro* production of viable mature *P. falciparum* gametocytes.

2. To produce purified mature *P. falciparum* gametocytes to use for exflagellation tests.

3. To standardize techniques for holding mature *P. falciparum* gametocytes in a suspended animation and still retain their exflagellation ability.

4. To examine the role of human serum, temperature, pH, and bicarbonate ion on exflagellation .

5. To determine from selected compounds, conditions that will inhibit or trigger exflagellation.

1.5. DEFINITIONS:

The following are definitions of the terms and abbreviations used in the present study based on Nijhout & Carter (1978):

Suspended animation (SA): State of inactivation induced by a solution that temporarily inhibits exflagellation until the gametocytes have been exposed to the appropriate physiological triggers. It is used in the test as a negative control at pH 7.4.

Physiologic pH-7.4

Alkaline pH-7.9

Isolate: a single sample of parasite material derived from a naturally infected host specimen on one occasion and preserved in the laboratory either by continuous *in vitro* cultivation or cryopreserved in liquid nitrogen.

COM	Centres of movement
HCO_3 -	Bicarbonate ion (25mM).
Neo -	Neomycin.
Gen-	Gentamycin.
Caf -	Caffeine.
Theo -	Theobromine.
2,3-DPG -	2,3 - Diphosphoglycerate.
Mg -	Magnesium ion.
Hep -	Heparin.
CQ -	Chloroquine
Qn -	Quinine
OAG -	Oleoyl -2-Acetyl- Glycerol.
A-23187-	Calcium ionophore.

pCO_2- Carbon dioxide tension

Exflagellation index- The average of the highest three scores of the replicate tests run for each experiment.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Isolates:

Three isolates, known gametocytes producers, were used for this work. These included two isolates collected from malaria patients as part of field studies and cryopreserved in liquid nitrogen, namely Kisumu isolate from Nyanza province (K-67) and another isolate from Coast province, Jilore primary school, (JP119). Another good gametocyte producer, NF-54, was obtained from John Hopkins University (courtesy of Dr. John Beier). These isolates were preserved in liquid nitrogen, using Rowes cryo solution (2.5% glycerol, 3% sorbitol, 0.65% sodium chloride) or dimethyl sulfoxide (DMSO) (Trager and Jensen, 1976;Diggs *et al*., 1977; Strome *et al*., 1977; Wilson *et al*., 1977; Siddiqui *et al* 1979;WHO, 1981;). The isolates used were all chloroquine sensitive(Ofula,1989).

2.2 *In vitro* cultivation of *Plasmodium falciparum* gametocytes.

The parasite isolates were thawed from liquid nitrogen (WHO, 1981; Trager and Jensen, 1976) and cultivated *in vitro* in blood group O^+ human erythrocytes using a modification of Ifediba and Vanderberg (1981) as described by other workers (Watkins *et al*., 1984; Spencer *et al*.,1985). A 50% haematocrit (erythrocyte suspension) of human type O^+ erythrocytes was prepared in wash medium consisting of special RPMI 1640 (GIBCO laboratories, Grand Island, N.Y with L-Glutamine, without sodium bicarbonate and antibiotics) diluted in sterile distilled, deionized water with

25mM Hepes buffer and 25mM sodium bicarbonate (Sigma Co. Ltd., USA). The cultures were maintained in tissue culture flasks at 6% haematocrit, in RPMI 1640 medium diluted in sterile distilled deionized water with 25mM Hepes buffer, 25mM $NaHCO_3$ 10% heat inactivated (40 minutes at 56^oC) human serum, and 50µg/ml hypoxanthine (Sigma Co. Ltd., USA). Two cultures were set up at 0.2% parasitaemia and the flasks were flushed with a gas mixture consisting of 3% CO_2, 5% O_2 and 92% N_2 (East African Oxygen, Nairobi, Kenya) at 37^oC.

Giemsa-stained thin blood films of the parasite cultures were made every two days to check for parasite developmental stages, possible contaminants, and parasitaemia. Daily culture medium changes were carried out and fresh erythrocytes were added to give starting parasitaemia of 0.2% every two to three days until an asexual parasitemia above 3% was achieved. Thereafter, asexual parasites were left without adding fresh red blood cells (undiluted) for fourteen days with daily medium changes and making of smears after every two days to monitor the parasitaemia and development of gametocytes. From day twelve, exflagellation was monitored using the petri-dish method (Jensen *et al* 1979). Multiple flasks of the cultures were staggered to allow for maturation of gametocytes at intervals of 2 to 3 days. A minimum of two weeks was necessary to achieve mature gametocytes ready for testing.(Smalley,1976; Lambros and Vanderberg,1979; Campbell,1982; Sinden,1985; Vermeulen,*et al*, 1986). When irregular medium changes were carried out (between 24 to 48 hours), gametocytes appeared in the flasks earlier and the asexual stages disappeared.

2.3 Purification of gametocytes.

This was carried out using 14 day old cultures (NF54) and 16 day old cultures (JP119 and K67) which were placed on a percoll gradient (Knight and Sinden, 1982; Carter *et al* 1987) and compared with gametocytes produced without purification (Sinden *et al*., 1984) for their exflagellation ability. The purified gametocytes lost their exflagellation ability after passing through the percoll. After returning the purified gametocytes into culture for three hours, they regained their exflagellation ability in a time dependent manner. The absence of red blood cells in purified gametocyte preparations made it difficult to count centres of movements(Knight and Sinden,1982; Sinden,*et al* 1984; Carter *et al*, 1987; Kawamoto *et al* 1990; Martin *et al* 1990) . Consequently the unpurified gametocytes obtained after cultivation for two weeks were used for quantifying exflagellation (Martin *et al*, 1978; Nijhout and carter, 1978; Kawamoto *et al*., 1990, 1991).

2.4 Preparation of Suspended Animation (SA) Solution and decarbonation of Serum

A suspended animation solution was prepared by using RPMI 1640, 25mM Hepes buffer, 1µM $CaCl_2$ and 1% decarbonated serum, pH 7.4 was warmed to 37°C. The SA solution was used throughout the tests as it prevented exflagellation of the mature gametocytes at room temperature (23°C) and at the same time kept the gametocytes viable for a long time (24 hrs) without damaging their exflagellation ability at 37°C. All test solutions and dilutions were made with this buffer which also served as a negative control.

Decarbonation of human serum was carried out to remove the bicarbonate effect (Nijhout and Carter, 1978, Kawamoto *et al.*, 1990). The procedure involved addition of drops IN solution of fresh hydrochloric acid until the pH of the serum dropped to 4.5 with shaking to remove the dissociated bicarbonate in form of carbon dioxide after which the pH was raised using freshly prepared IN sodium hydroxide (NaoH). The decarbonation was important in establishing suspended animation because when 1% whole serum (non-decarbonated) was used in suspended animation, low exflagellation (+) was observed.

2.5.0 Preparation of Test solutions (chemicals) and setting of test cultures

2.5.1 Test solutions:

Stock solutions for the chemicals tested were prepared in sterile distilled deionized water at room temperature except quinine which was prepared in water heated to 60°C. Gentamycin, chloroquine, neomycin and quinine, were tested at 2mM concentrations (Foster, *et al.*, 1989; Fujita *et al.*, 1984; Shute and Smith, 1985 respectively), heparin at 10μg/ml (Bird, *et al.*, 1991); 2,3 diphosphoglycerate and magnesium were tested at 2.5mM and 0.25mM concentrations respectively (Joseph and Williamson, 1986; Scheibel *et al.*, 1987). Caffeine and theobromine (Xanthine oxidase inhibitors) were tested at 5mM concentrations (Martin *et al.*, 1978; Kawamoto *et al.*, 1990). The other compounds, calcium ionophore - A23187 and diacylgycerol analogue - the lipid soluble - Oleoyl -2-Aceyl, Glycerol (OAG) were both

prepared in DMSO and tested at 2μM and 1μM concentrations respectively. (Rink *et al.*, 1983;Beaven et al.1984; Kawamoto *et al.*, 1990).

All the stock solutions were aliquoted in Bijou bottles and stored at -20°C, except for2,3 diphosphoglycerate whose activity on exflagellation deteriorated on thawing, so it was always prepared fresh for each test. The test solutions were made up fresh from the stock solutions for every test, diluted in SA. the pH of all test solutions was tested and adjustedto appropriate pH values with one normal sodium hydroxide or hydrochloric acid just prior to use. Note that the above tested compounds can be categorized as :- xanthine oxidase inhibitors (caffeine and theobromine); 2,3-Diphosphoglycerate; magnesium ions ; aminoglycosides (gentamycin and neomycin) ; anti-malarial drugs (chloroquine and quinine); heparin; ionophore A-23187 and diacylglycerol lipid analogue- Oleoyl-2-acetyl- Glycerol (OAG).

2.5.2 Preparation of Culture for exflagellation tests

From day 10 of culturing *P.falciparum* gametocytes, they were tested daily for exflagellation by petri-dish method (exposure to air). When good exflagellation was noted in a culture(2-3 exflagellating bodies per field), spent medium was removed as per normal medium change and warm (37°C) suspended animation buffer immediately added so as to achieve a 1:100 dilution. Gametocytes in the SA were then spun at 37°C at 750g for 5 minutes and the supernatant removed. The pellet was resuspended in 5ml of warm suspended animation buffer. Gametocytes prepared in this manner could be held for eight hours at 37°C before testing without loss of exflagellation capacity.

Gametocytes to be tested were aliquoted into 3 mls borosilicate (12x75mm) tubes: rapidly spun at 500g for 30 seconds in a Dade Serofuge II, and the supernatant decanted. 2 ml of test solution was then added to the pellet, cells resuspended by vortexing, respun for 30 seconds, test solution decanted and a hanging drop prepared on a vaseline rimmed coverslip using 9" plugged pasteur pipette (length approx. 230mm). All the experiments were done in replicate and repeated independently more than three times.

2.6 Negative and Positive Controls

The negative control consisted of a solution in which the gametocytes could be kept viable for as long as eight hours without exflagellation. Gametocytes in suspended animation solution made up as described in section 2.4 above was used in all the tests as negative control. In this solution, gametocytes were consistently inactive but exflagellated when the triggers were added. In the experiments carried out either increase in pH (7.9) of SA solution or addition of HCO_3^- to the SA solution was used as positive control, depending on which test was being carried out.

2.7 Scoring exflagellation:

Scoring of exflagellation was done by looking at ten random fields through the microscope (Kawamoto *et al.*, 1990). Slides were then viewed under phase contrast microscopy (400x) with closed circuit television. For television viewing, a 1 kappa S VHS TV camera was connected to a Carl Zeiss Axioskop research teaching microscope and a CFM/15/2 high resolution

color 14" monitor with S VHS input. The observer counted the number of exflagellating centres per unit screen. All slides were scanned every 5 minutes for 30 minutes and the average of the highest 3 scores for each slide was taken to be the exflagellation index. The following scoring system was used: no COM per 10 screens-0; an occasional COM per 10 screens-1; 0 to 1 COM per screen-5; 1 to 2 COM per screen-10; 2 to 3 COMs per screen-15 ; 3 to 4 COMs per screen-20; 4 to 5 COMs per screen-25; 5 to 6 COMs per screen-30; 6 to 7 COMs per screen-35; 7 to 8 COMs per screen-40; 8 to 9 COMs per screen-45; 9 to 10 COMs per screen-50. A COM can represent the presence of either a single microgamete or an exflagellating body with several microgametes. Each slide was scored sequentially every 5 minutes for 30 minutes and the avarage of the highest three scores taken as the exflagellation index. After each experiment .slides with a score of zero were thoroughly scanned for the presence of an occasional centre of movement. the presence of which changed the score from 0 to 1. Each experiment was repeated at least three times before being accepted. After observation of the slides for a maximum of 30 minutes. only 3 highest exflagellation scores were taken and the averages calculated the exfagellation index. Comparable indices were those from test solutions tested at the same conditions at the same time using the same isolates.

2.7.1 Effect of decarbonated human serum on the exflagellation of cultured *P. falciparum* gametocytes

Decarbonated human serum was prepared by adding 1N hydrochloric acid dropwise to agitated serum until the pH dropped to 4.5 and kept to equilibrate overnight. The following day the pH of the serum was brought up

to 7.4 with sodium hydroxide solution. Gametocytes were added to suspended animation media with varying concentrations of human serum (pH 7.4) and held at 37°C for 1 hr. Temperature was then allowed to drop to room temperature (23°C-26°C) and exflagellation scored.

2.7.2 Effect of pH and bicarbonate (HCO_3^-) on exflagellation of gametocytes held in suspended animation (SA) at room temperature (23° C)

Gametocytes were held in suspended animation at room temperature (23°C) by using 1% decarbonated serum and physiologic pH (7.4). Test solutions were tested in the presence and absence of HCO_3^- at pH 7.4 and pH 7.9. Test solutions, pH 7.4 + HCO_3^-, pH 7.9, pH 7.9 + HCO_3 and control pH 7.4 were prepared and held at RT (23° C). Solutions were added to gametocyte pellet, vortex spun for 30 seconds at 500g and exflagellation scored.

2.7.3 Effect of PCO_2 tension, pH and HCO_3^- on exflagellation at 37° C and 23°C.

Gametocytes were held at 37°C in a medium containing 10% decarbonated serum in an atmosphere of 5% CO_2 or ambient air, high pH , (7.9) and 25mM $NaHCO_3$ for one hour. Temperature was then allowed to drop to room temperature (23°C) and exflagellation scored.

2.7.4 pH changes in culture media on exposure to ambient air

A 16-day old gametocyte culture was divided into two parts: one part centrifuged and the cells excluded - 1. spent medium without cells; 2. spent medium with cells. Uninfected red blood cells were added to fresh culture medium in flasks to a haematocrit of 5% and 20%. All 4 flasks were flushed with a gas mixture of 5% CO_2, 3% O_2, and 92% N_2 and incubated at 37°C for 1 hour. Thereafter, the contents of each flask were separately poured into 9 cm diameter petri-dishes exposed to ambient air and pH measurement taken every 10 minutes. The change in hydrogen ion concentration was then calculated.

2.7.5 Effect of temperature on exflagellation using two isolates NF-54 and K-67

14 and 16 day old gametocytes of two isolates NF54 and K67, respectively, were held in suspended animation. 1.5ml aliquots of these gametocytes were transferred into exflagellating medium containing HCO_3^- at pH 7.4 and one sample each incubated at the following temperatures: 37°C, 35°C, 32°C, 30°C and 28°C, for 1 hour. Temperature was then allowed to drop to room temperature (23°C) and samples examined for exflagellation.

2.8.0 Effect of non-physiologic triggers of exflagellation

2.8.1 Ranking of Triggers of exflagellation using *P. falciparum* K-67 gametocytes

16-day old K-67 gametocytes were held in suspended animation (SA) and tested for exflagellation using the following compounds: HCO_3^- 25mM,

Caf 5mM, Theo 5mM, Ca^{2+}, A-23187 2μM, OAG 1μM, Mg^{2+} 0.25mM, 2,3 DPG 2.5mM and Gen 2mM. The other chemical triggers, Ca^{2+}, Mg^{2+}, 2,3 DPG, A-23187, theobromine, caffeine, OAG, and gentamycin were tested by running a concentration curve for each compound using 16-day old K67 or 14-day old NF54 gametocytes. The gametocytes were spun in SA at room temperature, test solution prepared and added to gametocytes pellet, vortex spun for 30 seconds at 500g and exflagellation scored. The other two compounds, neomycin and heparin were tested by adding the compounds to exflagellating media containing known triggers

2.8.2 Inhibition of exflagellation by chloroquine (0.5mM) at pH 7.4 and 7.9 using *P. falciparum* NF-54 gametocytes.

The effect of chloroquine as an inhibitor of exflagellation was tested by adding 0.5mM chloroquine to exflagellating media containing triggers. 14-day old NF-54 gametocytes were held in SA. Test solutions were made in SA and used to check for exflagellation in the presence or absence of chloroquine at .5mM, pH 7.4 and pH 7.9.

2.8.3 Effect of quinine on exflagellation of *P. falciparum* K-67 gametocytes

The effect of quinine as an inhibitor of exflagellation was tested by adding 0.5mM quinine to exflagellating media containing known triggers. 16-day old K-67 gametocytes were held in SA then tested for exflagellation with different concentrations of quinine in SA at pH 7.4 and pH7.9.

2.8.4 Effect of incubation with chloroquine and quinine (0.5mM) on exflagellation

Gametocytes were incubated in SA solution containing either 0.5 or 2mM of quinine or chloroquine. Thereafter, the gametocytes were centrifuged and washed twice with SA, and stimulated to exflagellate using both physiologic and chemical triggers.

2.9.0 Statistical analysis

This was done by using Kruskal Wallis one-way analysis of variance (ANOVA) together with multiple range test. Both parametric and non parametric statistics were used to confirm differences between and within groups of data. Results were considered significant if the $p < 0.05$.

CHAPTER 3

3.0 RESULTS

3.1. *In vitro* Cultivation of *Plasmodium falciparum* gametocytes

During cultivation of the gametocytes, contaminantion with bacteria and fungi was encountered at times in the culture (Plate I). The growth rate of the parasites was not always good, sometimes this was observed after a change of blood or serum donors and other times for unknown reasons. It was not always easy to predict the percentage gametocytaemia that would be realised from a culture flask that had a good growth rate. Gametocytes appeared earlier in flasks where irregular medium changes were carried out. From stage II of development , most gametocytes progressed well to the other stages if the medium changes were done between 24 and 48 hours. Irregular medium changes, however, easily caused the disappearance of the asexual stages from the culture flasks (Plate II). The gametocytes of the foreign isolate NF54 matured faster (12-14 days) compared to the local isolates K67 and JP119 (14-16 days). This was also observed in their rates of growth, the foreign isolate, if not diluted after two days, all the asexual stages died while the local isolate could survive even at high parasitaemia.

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3. 2 Purification of gametocytes

Purified gametocytes lost their exflagellation ability in percoll immediately after purification ,exflagellation recorded was low (40%)than in

the unpurified ones. The results from this procedure revealed that there was partial stimulation of gametocytes when undergoing purification in percoll (Plate III). They rounded and some even exflagellated in this gradient, a condition which made the procedure unsuitable for observing exflagellation.

Plate I: Bacteria contaminants in cultured *P. falciparum* gametocytes

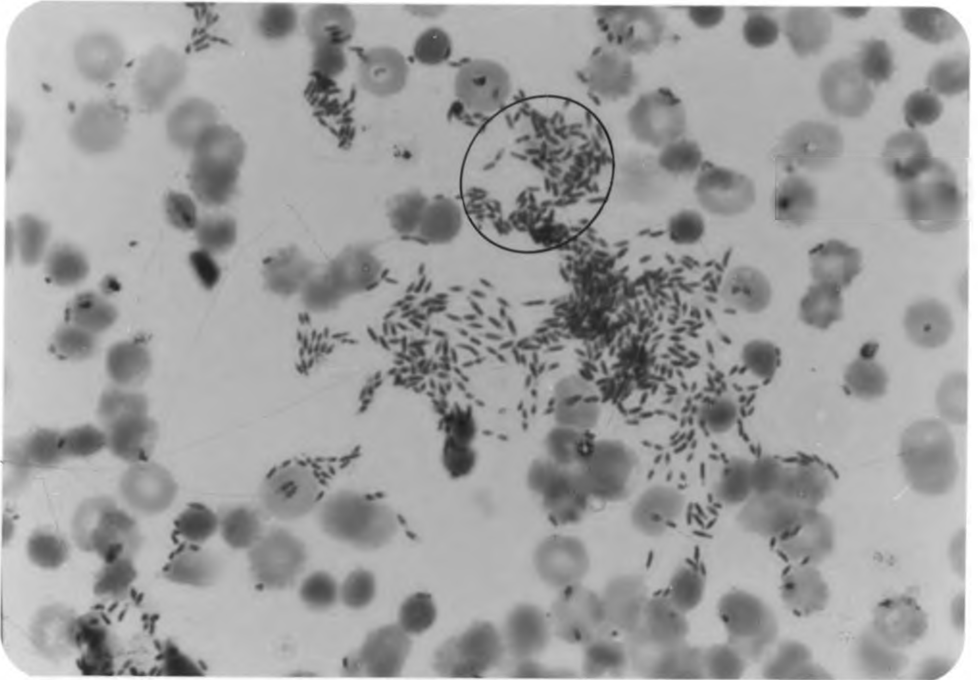


Plate II: Unstimulated mature cultured *P. falciparum* gametocytes in culture medium (irregular medium changes):- note the elongated forms.

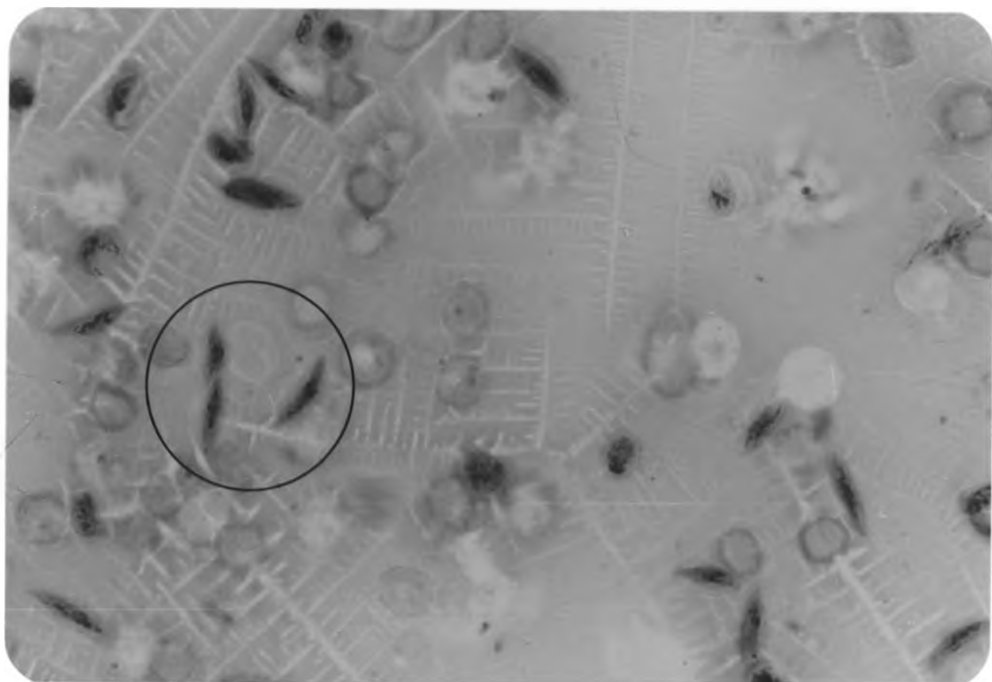
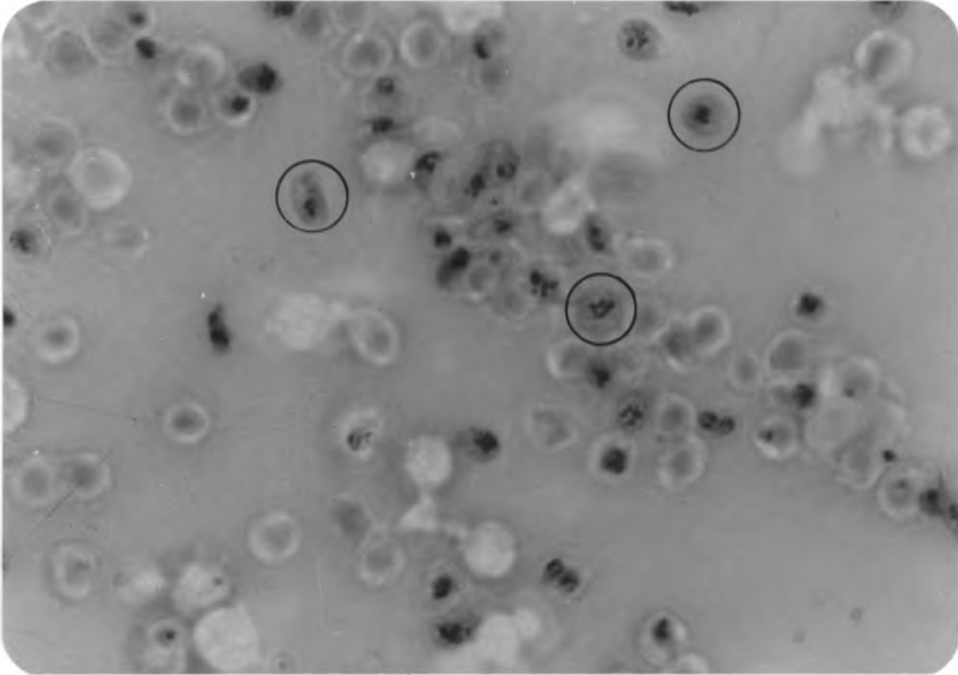


Plate III: Partial stimulation of exflagellation of *P. falciparum* gametocytes in percoll:-note the rounded forms.



3.3 Scoring exflagellation

Preliminary observations on exflagellation were made in wet preparations of mature cultures (14- to 16- days old) on a slide under a vaseline-rimmed coverslip. Just before exflagellation, the mature gametocytes were seen to change from the elongate shape within the human red cell to rounded shape. The host red cell membrane lysing and the spherical parasite emerged into the medium, with the flagellae beating about, creating centres of movements. The vibrations of the emerging gametes drew surrounding red cells into a rossette (Plate IV). Exflagellation of the microgametocytes was observed in all cases for a maximum of 30 minutes, the peak in numbers of exflagellations occurred between 8-15 minutes and then declined. By thirty minutes, most exflagellations had stopped and no more movements could be observed (Plate V). Plate IV also shows clumped red blood cells due to exflagellating microgametocytes while Plate V shows a monolayer of red blood cells with inactive gametocytes. Alkaline pH always triggered exflagellation thus all compounds were tested at pH 7.4 to remove the effect of alkalinity on the tested compounds.

3.4.0 Effect of physiologic triggers on exflagellation

3. 4.1 Effect of decarbonated human serum on the exflagellation of cultured *P. falciparum* gametocytes

Using varying concentrations of decarbonated human serum, it was found that concentrations of 2% and above could trigger exflagellation so long as the temperature dropped below 30°C;our working room temperature was

fortunately 23-26°C. No exflagellation was observed at the same concentration at 37°C(exflagellation index=0). At 37°C, mature microgametocytes did not exflagellate even if the concentration of serum was increased to 100%. (Fig.2) shows the effect of serum on exflagellation. No exflagellation occurred with 10% bovine serum albumin or human serum concentrations below 1.5%. Exflagellation was noted at serum concentrations above 2% even though, the indices were much lower than those observed in media containing serum and bicarbonate ion or serum at alkaline pH. Similar results were obtained on substiting decarbonated human with fetal bovine, horse, or chicken serum. Increasing decarbonated serum concentrations did not affect the number of exflagellating bodies counted but only affected the duration of movements of the microgametes. The values(1.0-maximum) indicate how low the indices were. Keeping the percentage of human serum to below 2% maintained suspended animation at normal room temperature (below 30°C), a condition at which gametocytes remained inactive for as long as 24 hours. Using Kruskal-Wallis one-way analysis of variance (ANOVA) together with multiple range test, whole serum concentrations of 2%, 5%, 7.5% and 10% produced significantly higher exflagellation indices than that of 1.5% (exflagellation index=0.5)but below 1.5% of serum there was no exflagellation($P<0.05$) [Table 1].

3.4.2 Effect of pH and bicarbonate (HCO_3^-) on exflagellation of gametocytes held in suspended animation (SA) at room temperature (23° C)

These two conditions were tested independently to find out the role each played in exflagellation. Addition of HCO_3 to the gametocytes at

pH 7.4 caused exflagellation. while absence of HCO_3 at pH 7.4 showed no exflagellation. Raising pH alone, from 7.4 to 7.9 in the absence of HCO_3 caused exflagellation. Although gametocytes also exflagellated in the presence of both bicarbonate ion and alkaline pH.the exflagellation index was always lower than that observed with either alkaline pH or bicarbonate ion alone (Figure 3).

Plate IV: Rosetting of red blood cells due to exflagellation

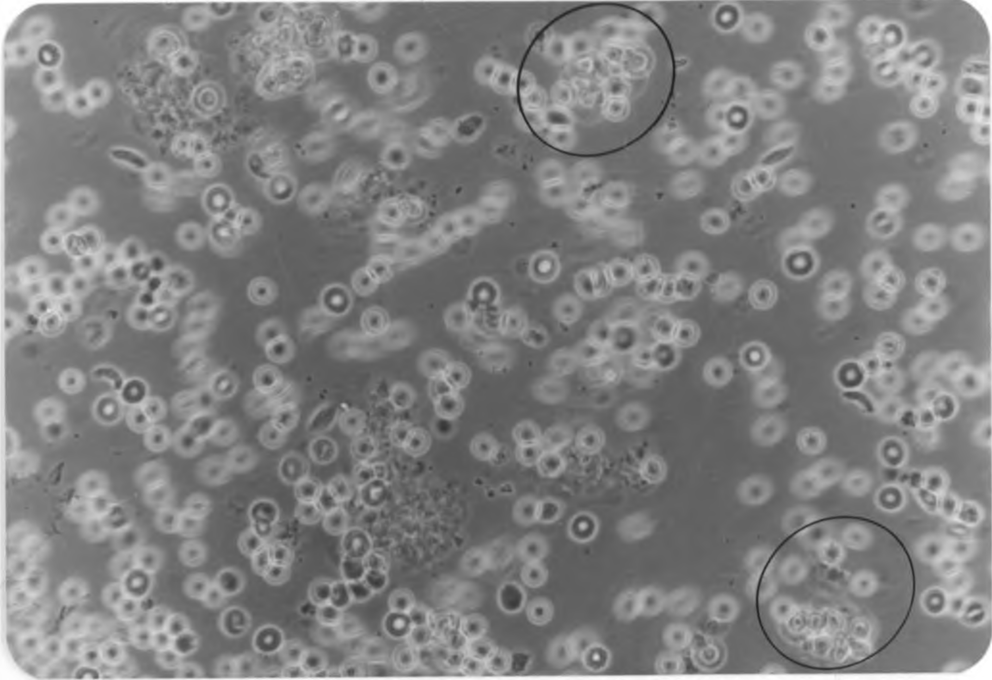


Plate V: No rosetting of cells in culture - monolayer red blood cells.

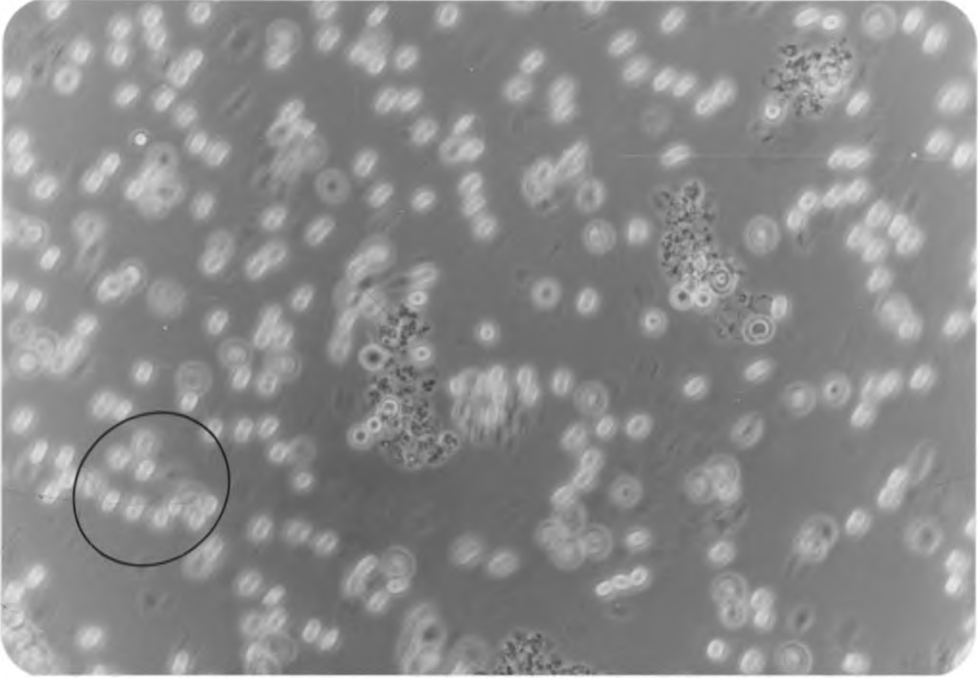


Figure 2: Effect of decarbonated human serum on exflagellation of cultured *P. falciparum* gametocytes.

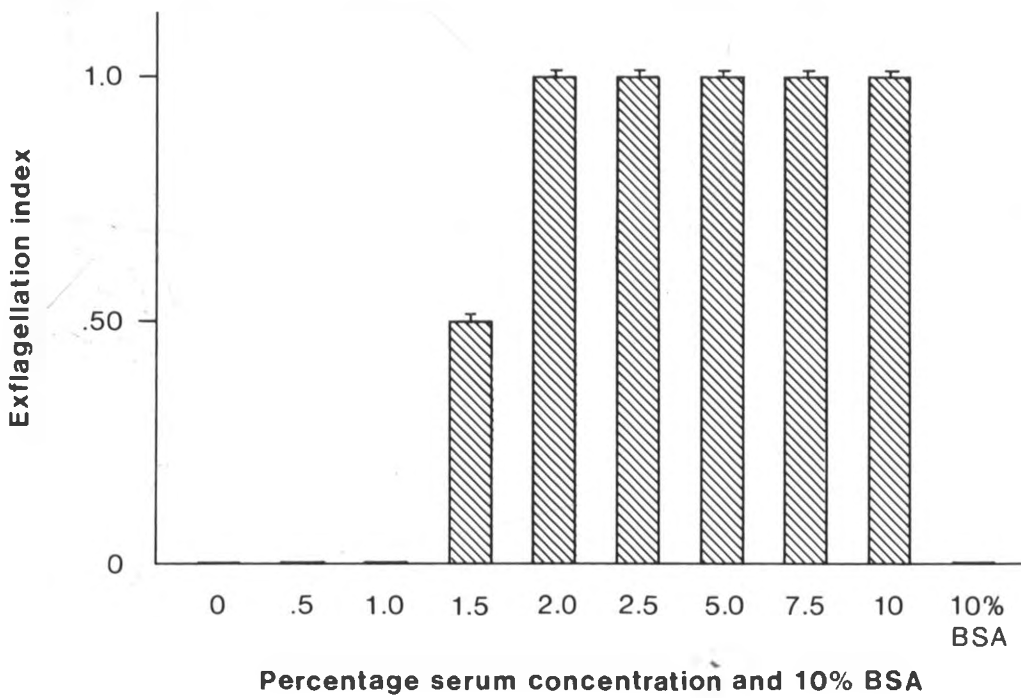
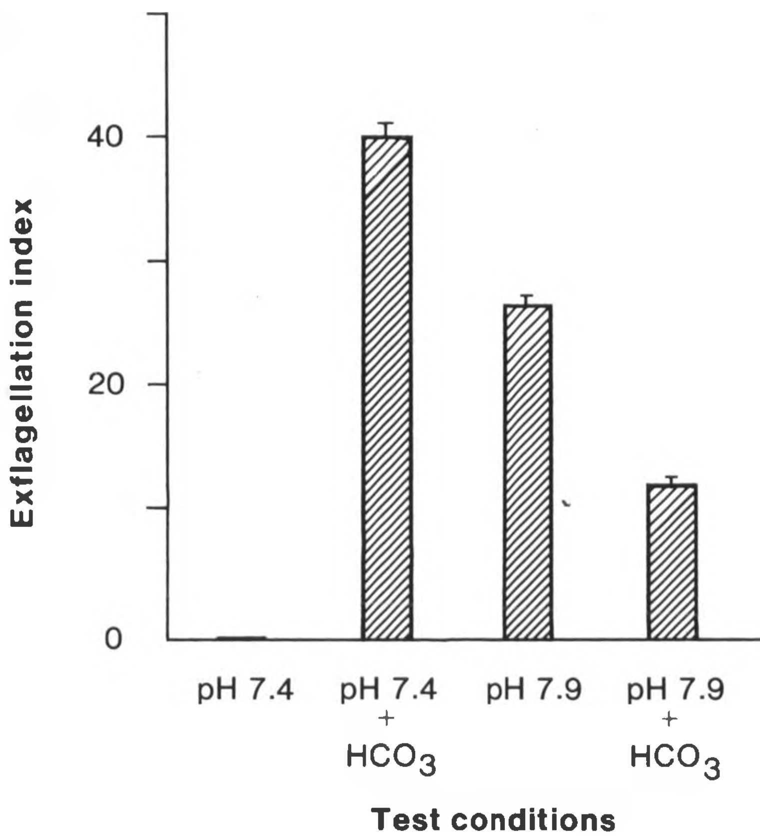


Table 1: Exflagellation index of cultured *P. falciparum* gametocytes in differing percentages of whole human serum at 23°C.

	Human Serum Concentrations									
Experiment	0%	0.5%	1%	1.5%	2%	2.5%	5%	7.5%	10%	10% BSA
1	0	0	0	0.67	1.0	1.0	1.0	1.0	1.0	0
2	0	0	0	0.67	3.7	1.0	6.7	5.0	6.7	0
3	0	0	0	0.3	5.0	5.0	8.3	7.0	10	0
4	0	0	0	0.3	7.0	6.7	3.7	5.0	8.3	0
5	0	0	0	0.67	8.3	5.0	6.7	5.0	5.0	0
6	0	0	0	0.3	8.3	1.0	0.67	0.67	0.67	0

Figure 3: Effect of pH and bicarbonate (HCO_3^-) on exflagellation of gametocytes held in suspended animation (SA) at room temperature (23° C) Exflagellation indices ± S.D.



3.4.3 Effect of PCO_2 tension, pH and HCO_3^- on exflagellation at 37° C and 23°C.

Neither alkaline pH nor HCO_3 induced exflagellation at 37°C in the presence or absence of 5% CO_2. In contrast, gametocytes exflagellated when temperature was dropped from 37°C to 23°C both in high and low pCO_2 tension. 10%, decarbonated serum at pH 7.4 (+) triggered weak exflagellation . Increasing the pCO_2 tension increased the rounding of the gametocytes leading to faster exflagellation. 5% CO_2 had no effect on exflagellation since samples exflagellated just as readily in ambient air as in 5% CO_2 environment (Figure 4).

3.4.4 pH changes of media on transfer from culture gas condition to ambient air.

Transferring the culture medium from gas condition of 5% CO_2, 3% O_2, 92% N_2 to ambient air, caused a rise in pH (Fig. 5). The pH rise was time dependent, and by 1 hour, the pH had risen to above 8.0, the optimum pH for exflagellation. The same results were observed when human blood was diluted out to 5%, 20% haematocrit, gassed and then exposed to ambient air. A less rapid rise in pH was obtained in media with higher haematocrit. Measurements of pH every 10 minutes showed inverse relationship between haematocrit and rate of rise and final pH of the solution. It confirmed that the lower haematocrit (5% red blood cells, spent medium and culture at 6% Haematocrit) reached the highest pHs faster than higher ones (40% Hct). Presence of a high percentage of the red blood cells in culture (40% and above), lowered the pH of the solutions (Fig. 5) and hence the change in

hydrogen ion concentration (Table 2.a and b). The pH of the spent medium with and without parasitized cells rose to pH 8.3± 0.05 within one hour. The rate of rise and maximum pH of fresh media was less than that with spent media with or without cells and increase in haematocrit further decreased the final alkalinepH of the medium.

Table 2: pH changes of media on transfer from culture gas condition to ambient air: [ΔH^+ concentration].

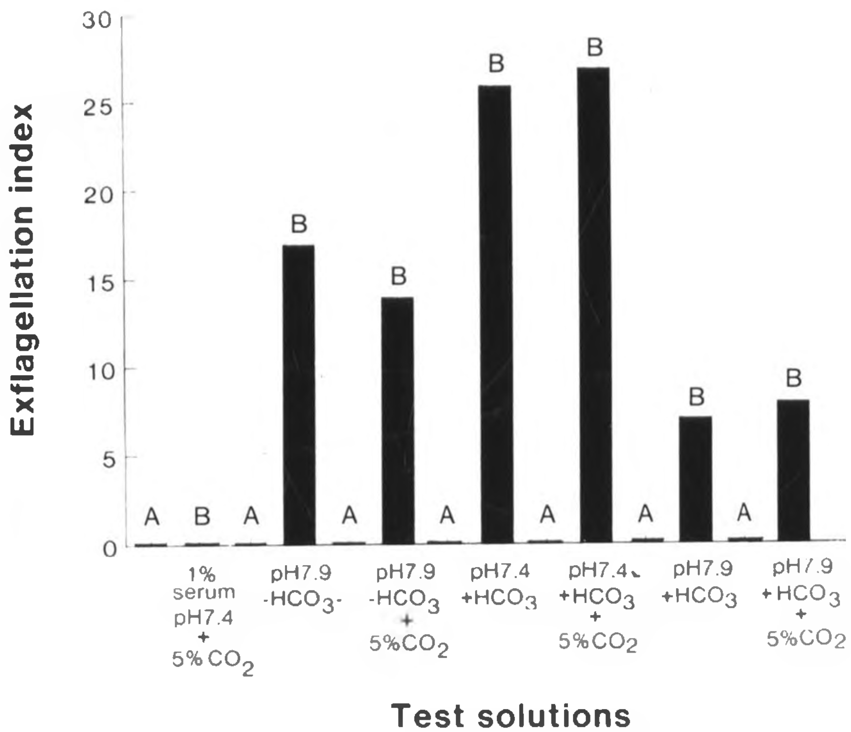
a) Isolate-K-67

Time after exposure (min)	Spent medium without cells	Spent medium without cells	5% RBC's	10% RBC's	20% RBC's	30% RBC's	40% RBC's	50% RBC's
10	0.31	0.38	0.37	0.37	0.34	0.35	0.29	0.27
20	0.54	0.60	0.61	0.60	0.54	0.48	0.41	0.38
30	0.66	0.69	0.74	0.72	0.65	0.52	0.42	0.37
40	0.72	0.72	0.84	0.83	0.72	0.54	0.42	0.41
50	0.74	0.76	0.90	0.88	0.74	0.57	0.43	0.39

b) Isolate-NF-54

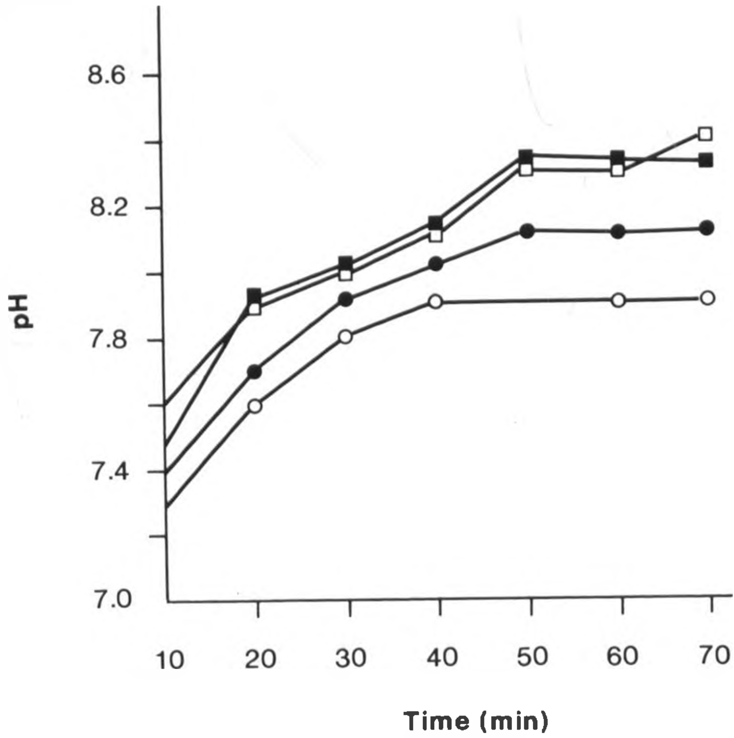
Time after exposure (min)	Spent medium without cells	Spent medium without cells	5% RBC's	10% RBC's	20% RBC's	30% RBC's	40% RBC's	50% RBC's
10	0.29	0.33	0.34	0.36	0.30	0.23	0.18	0.17
20	0.47	0.51	0.53	0.55	0.46	0.36	0.28	0.23
30	0.57	0.62	0.65	0.66	0.54	0.43	0.34	o.27
40	0.70	0.74	0.71	0.73	0.56	0.44	0.34	0.27
50	0.75	0.77	0.74	0.74	0.59	0.43	0.34	0.27
60	0.81	0.82	0.75	0.77	0.61	0.46	0.32	0.27

Figure 4 Effect of pCO_2 tension, pH and HCO_3^- on exflagellation at 37°C and 23°C.



Key
A 37°C
B 23°C

Figure 5 pH changes of media on transfer from culture gas condition to ambient air.



Key

- □ spent medium
- ■ spent medium with cells
- ● 5% fresh red blood cells
- ○ 20% fresh red blood cells

3.4.5 Effect of temperature on exflagellation using two isolates NF-54 and K-67

Gametocytes held in media known to induce exflagellation 25mM sodium bicarbonate, pH 7.4 or pH 7.9 without bicarbonate ion-for one hour remained in suspended animation if temperature was kept above 30°C (Figure 6). In contrast, gametocytes kept at temperatures below 30°C exflagellated during the one hour incubation period. Centres of movements (COMs) were not seen in samples held at temperatures below 30°C for one hour and then dropped to ambient temperatures, since exflagellation had already taken place during the one hour incubation period. for samples held at temperatures above30oC gamete formation including the early stages of rounding and erythrocyte lysis were observed after the one hour incubation period. There was not significant difference in exflagellation index within temperatures above 30oCand between the two strains tested. Therefore, temperatures above 30°C blocked both pH and bicarbonate ion from inducing gametocyte activation.

3.5. 0 Non-physiologic triggers of exflagellation

At room temperatures (21-28°C), the cultures remained in suspended animation when the decarbonated serum concentration was kept below 2%. From these results the physiologic suspended animation was temperature (37°C) dependent. At room temperature (21-26°C) gametocytes could be held in SA by using a lower concentration (1%) of serum in a solution of RPMI 1640 at pH 7.4. Any tested chemical that caused exflagellation at room temperature with 1% decarbonated serum at pH 7.4 was called a trigger of

exflagellation as reported in the present study. These compounds substituted for extra serum, bicarbonate, or rise in pH (7.9) as they caused exflagellation under non-permissive conditions (SA).

In this study, the following compounds were tested and found to be triggers: Xanthine oxidase inhibitors (caffeine, theobromine, tested at 5mM), bicarbonate (25mM), oleoyl-2-acetyl-glycerol (1µM), calcium ionophore -A23187 (2µM), 2,3 diphosphoglycerate (2.5mM),magnesium (.25mM) and gentamycin (2mM). All of the above triggers induced exflagellation in SA at room temperature but not at 37°C. The following compounds were found to be inhibitors of exflagellation: heparin (10µg/ml), neomycin (2mM), chloroquine and quinine (both tested at .5mM). These inhibitors did not trigger exflagellation in SA at room temperature.

3.5.1 Ranking of triggers of exflagellation using *P. falciparum* K-67 gametocytes:

All the chemicals that caused exflagellation under the non-permissive conditions at room temperature were compared and ranked according to their exflagellation indices (Fig. 7). Bicarbonate and alkaline pH, both physiologic conditions gave the highest exflagellation indices compared to chemical agents. Exflagellation was completely inhibited at 37°C. Hence temperature seemed to be a key factor preventing initiation of exflagellation. The xanthine oxidase inhibitors, caffeine and theobromine, caused exflagellation in SA at pH 7.4 with caffeine giving higher exflagellation indices than theobromine. Of the non-physiological triggers tested, caffeine gave the best stimulation of exflagellation though much weaker than the physiological triggers HCO_3^- and

alkaline pH. The other compounds tested varied in their stimulatory ability. Alkaline pH and HCO3- produced significantly higher exflagellation indices ($p < 0.05$) than the chemical triggers. The chemical triggers which caused exflagellation did so at significantly lower indices than the physiological triggers ($P>0.05$) [Table 3].

3.5.2 Effect of calcium ions on exflagellation at pH 7.4 using *P. falciparum* K-67 gametocytes

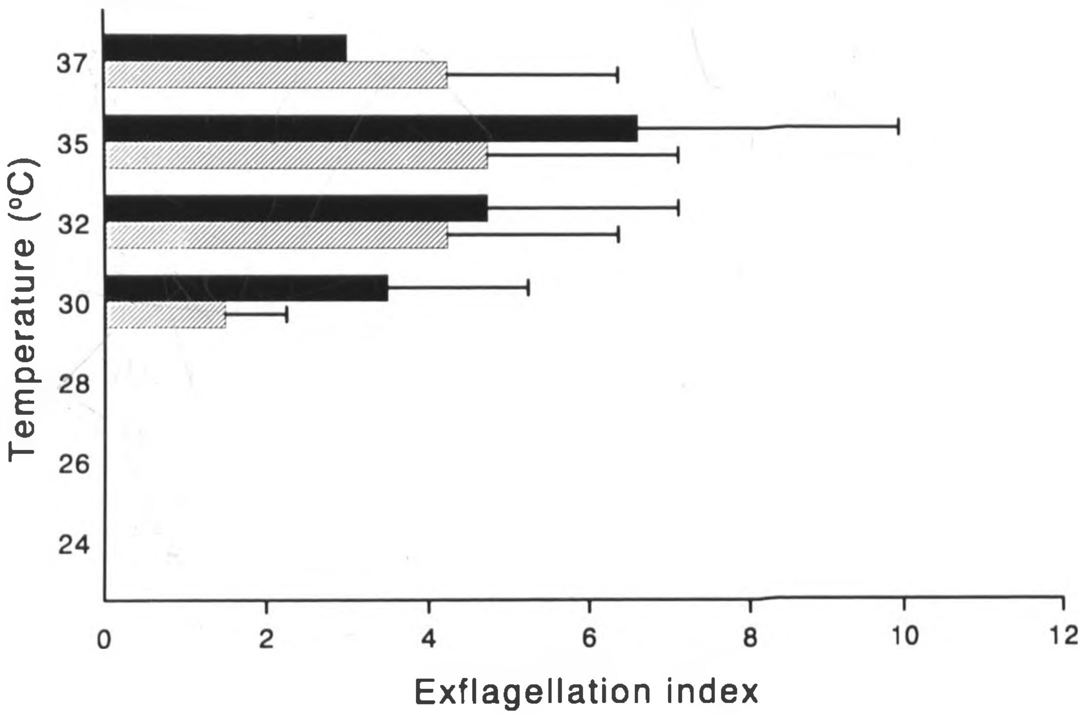
The exflagellation index increased with increasing extracellular calcium concentration (Ca^{2+}) up to an optimum Concentration curve for calcium ions showed optimal concentration at 2.5mM (Figure 8). Thereafter, further increases in external calcium resulted in a decrease in the exflagellation index. Red blood cell lysis was noted at calcium concentrations above 10mM, therefore, the decrease in exflagellation noted could have been a toxic effect of external calcium. Exflagellation was consistently observed at 0.1mM (+) but not at 0.05mM or 0.5mM, a condition which could not be explained.

3.5.3 Effect of magnesium ions:

When 16-day old K-67 cultured gametocytes were held in suspended animation and tested for exflagellation using varying concentrations of magnesium ions (.05-2.0mM), the results showed that, magnesium ions could trigger exflagellation in SA at pH 7.4 . At 0.25mM, magnesium triggered exflagellation in the same magnitude as at concentration of 2mM

(exflagellation index = 3.7). Magnesium ion induced exfagellation in a biphasic manner. the reason for this biphasic response was not known. Hence other tests carried out with magnesium were done at 0.25mM (Fig. 9). Magnesium, was a weak trigger of exflagellation when tested alone at pH 7.4 (Figure 9). In the presence of bicarbonate, magnesium synergised to give even higher exflagellation indices (Fig. 10). Combination of Mg^{2+}, HCO_3- and alkaline pH completely inhibited exflagellation. The reason for this observation was unknown. Mg^{2+} also synergised with caffeine but not in alkaline pH. 2,3,DPG synergised with Mg^{2+} in alkaline pH, a unique condition observed.

Figure 6: Effect of temperature on exflagellation using two isolates NF-54 and K-67.



Key

■ Isolate K67

▨ Isolate NF-54

Figure 7 Ranking of triggers of exflagellation using K-67 gametocytes at pH 7.4.

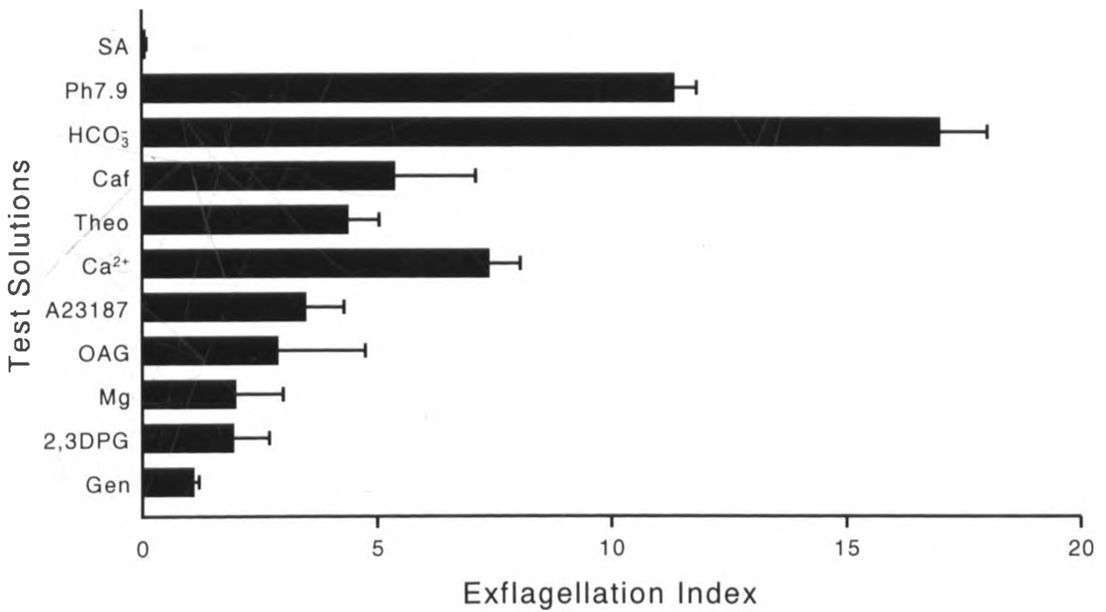
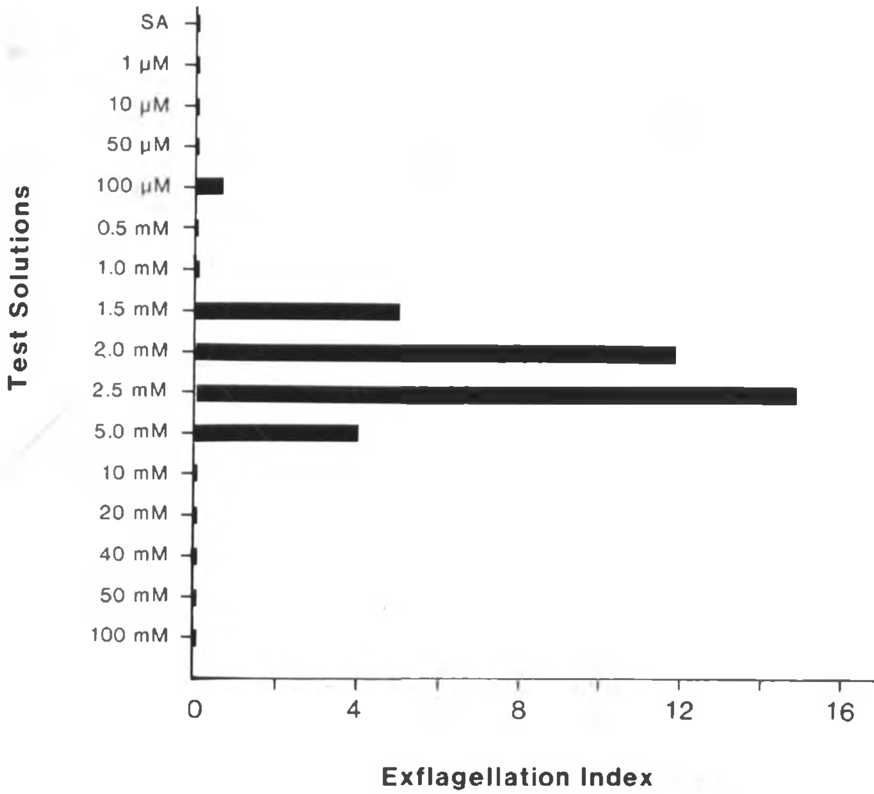


Table 3: Ranking of triggers of exflagellation using *P. falciparum* K-67 gametocytes using exflagellation indices.

EXP.	pH 7.9	HCO_3^-	Caf	Theo	Ca^{2+}	A-23187	OAG	Mg^{2+}	2,3DPG	Gen
1	12.5	18.3	6.0	5.0	6.7	2.3	2.0	0.3	1.0	0.67
2	12.5	13.0	7.0	3.7	3.7	5.0	5.0	1.7	1.0	3.0
3	23.3	15.0	5.0	4.0	12.0	3.7	7.0	1.0	5.0	1.0
4	32.5	40.0	5.0	3.7	1.0	0.3	11.0	5.0	11.0	1.7
5	7.0	20.0	11.0	10.0	3.7	1.0	3.7	7.0	0.3	7.0
6	3.7	18.5	6.0	5.0	10.0	12.5	0.3	0.67	0.67	0.67
7	5.0	11.0	2.0	3.7	12.5	5.0	1.0	1.0	0.3	-
8	-	15.0	6.7	-	-	2.3	1.0	1.0	0	-
9	-	6.8	8.3	-	-	0.3	0.67	-	-	-
10	-	18.3	2.0	-	-	6.7	0.3	-	-	-

Test were used to identify the factors which significantly trigger the gametocytes to produce higher exflagellation scores. pH 7.9 and HCO_3^- gave the highest readings ($p<0.05$). The other triggers were not significantly different from one another.

Figure 8 Effect of calcium ions on exflagellation of *P. falciparum* K-67 gametocytes



3.5.4 Effect of 2,3 Diphosphoglycerate (2,3 DPG) on exflagellation using *P. falciparum* NF-54 gametocytes

A concentration curve for 2,3 DPG showed 2.5mM as the optimal concentration for exflagellation (same data as for calcium ions, figure 8). 14-day old NF54 gametocytes were held in suspended animation and tested for exflagellation using 2,3 DPG at 2.5mM at both pH 7.4 and pH 7.9. Tests were also run in combination with other compounds and synergism was realised with HCO_3^-, alkaline pH, caffeine, but not with neomycin (Fig. 10). Hence 2,3 DPG featured as a weak trigger of exflagellation when tested alone at pH 7.4 but in the presence of HCO_3^- or alkaline pH (7.9), it was even a better trigger for exflagellation. Statistical analysis showed that exflagellation indices of 2,3,DPG at pH 7.4 were significantly lower than the indices got with 2,3,DPG at pH 7.9($p < 0.05$) [Figure 11]. Comparison of the concentration curve for the three compounds,magnecium, 2,3DPG, and gentamycin revealed that the three had different optimal, minimum and maximum doses in initiating exflagellation, (figure 12).

Figure 9 Effect of magnesium ions on exflagellation of P. falciparum K-67 gametocytes at pH 7.4.

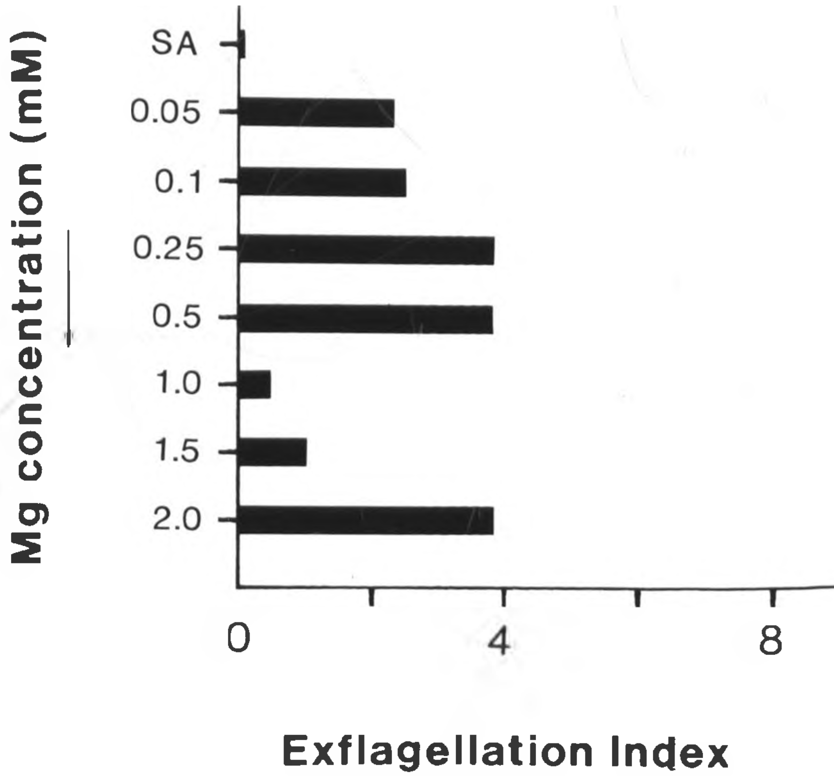


Figure 10 Effect of magnesium ions, in combination with other triggers on exflagellation of *P. falciparum* K-67 gametocytes

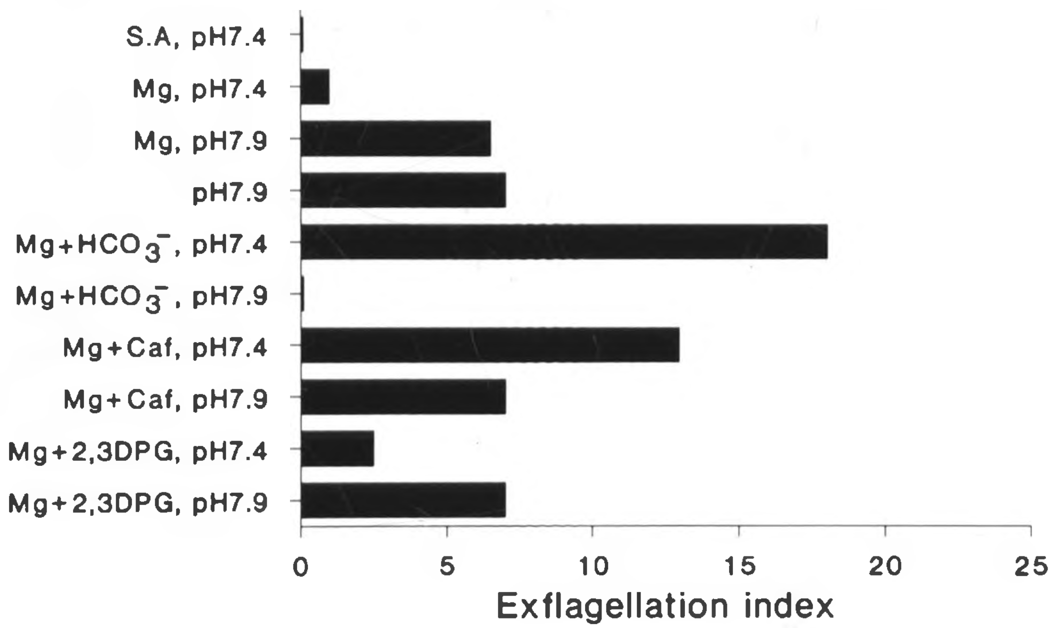
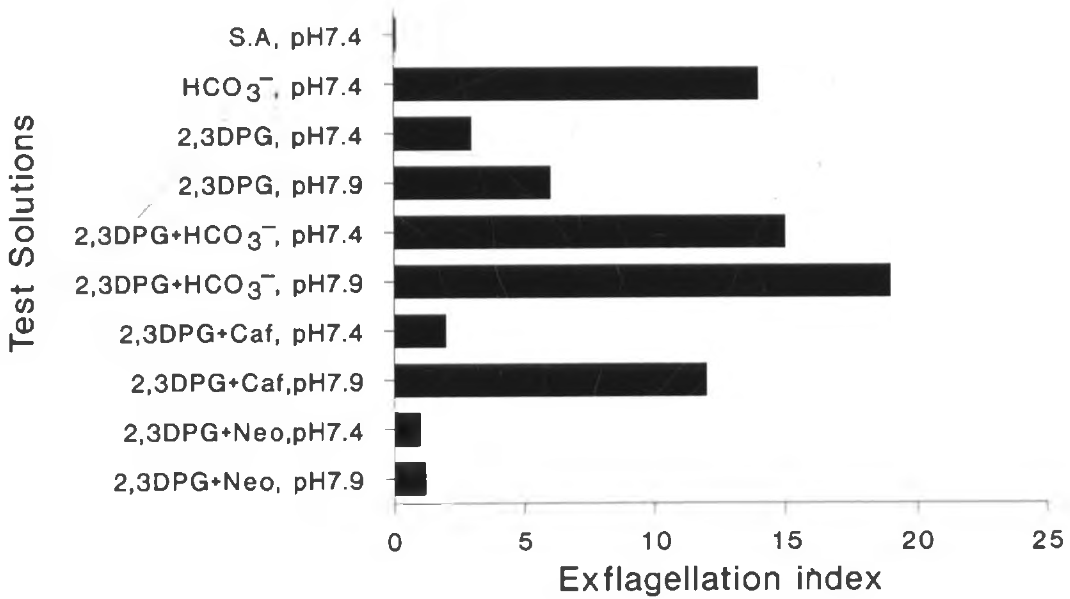


Figure 11 Effect of 2, 3 Diphosphogycerate (2.5mM) in combination with other chemicals on exflagellation of *P falciparum* NF-54 gametocytes



3.5.5 Effect of calcium ionophore-A23187 caffeine and theobromine on exflagellation using *P. falciparum* K-67 gametocytes at pH 7.4.

Sixteen-day old K-167 cultured gametocytes were held in suspended animation and tested for exflagellation using the ionophore at concentrations ranging from 2µm to 100µM. A-23187 was found to be a weak trigger of exflagellation at pH 7.4 in comparison with other chemical triggers. The differences in exflagellation indices produced by caffeine, theobromine and ionophore-A23187 were statistically non significant (Figure7). The xanthine oxidase inhibitors also stimulated weak exflagellation at 5mM, a concentration at which they have been effective in other cell systems (Figure 13).

3.5.6 Effect of Oleoyl-2 acetyl-Glycerol (OAG) on exflagellation.

Sixteen-day old K-67 cultured gametocytes were held in suspended animation and tested for exflagellation using OAG at 1µM at both pH 7.4 and 7.9. This synthetic diglyceride was tested at 1µM, the concentration at which it is effective in other signal transduction systems. The compound triggered very weak exflagellation at pH 7.4. Increasing the concentration of this compound did not cause increase or reduction in exflagellation even up to very high concentrations (25mM). This compound behaved at all tested concentrations as a weak trigger of exflagellation. At high concentrations (25mM), exflagellation was not observed. In comparison with the physiologic triggers, OAG was a weak trigger of exflagellation at the physiologic pH 7.4 (Table 4.)

3.5.7 Effect of gentamycin on exflagellation:

Gentamycin turned out to be a weak trigger of exflagellation.at 2mM. In all experiments, gentamycin induced only weak exflagellation . The compound also did not inhibit exflagellation when tested in combination with other triggers . Running a concentration gradient for this antibiotic showed that Gentamycin was a trigger of exflagellation even at low concentrations but at the concentration at which the compound was tested (2mM) exflagellation was consistently positive (Fig. 14). Gentamycin was not tested at concentration greater than 2mM as at this concentration is far more than the concentration which is used in cultures (40μg/ml).

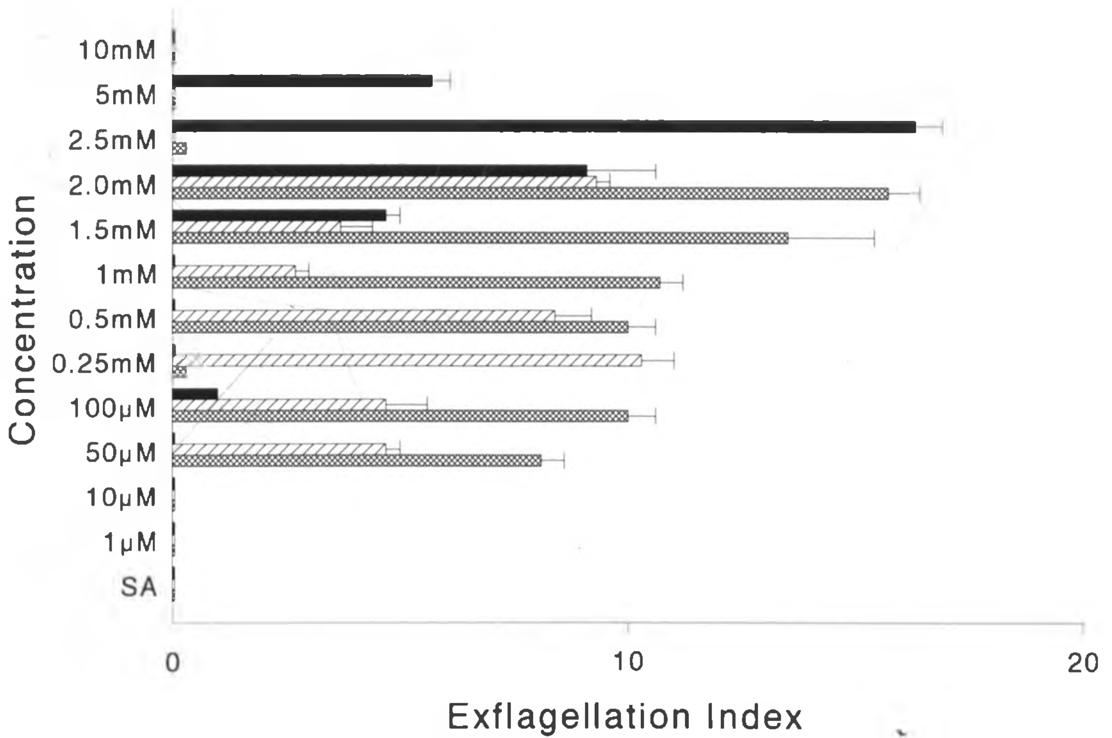
3.5.8 Effect of neomycin on exflagellation

The effect of Neomycin as an inhibitor of exflagellation was tested by adding 2mM Neomycin to exflagellating media containing known triggers - Caffeine 5mM, 2.3 DPG 2.5mM and HCO_3^- 25mM. In every case, the presence of Neomycin decreased the exflagellation index observed with the trigger alone (Fig. 15). It did not trigger exflagellation when tested alone at the physiological pH (7.4) in SA. It reduced exflagellation at pH 7.9 but did not completely inhibit it. When neomycin was added to physiologic triggers, alkaline pH and HCO_3^- it reduced exflagellation. Neomycin significantly reduced exflagellation in all the conditions tested except in caffeine at pH 7.9 and 2.3 DPG at pH 7.9 ($P<0.05$). The compound was classified as an inhibitor of exflagellation (Table 4).

3.6.0 Other non-physiologic inhibitors of exflagellation

In culture conditions, mature gametocytes were kept in suspended animation by temperature (37°C). Of the compounds tested, the ones that did not stimulate exflagellation in SA at room temperature were neomycin, heparin, chloroquine and quinine.

Figure 12: Effect of 2,3DPG; magnesium; and gentamycin on exflagellation of *P falciparum* gametocytes at pH 7.4.



Key

- Magnesium
- 2,3DPG
- Gentamycin

Figure 13: Effect of A-23187 (2μM), caffeine (5mM), and theobromine (5mM) on exflagellation of *P. falciparum* gametocytes.

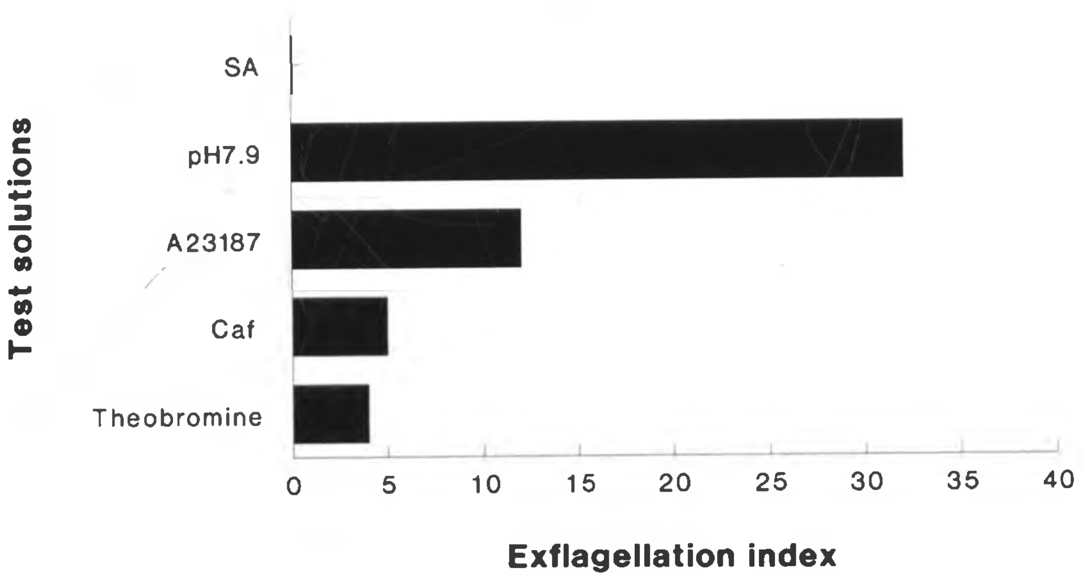


Table 4 Effect of oleoyl-2-acetyl-glycerol (1μM) on exflagellation of *P. falciparum* gametocytes

Test solutions	SA pH 7.4	SA pH 7.9	OAG pH 7.4	OAG pH 7.9	HCO_3-pH 7.4
Exflagellation	-	1-2	0-1	1-2	2-3
Scores	-	2-3	0-1	2-3	3-4
	-	1-2	+	2-3	3-4
Exflagellation index	0	11.7	3.7	13.3	18.3

The three scores were the best scores obtained in three replicate experiments run with the same harvest of same isolate.

Figure 14 Effect of gentamycin on exflagellation of *P. falciparum* K-67 gametocytes at pH 7.4

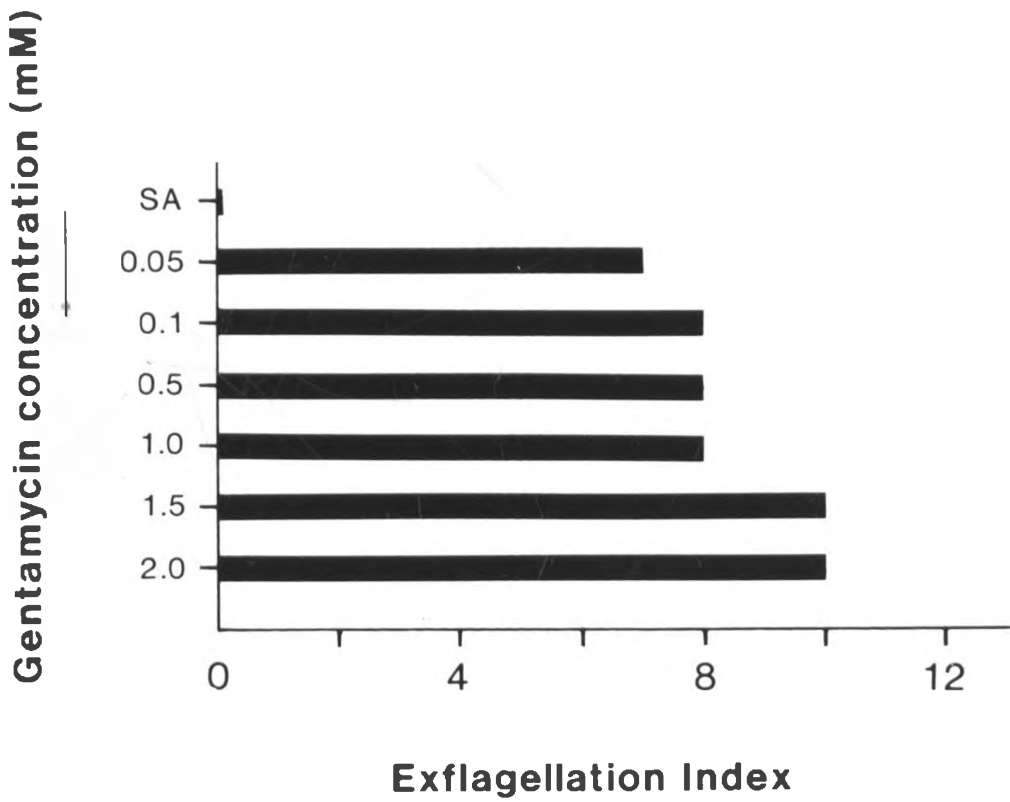


Figure 15 Effect of neomycin (2mM) on exflagellation of *P. falciparum* NF-54 gametocytes.

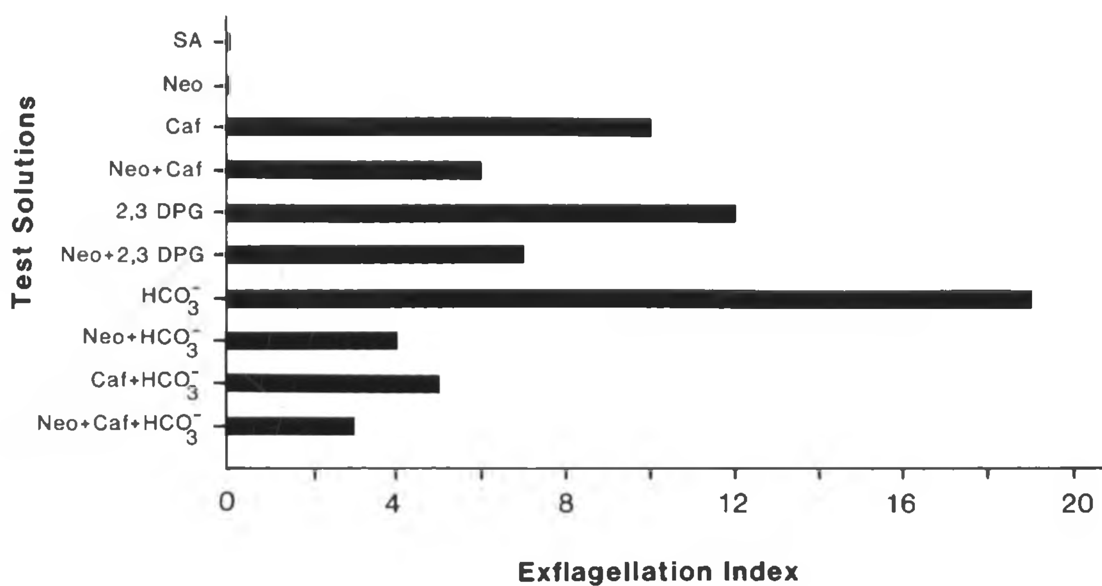


Table 5: Effect of neomycin on exflagellation of K-67 *P. falciparum* gametocytes

SA	Neo, pH7.4	HCO_3^-,pH 7.9	Neo+HCO_3^-, pH 7.9	pH 7.9	Neo+ pH 7.9
0	0	16	8.3	12.5	5.0
0	0	3.7	1.0	12.5	7.0
0	0	9	0.3	3.7	0.67
0	0	0.3	0	5.0	1.0
0	0	1.0	0.3	7.0	3.5
0	0	3.5	1.0	13.3	8.3

Neomycin significantly reduced exflagellation in all the positive conditions tested ($p<0.05$).[HCO_3, pH7.9 and pH 7.9 alone] and never stimulated exflagellation at pH 7.4.

3.6.1 Effect of Heparin on exflagellation

The effect of Heparin as an inhibitor of exflagellation was tested by adding 10µg/ml of Heparin to exflagellating media containing known triggers HCO_3^- 25mM, Caffeine 5mM, Gentamycin 2mM, 2,3DPG 2.5mM, and Mg^{2+} 0.25mM. In every case the presence of heparin decreased the exflagellation index observed with the triggers alone(Fig. 16). When tested alone at pH 7.4, heparin was not a trigger of exflagellation but it reduced exflagellation caused by alkaline pH 7.9. The same experiment was carried out using two other isolates, NF-54 and JP119 and the inhibition was the same as in K-67. Partial inhibition of exflagellation caused by heparin, could not be eliminated by adding whole serum, a factor that on its own enhanced exflagellation.

3.6.2 Effect of chloroquine on exflagellation.

Sixteen-day old K-67 gametocytes were held in suspended animation then tested for exflagellation using varying concentrations of chloroquine in SA at pH 7.9. The results (Fig. 17) clearly showed that chloroquine at low concentrations did not completely block exflagellation (0.05-.25mM) while at concentration above 0.5mM, exflagellation was completely blocked. Therefore chloroquine at 0.5mM was used in subsequent experiments. The concentration of chloroquine required to block trigger of exflagellation by Mg^{2+} (0.25mM) at pH 7.9 was determined by adding increasing amounts of chloroquine to exflagellating medium with Mg2+(Figure17).

Figure 16 Effect of heparin (10μg/ml) on exflagellation of *P. falciparum* gametocytes at pH 7.4.

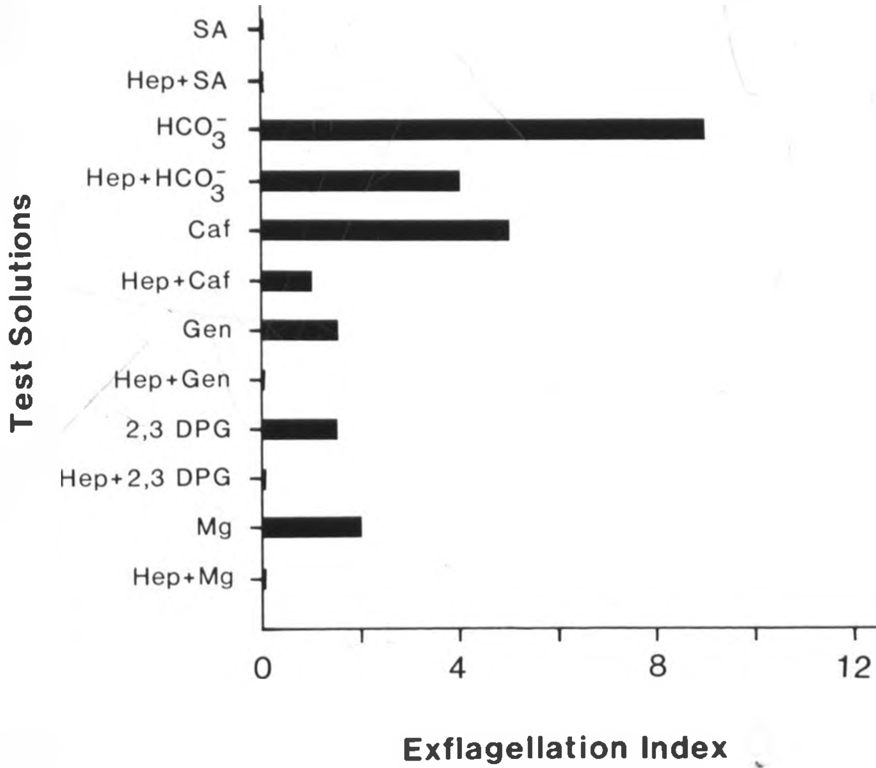
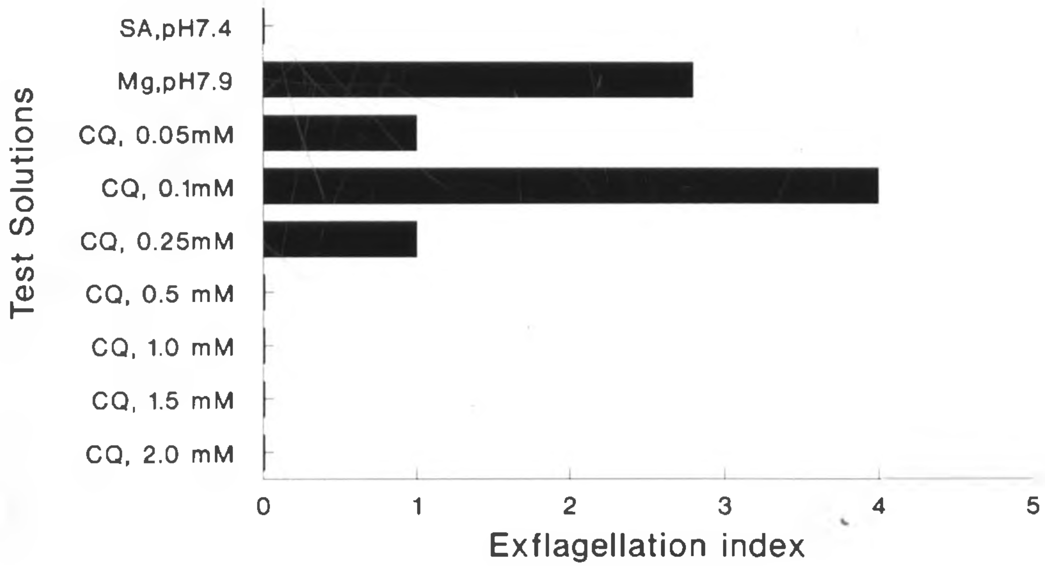


Figure 17: Effect of chloroquine on exflagellation of *P. falciparum* K-67 gametocytes.



Mg,pH 7.9 was used as the positive control for this experiment, and varying concentrations of chloroquine used to block stimulation caused by the positive control.

3.6.3 Inhibition of exflagellation by chloroquine (0.5mM) at pH 7.9 using *P. falciparum* NF-54 gametocytes.

Chloroquine caused complete inhibition of exflagellation. In every case, the presence of chloroquine inhibited the exflagellation observed with the triggers alone(Fig. 18). The same results were found when chloroquine was tested at 2mM

3.6.4 Inhibition of exflagellation by chloroquine (0.5mM) at pH 7.4 using *P.falciparum* NF-54 gametocytes.

Chloroquine was also tested at pH 7.4, where it completely inhibited exflagellation by all compounds that had triggered exflagellation at this pH (Fig.19). The mature *P. falciparum* gametocytes did not exflagellate in SA at pH 7.4 but some gametocytes rounded up without disruption of the cell membrane. When mature gametocytes were incubated with 0.5mM chloroquine for 1 hr at 37°C, then washed in SA twice they retained their ability to exflagellate in the presence of non-physiologic triggers. Moreover, gametocytes incubated in 0.5mM of chloroquine for one hour in SA exflagellated in SA, a condition which was not observed with other gametocytes not incubated with chloroquine. This observation could not be explained.

3.6.5 Effect of quinine on exflagellation of *P. falciparum* K-67 gametocytes

Quinine inhibited exflagellation caused by alkaline pH in a dose-dependent manner (minimum dose being 0.25mM and maximum non toxic dose,2mM) in all the 3 isolates tested (Figure. 20). Alkaline pH, which consistently triggered exflagellation did not do so in the presence of quinine. Addition of other non-physiologic triggers like caffeine, 2,3 DPG, magnesium, or HCO_3 to the alkaline pH, did not cause exflagellation in the presence of quinine. Similar results were obtained with quinine tested at pH 7.9 (Fig. 21). Testing mature gametocytes in quinine, showed that the drug completely inhibited exflagellation in all the conditions that had caused exflagellation at pH 7.4 (Figure 22). In the presence of both physiologic and non-physiologic triggers of exflagellation, quinine completely blocked exflagellation when the triggers were used singly or in combinations that synergised (Fig. 22).

Similar results were obtained when chloroquine and quinine, were tested using the three different isolates, K-67, NF54 and JP119, indicating that these results are common to all *P. falciparum* gametocytes. In the presence of these antimalarial drugs, drop in temperature did not cause exflagellation, but when the drugs were diluted out by washing in SA, the temperature dropping to below 30^oC triggered exflagellation.

3.6.6 Effect of incubation with chloroquine and quinine (0.5mM) on exflagellation

In order to determine if the absence of exflagellation in the presence of the two antimalarial drugs was due to a toxic rather than a physiologic effect of the drugs, a toxicity test was performed. The same tests were carried out using chloroquine and quinine with the same results realised (Table 6a and b). The results showed that incubation with the drugs did not cause permanent inhibition on exflagellation. After washing, the gametocytes spontaneously exflagellated even in SA at pH 7.4. Addition of chemical triggers, Mg^{2+} and 2,3, DPG at alkaline pH also triggered these washed gametocytes.

Figure 18: Inhibition of exflagellation by chloroquine (0.5mM) at pH 7.9 using *P. falciparum* NF-54 gametocytes.

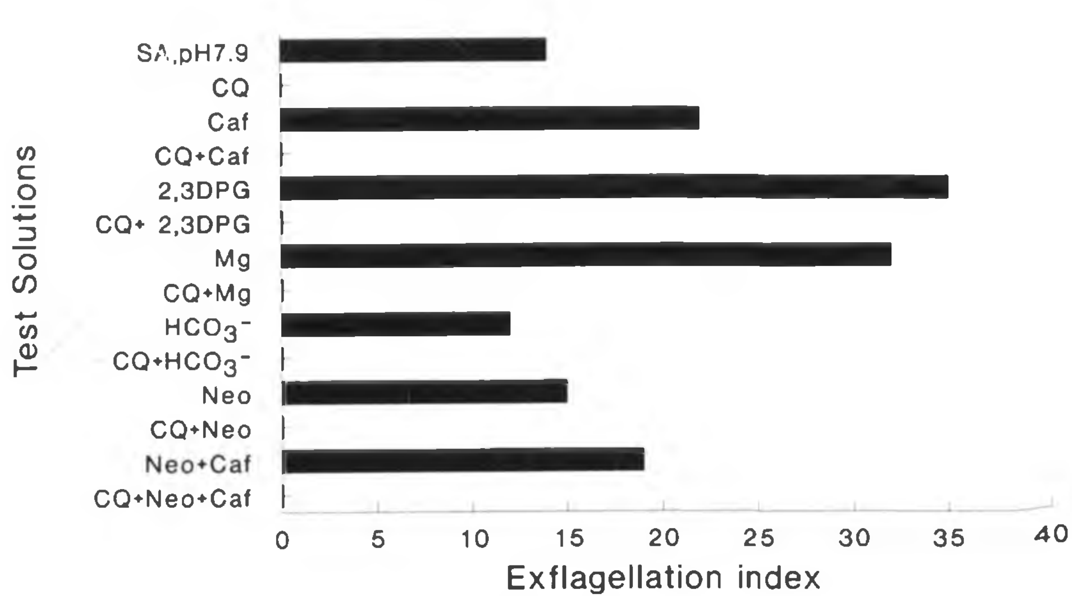


Figure 19: Inhibition of exflagellation by chloroquine (0.5mM) at pH 7.4 using *P. falciparum* NF-54 gametocytes.

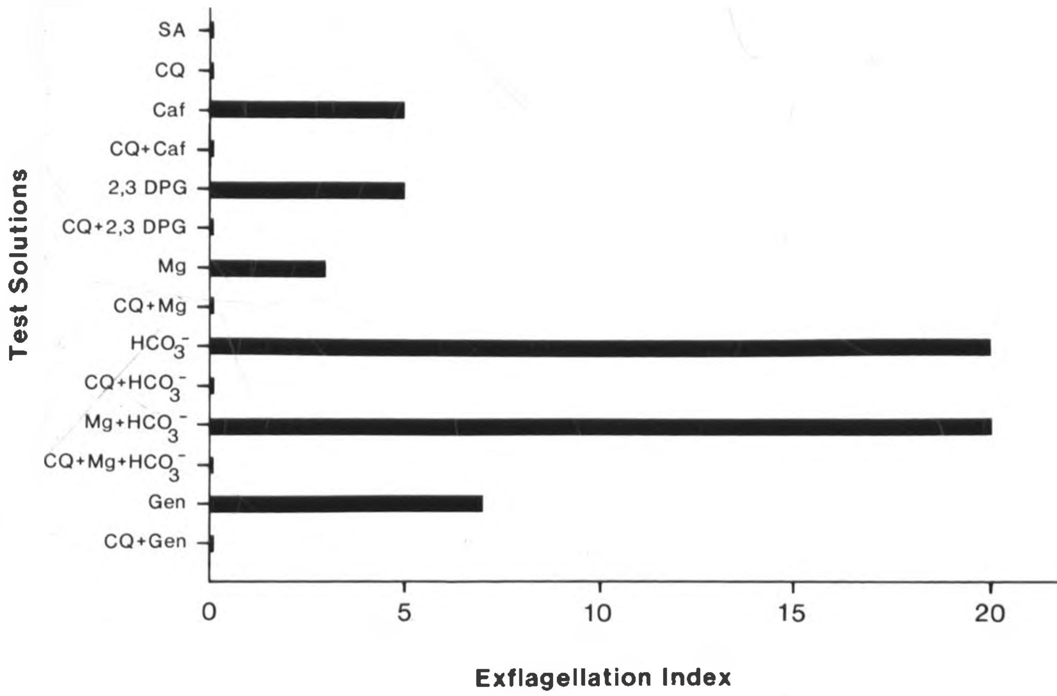
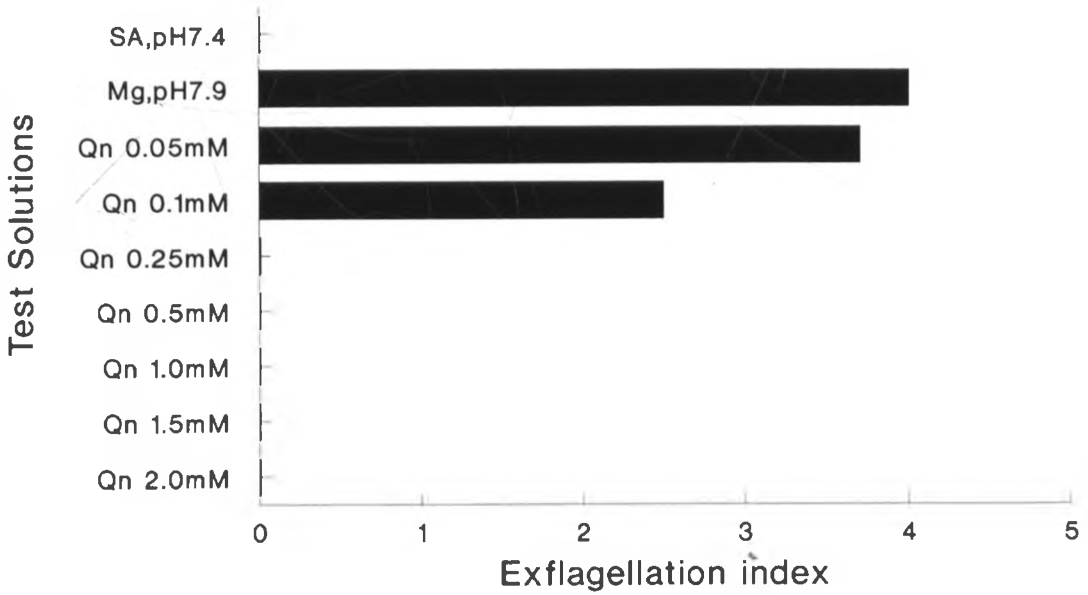


Figure 20 Inhibition of exflagellation by quinine at pH 7.4 using *P. falciparum* NF-54 gametocytes



Mg,pH 7.9 was used as the positive control for this experiment, and varying concentrations of quinine used to block stimulation caused by the positive control.

Figure 21 Inhibition of exflagellation by quinine (0.5mM) at pH 7.9

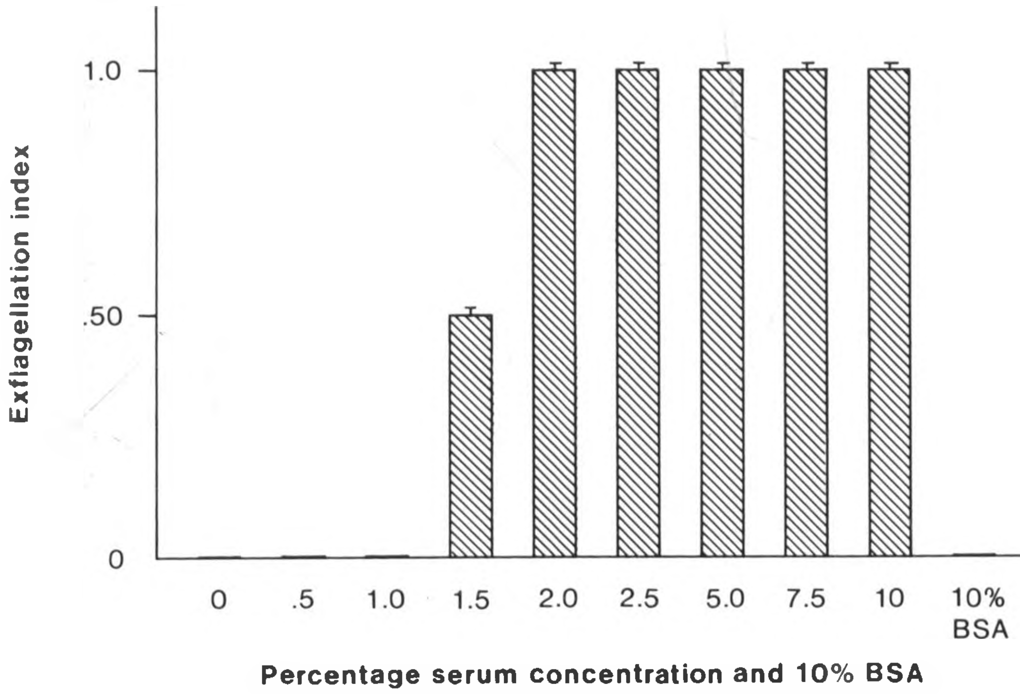


Figure 22: Inhibition of exflagellation by quinine (0.5mM) at pH 7.4 using *P. falciparum* NF-54 gametocytes.

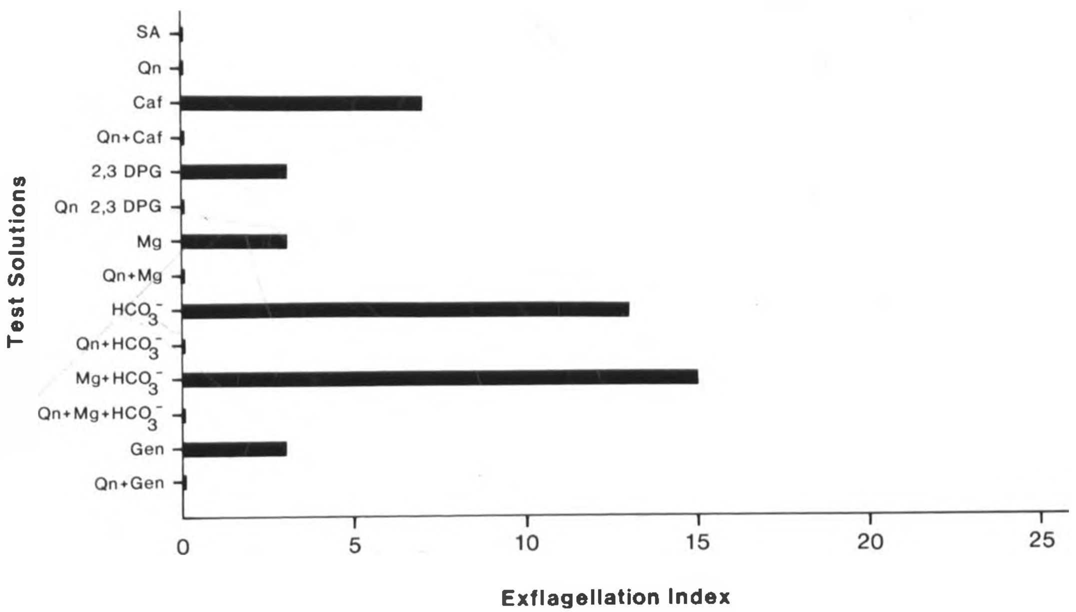


Table 6 *P. falciparum* gametocytes tested for exflagellation after one hour incubation with :

a: 0.5mM of chloroquine

Test Solution	SA pH 7.4	Mg pH 7.4	2.3 DPG pH 7.4	pH 7.9	HCO_3 pH 7.4
Exflagellation Index	2.0	6.2	7.0	12.5	13.0

b. 0.5 mM of quinine

Test Solution	SA pH 7.4	Mg pH 7.4	2.3 DPG pH 7.4	pH 7.9	HCO_3 pH 7.4
Exflagellation Index	3.7	6.8	8.3	12.5	18.3

CHAPTER 4

4.0 DISCUSSION AND CONCLUSIONS.

4.1 Effect of physiologic triggers on exflagellation of cultured *P. falciparum* gametocytes.

Early observers of exflagellation in *Haemoproteus* highlighted the role played by temperature in gamete development (reviewed by Micks *et al.*, 1948). Kligler and Mer (1937) observed exflagellation of *P. vivax*, *P. falciparum* and *P. malariae* at 21°C and somewhat beyond depending upon the species (reported by Bishop and McConnachie1956). However, later research, using avian plasmodia attributed gametogenesis to pH control, presence of bicarbonate and PCO_2 tension (Micks *et al.*, 1948; Bishop and McConnachie, 1960; Carter and Nijhout, 1977, and Nijhout and Carter, 1978). The results of the present study proved that temperature of 37°C is the physiologic factor that prevents mature cultured *P. falciparum* gametocytes from exflagellation *in vitro*. All the physiologic and chemical triggers of exflagellation tested failed to trigger exflagellation at 37°C. The results indicated that above 30°C, cultured *P. falciparum* mature gametocytes would remain inactive and could only be activated after the temperature was dropped. This clearly defined the role of temperature in maintaining suspended animation, being independent of other physiologic triggers of exflagellation. In keeping with the findings of the earliest students of gametogenesis, Kawamoto *et al.* in 1990 used high temperature (37°C) to achieve suspended animation. They confirmed that 37°C is the physiologic trigger that keeps cultured gametocytes in suspended animation *in vitro* . They also found that *P. berghei* microgametocytes synthesize DNA at pH 7.3

(physiologic) and 20ºC (room temperature), but not at pH 8.0 (permissive) at 37ºC, suggesting that DNA synthesis during microgametogenesis is regulated by a temperature-dependent and pH independent mechanism.

The results of the present work showed that exposing diluted blood or cultures to room temperature after gassing with carbon dioxide resulted in a rise in pH from phyisologic to over 8.0, and a corresponding drop in hydrogen ion concentration (Green,1978). As the rise in pH was concomitant with drop in temperature, it was important to find out whether it was the pH rise or drop in temperature that caused exflagellation. When temperature was held constant (37ºC) and pH raised (7.9) no exflagellation was observed, while at physiologic pH (7.4) with drop in temperature (24ºC), exflagellation was observed. Early investigators reported the importance of bicarbonate which caused a rise in pH and hence stimulation of gametogenesis in avian plasmodia (Bishop and McConnachie, 1960; Carter and Nijhout, 1977; Martin *et al.*, 1978 and Nijhout and Carter, 1978). The present work revealed that temperature was the over-riding factor in stimulation of exflagellation in cultured *P. falciparum* gametocytes, not the presence of bicarbonate.

After testing various physiologic triggers such as pH and HCO_3^- separately, it became clear that in cultured *P. falciparum* gametocytes in contrast to *P. gallinaceum*, the rise in pH alone is sufficient to trigger gametogenesis independent of bicarbonate ions. In all experiments, bicarbonate was always removed from serum before carrying out any test for exflagellation. Addition of bicarbonate without rise in pH was enough to cause exflagellation. This finding is different from earlier researchers who used both bicarbonate and rise in pH to induce exflagellation in avian plasmodia (Martin *et al.*, 1978; Nijhout and Carter, 1978). These results

indicate that in cultured *P. falciparum* gametocytes it is possible to induce exflagellation by using either bicarbonate or alkaline pH independent of each other so long as the temperature drops below 30°C. Micks *et al* (1948), using the avian parasite *P. elongatum* found no correlation between exflagellation and pH. However, Kawamoto *et al.* in 1990 reported that there was evidence that exflagellation of *P. berghei* differed from *P. gallinaceum* in that it occurs in the absence of $NaHCO_3$ and that its induction mechanisms may consist of a low-temperature-dependent DNA synthesis and a pH-dependent control of cytoplasmic morphogenesis leading to emergence and exflagellation. The present work pointed to the fact that in cultured *P. falciparum* gametocytes exflagellation could be induced by either alkaline pH or HCO_3 in the presence of at least 1% decarbonated serum at room temperature (21-26°C).

There is evidence that upon uptake by the female anopheline mosquito, the pH of the blood in the mosquito bloodmeal rises to about pH 7.7 (Bishop and McConnachie, 1960), hence alkaline media have been used *in vitro* to mimic this natural physiological stimulus. Other observers, suggested that alkaline conditions may not represent the true "trigger" for gametogenesis in the mosquitoes, since it too may mimic the action of natural trigger(s) present in the mosquito midgut by activating Ca^{2+} mobilization and increasing cGMP levels (Scheibel, 1987; Tanabe *et al.*, 1989). Work by Nijhout and Carter (1978) revealed that gametogenesis in *P. gallinaceum* involves a bicarbonate dependent process and requires a continuous supply of glucose. They observed that emergence and exflagellation of gametocytes *in vitro*, occur independently of the CO_2 tension but are rigidly correlated with the pH of the external medium. Their results suggested that gamete development of malaria parasites is stimulated when infected blood is exposed to air because the

decrease in the CO_2 tension of the blood causes its pH to rise. The present study with *P. falciparum* gametocytes shows that emergence and exflagellation of gametocytes *in vitro* also occurs independent of the CO_2 tension but a rise in pH triggers exflagellation so long as the temperature is below 30°C. Rise in pH alone without a drop in temperature does not cause exflagellation in cultured *P. falciparum* gametocytes and the presence of bicarbonate only permits exflagellation when there is a drop in temperature (Figures 2 and 3).

Serum, was found to be another important factor for exflagellation. Of all the compounds that caused exflagellation, none did so at serum concentration below 1%. In all the experiments, serum was decarbonated to remove the bicarbonate effect, yet, the decarbonated serum (10%) was enough to causeweak (+) exflagellation below 30°C. This finding differs from what was observed in *P. gallinaceum*, where decarbonated serum did not cause exflagellation (Nijhout and Carter, 1978). In all tests run, addition of decarbonated serum increased the exflagellation ability of the *P. falciparum* gametocytes and the free microgametes were active for a longer period of time. Therefore serum may contain factors (other than bicarbonate) that maintain the motility of the microgametes.

By increasing the concentration of decarbonated serum to 2% and above, it was possible to trigger exflagellation by only a drop in temperature, in the absence of HCO_3^- and pH rise. Below 2% of decarbonated serum, drop in temperature alone was not enough to trigger exflagellation hence a drop in temperature was necessary but not sufficient to trigger exflagellation. Unknown serum factors are also involved in the induction of exflagellation. Earlier work incriminated chicken, human and horse sera in induction of

gametogenesis in avian malaria (Micks *et al.*, 1948; Bishop and McConnachie, 1960; Nijhout and Carter, 1978). Our results show, the two factors, alkaline pH and bicarbonate, stimulated exflagellation independently in *P. falciparum* but each needed the presence of small percentage of decarbonated serum (1%). When human serum was replaced by 10% bovine serum albumin (BSA), no exflagellation was observed. Gametocytes that were cultured in BSA grew well and matured but did not exflagellate when triggered. From this observation, it was interpreted to mean that there is something in serum, other than albumin which was absolutely necessary for triggering exflagellation in *P. falciparum*.

4.2.0 Non-Physiologic Triggers of Exflagellation

Despite the recognition of important extracellular inducers of exflagellation it is clear that the true nature of the secondary messenger systems involved in signal transduction are inadequately characterized. Therefore the effects of various chemicals which affect IP3 dependent pathways-upon the induction of exflagellation of cultured *in vitro* were examined. Results point to the evidence that exflagellation of *P. falciparum* may be regulated by PI hydrolysis products.

4..2.1 Effect of magnesium ions and 2,3 Diphosphoglycerate on exflagellation

Mg^+ and 2.3.DPG. were tested for their known inhibition of the enzyme Ins (3.4.5.) P3 phosphatase in other cell systems (Brass *et al.*, 1985;

Shute and Smith, 1985; Joseph and Williamson, 1986). Results from those compounds suggest that they are triggers of exflagellation in that they cause exflagellation at the non-permissive condition of pH 7.4 in the presence of only 1% decarbonated serum at room temperature (23-26°C). This suggests that exflagellation could be a PI hydrolysis related system in that accumulation of Ins-(1, 4,5) P3 after the enzyme phosphatase has been inhibited by magnesium or 2,3 diphosphoglycerate, causes exflagellation to take place in mature *P. falciparum* gametocytes. However, the exflagellation caused by these two compounds independently is in no way comparable to that caused by the physiologic triggers like 100% serum, bicarbonate or alkaline pH (7.9).

A normal red cell maintains a high level of the organic phosphate termed 2,3-DPG (2,3-diphosphoglyceric acid). This allows oxygen to be given up more readily by the haemoglobin when the blood arrives at the tissues. The 2,3-DPG in the cells falls rapidly when the blood is stored (Green, 1979). Micks *et al.* in 1948 demonstrated that the factor which initiates exflagellation of *P. elongatum* in *C. pipiens* appears to be associated with secretions produced by the ingestion of blood, since unfed (non-blooded) stomach contents from the species do not induce exflagellation upon their addition to infected blood from the bird. Many scientists, in infecting mosquitoes in the laboratory, add fresh red blood cells to the cultured gametocytes for optimum infectivity (Smalley, and Sinden 1977; Burkot *et al.*, 1984; Ponnudurai *et al.*, 1989). In the presence of this compound, exflagellation occured at pH 7.4, although its rate was 33% lower than in serum. The enzyme responsible for the first step in breakdown of Ins(1,4,5) P3 - phosphatase, has been studied in other preparations and has been found to be inhibited by Mg^{2+} or as 2-3 bis phosphoglycerate (Vergara *et al.* 1985).

An earlier finding was consistent with this in that exflagellation showed well with a low concentration of Mg^{2+} (0.25 mM) than high concentration (2mM) at pH 7.4. Downes (1983) also found that in excitation-contraction of muscle fibres, the effect of lowering Mg^{2+} concentration was too dramatic to be explained solely by the known effect of Mg^{2+} on the sensitivity of the contractile proteins to Ca^{2+}. Therefore presence of 2,3DPG, probably could be the factor that causes fresh RBCs to trigger exflagellation hence high infectivity in *in vitro* mosquito infection. Running a concentration gradient for both 2,3DPG and Mg^{2+}, showed that at low concentration, (2.5mM and .25mM respectively) exflagellation was as good as at concentrations at which they have been tested in other cell systems (5mM and 2mM respectively) [Figure12].

As stated earlier (section 1.0), Ins(3,4,5) P3 binding increases 6- fold between pH 7.0 and 8.5 and remains constant between pH 8.5 and 9.5 (Worley *et al* ., 1987). In the present study increasing pH to7.9 significantly increased exflagellation in solutions containing chemical trigger compounds, suggesting that there was increased Ins-(1,4,5)P3-binding. From the results, it was not possible to postulate the mechanism of action of the two compounds that permits exflagellation, but it was clear that the compounds triggered exflagellation at the stated concentrations. Work by Foster *et al.* (1989) suggested that the dephosphorylation of Ins-(1,4,5)P3 to Ins (1,4) P2 is magnesium dependent and Mg^{2+} tested at 2mM was less effective while at .25mM was more effective.This situation was not realized in stimulation of *P. falciparum* gametocytes as both concentrations gave same exflagellation indices(Figure 9). From this work, rise in pH and bicarbonate concentration were factors that regulated exflagellation but could not cause exflagellation at high temperature (37°C). Rise in pH (8.0) increased exflagellation. This is in

contrast to the finding of Joseph and Williamson (1984) who reported that amount of Ca^{2+} released by Ins (1,4,5) P3 was not affected by pH rise (6.5-8.0) and initial rate of Ca^{2+} release was not greatly affected by the incubation temperature (30°C and 40°C) in permeabilized hepatocytes. A temperature of 37°C remained an inhibiting factor in triggering exflagellation, while alkaline pH (7.9) synergized the effect of the compounds that acted as triggers.

4.2.2 Effect of calcium ionophore A-23187 on exflagellation

The results showed that this compound A-23187, was capable of initiating exflagellation at pH 7.4 in SA. This finding is in contrast to the recent finding of Kawamoto *et al.*, in 1990 who reported that the compound had no significant effect on exflagellation. Using Ca^{2+} ionophores such as A-23187 (1μM) and lasalocid (5μM), they reported that the two compounds strongly inhibited exflagellation of both *P. falciparum* and *P. berghei* at pH 8.0, a finding which contradicts the observations of this present study. Calcium ionophore, tested at pH 7.9 caused high exflagellation indices in the present study (>10). The role of Ca^{2+} as a secondary messenger for external 'positive signals have been widely reported in various cellular responses of eukaryotic cells such as proliferation, division and motility. Kawamoto *et al.* (1990) reported that treatment with Ca^{2+} ionophores which may induce an increase in Ca^{2+} mobilization, inhibited exflagellation in both *P. berghei* and *P. falciparum* parasites. Beaven and co-workers (1984) had also reported that stimulation by the ionophore A-23187 caused only a small increase in PI breakdown. Both PI breakdown and the Ca^{2+} signal appeared to depend on a common external Ca^{2+} site. Scheibel *et al.* in 1987 reported that addition of A-23187 inhibits the asexual growth of *P. falciparum* while Tanabe and Dois

(1989) also reported rapid clearance of *Plasmodium yoelii* - infected erythrocytes after exposure to the ionophore A-23187. Calcium ionophore A-23187, was found to enhance exflagellation at pH 7.9 and to initiate exflagellation at pH 7.4, a condition which qualifies it as a trigger of exflagellation. The magnitude of triggering caused by this compound was in no way comparable to that caused by the physiologic triggers such as alkaline pH (7.9), bicarbonate and serum. The fact that Kawamoto and co-workers never removed HCO_3- from their serum when testing exflagellation may explain the difference in our results.

4.2.3 Effect of Oleoyl-2-acetyl-Glycerol (OAG) on exflagellation.

This compound was tested for its effect on exflagellation due to the fact that it has been implicated in many cell systems (Rink *et al.*, 1983; Fujita *et al.*, 1984; Morgan, 1989; Berridge and Irvine, 1989). Stimulation of cell-surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers , diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins (1,4,5) P3). These messengers are generated by a membrane transduction process comprising three main compounds: a receptor, a coupling G protein and phosphoinositidase C. DAG acts by stimulating protein kinase C, whereas Ins (1,4,5) P3 releases calcium from internal stores (Berridge and Irvine, 1989). In this work OAG stimulated exflagellation. The compound OAG was tested by Kawamoto *et al.* in 1990, where it was found to have no significant effect on exflagellation of *P. berghei*.. From the present study, it was evident that OAG was a weak trigger of exflagellation of *P. falciparum* at pH 7.4 and enhanced exflagellation triggered by alkaline pH 7.9. The fact that this compound triggered

exflagellation weakly implicates PI hydrolysis in the signal transduction system of gamete activation.

4.2.4 Effect of Xanthine Oxidase Inhibitors on exflagellation.

Previous experiments with caffeine, a substance known to release bound calcium from membranes, showed that Xanthine oxidase inhibitors can bypass the obligate requirement for bicarbonate and trigger exflagellation of *P. gallinaceum* gametocytes (Martin *et al.*, 1978). Caffeine was also used in sarcoplasmic reticulum (SR) and application of 5mM caffeine after removal of neomycin showed that the SR still retained Ca^{2+} in sufficient amounts to elicit a large contraction. Kawamoto *et al.* in 1990, reported that caffeine at 5mM - strongly inhibited exflagellation of *P. berghei* at pH 8.0, and did not induce exflagellation at pH 7.3. In contrast, the results presented here indicate that caffeine and theobromine, (5mM) enhance exflagellation at alkaline pH of 7.9 and induce exflagellation at pH 7.4. Tanabe and Dois in 1989, reported that caffeine, a potent inhibitor of cyclic adenosine monophosphate-phosphodiesterase (cAMP-PDE), strongly inhibited the exflagellation of *P. berghei* and *P. falciparum*, while early work by Martin *et al.* (1978) reported caffeine as a strong trigger of exflagellation of *P. gallinaceum* at pH 8.0 and a weak trigger at pH 7.4. Our results indicate that the two Xanthine oxidase inhibitors, caffeine and theobromine, tested at 5mM are both strong triggers of exflagellation of *P. falciparum* gametocytes at pH 7.9 and weak triggers at pH 7.4. Since pH 7.9 alone is atrigger for exflagellation, only results of pH 7.4 were reported in the present study.

It was observed that when physiologic and non-physiologic triggers were mixed and exflagellation observed, the end result was not predictable. For example, when caffeine was tested in the presence of bicarbonate at pH

7.9, exflagellation was drastically reduced to insignificant levels ($P<0.05$). Whereas, while removing bicarbonate and testing caffeine at both pH 7.9 and 7.4 resulted in an increment in exflagellation index. Caffeine and HCO_3^- both being triggers of exflagellation when used together at the permissive pH of 7.9, gave a net negative effect. These results indicate that mixing triggers of exflagellation can lead to synergism or antagonism along the exflagellation cascade. There is a likelihood that in reported cases where caffeine did not trigger exflagellation, bicarbonate antagonized its reaction.

4.2.5 Effect of the Aminoglycosides on exflagellation

The aminoglycoside antibiotic, neomycin, has been shown to block neuromuscular transmission and also inhibit the enzyme - calcium-dependent phosphatidyl inositol phosphodiesterase (Shute and Smith, 1985). However, low concentrations of the antibiotic, stimulated the enzyme activity and the inhibiting effect observed at the higher concentrations of the drugs was more marked at lower Ca^{2+} concentration. After testing neomycin and gentamycin, the results indicated that the two acted differently. Neomycin turned out to be an inhibitor of exflagellation while gentamycin was a weak trigger of exflagellation (both tested at 2mM). It was reported that the effects of the antibiotics were dependent on the concentrations of Ca^{2+} but neomycin reportedly was a constant inhibitor of contractions in swine muscles at 2mM, while at lower concentrations (.5mM) it inhibited after equilibrium time was increased from 20 to 40 minutes (Foster *et al.*, 1989).

The inhibition of exflagellation by neomycin reported here is not unusual, as this polyamine antibiotic has been shown to bind tightly to

phosphatidylinositol phosphates to prevent their enzymatic degradation(Schacht,1976). Moreover, the Ca^{2+}-stimulated release of Ins(1,4,5) P3 from erythrocyte membranes has been shown to be severely blocked by this drug (Downes, 1983). Different concentrations of neomycin added to the solutions diffusing into the myoplasm, showed that the signal amplitudes subsequently diminished toward a lower steady level in response to neomycin at .15mM and virtually disappeared in response to higher doses (.3mM, .5mM). Neomycin, (1mM) applied to skinned fiber did not cause any tension, even with repeated applications and prolonged exposure (Vergara, *et al.*, 1985). These two compounds, belonging to the same group of aminoglycoside gave opposite response to gametocyte stimulation, which are consistent with their known mode of action on other systems. Neomycin has proved specific in blocking signals better than tetracaine or procaine, known blockers of excitation-contraction (Vergara, *et al.*, 1985). From the results it was not possible to define the route of action of gentamycin. Further work in this field would reveal more.

4.3.0 Non-physiologic inhibitors of exflagellation:

Apart from neomycin, heparin did not trigger exflagellation at pH 7.4 and partially (significantly) reduced exflagellation caused by alkaline pH as repoted in figure 16 ($P>0.05$).

4.3.1 Effect of heparin on exflagellation

This compound has been reported to displace Ins(1.4.5)P3 from binding to its receptor but is not an inhibitor of the enzyme phosphatase in membrane fractions (Worley *et al*., 1987). This same compound reportedly inhibits both the binding of Ins (1.4.5)P3 to its purified receptor and mobilization of calcium from liver cells (Berridge and Irvine, 1987). From the results, heparin does not trigger exflagellation in *P. falciparum* gametocytes at pH 7.4 and significantly reduces exflagellation at pH 7.9 ($P>0.05$).

4.3.2 The effect of Quinine and Chloroquine on exflagellation.

The two antimalarial drugs, chloroquine and quinine were tested because of their known action as phospholipase inhibitors. Chloroquine sulphate was tested on membrane-bound substrate at pH 7.4 and it was inhibitory on phosphodiesterase activity (Shute and Smith, 1985). When membrane-bound substrate was used, the drug was maximally effective at a concentration of 2mM but low activity remained in neuromuscular transmission (Shute and Smith, 1985). When chloroquine was used for testing exflagellation, it completely blocked the process. At 2mM, the drug completely blocked exflagellation at both pH 7.4 and pH 7.9. However, from the literature there is no evidence of chloroquine specific inhibition of enzyme phospholipase-C.

Quinine has been shown to block responses to stimulation of both nicotinic and muscarinic acetylcholine receptors and the effect at the neuromuscular junction was found to be due to channel blockade (Shute and Smith, 1985). The effect of quinine hydrochloride on the enzyme was realised

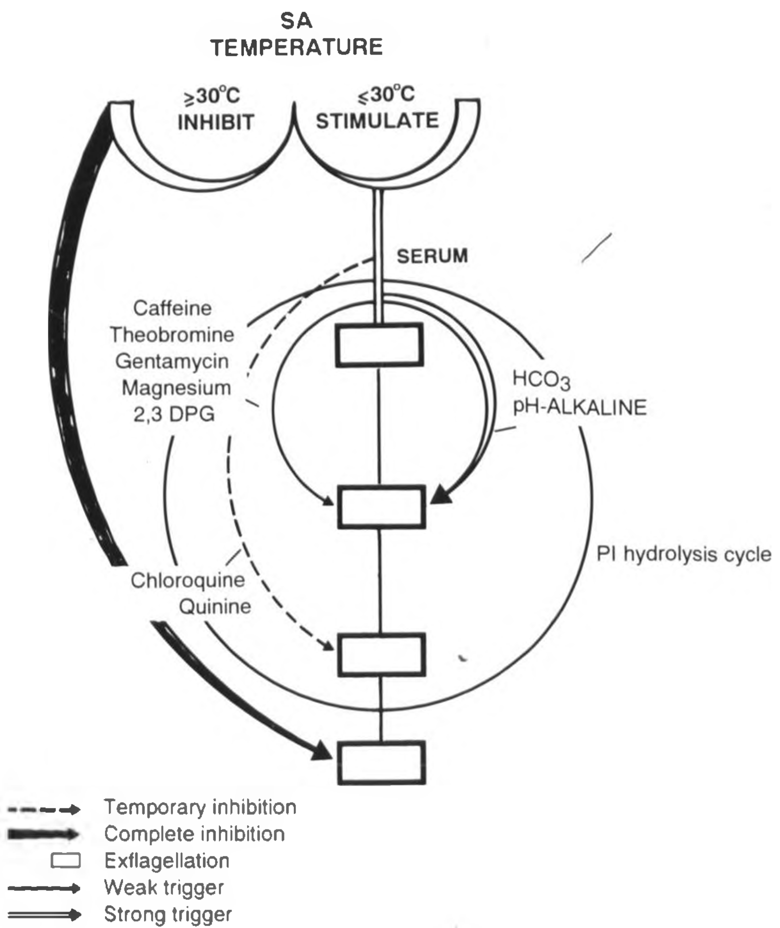
at 2mM but only at a maximum of 35%. In contrast, complete inhibition of exflagellation was observed when quinine was used at a concentration of 0.5mM. In order to rule out the toxicity of the drugs, mature gametocytes were incubated with chloroquine or quinine were washed with suspended animation solution and tested for exflagellation using various triggers. The results confirmed that the blockage was a reversible process and therefore physiologic instead of a toxic effect.

4.3.3 Involvement of Phosphoinositide (PI) hydrolysis in exflagellation:

Despite the involvement of physiologic triggers of exflagellation or the important extracellular inducers of exflagellation, it is clear that the true nature of the secondary messenger systems involved in signal transduction remain inadequately characterized. By examining the effects of various chemicals which are known to affect the Ins(1,4,5)P3 dependent pathways - on the induction of exflagellation of cultured *P. falciparum in vitro*, the evidence indicates that exflagellation of *P. falciparum* may be regulated by PI cycle. All the chemicals chosen which affect the cycle also affect ed exflagellation in a consistent manner, indicating that it is involved in the initiation of this cycle. Thus, of all the factors studied, temperature over-rode all the triggers of exflagellation in that at 37°C no trigger, physiologic or non-physiologic could cause exflagellation. This could point to the presence of a temperature sensitive protein which is triggered by a drop in temperature(Nurse,1990; Marx,1991; Reider,1991). From these results a working model explaining how the various physiologic and non-physiologic factors of exflagellation interact is proposed (Figure 23).

From the present study, the model given below, based on the present finding, shows that temperature is the over-riding factor in exflagellation. Serum also plays a vital role in this process, much more than HCO_3- and alkaline pH. The chemical triggers presumably act via the PI hydrolysis cycle, and the inhibition caused by chloroquine and quinine is removed when the gametocytes are washed in SA. The inhibition of exflagellation is paramountly temperature dependent (Figure 23).

Figure 23 Suggested model for signal transduction mechanisms involved in exflagellation of *P. falciparum* gametocytes.



4.4 CONCLUSIONS.

This study has shown that:

1. In cultured *P. falciparum* gametocytes:

    i. Temperature (37°C) is the physiologic factor that keeps cultured *P. falciparum* gametocytes inactive.

    ii. Neither bicarbonate ion, low pCO_2 nor alkaline pH are necessary for exflagellation to take place at temperatures below 30° C in the presence of >2% serum.

    iii. None of the compounds that trigger exflagellation at room temperature can activate gametocytes at 37° C.

    iv. SA developed for *P. falciparum* gametocytes maintains the washed sexual stages, viable for a minimum of 24 hours at 37° C, hence can allow easy manipulation of gametocytes when setting various *in vitro* ttests.

2. Of all the compounds studied:

    i. Xanthine oxidase inhibitors - caffeine and theobromine; calcium ions; DAG analogue, 1-oleoyl-2-acetyl-glycerol; Ins(1,4,5)P3 phosphatase inhibitors- magnesium, 2,3 DPG, gentamycin all triggered weak exflagellation at pH 7.4.

ii. Neomycin, heparin, quinine and chloroquine negated the ability of the triggers to induce exflagellation both at physiologic and alkaline pH. Chloroquine and quinine completely inhibited exflagellation while neomycin and heparin partially inhibited the process.

This work identified temperature and serum as the major physiologic determinants in the activation of *P. falciparum* gametocytes. It also implicated PI hydrolysis products Ins(1,4,5)P3 and DAG as second messengers in malaria gametocyte signal transduction. The study also implicates the two secondary messengers , Ins(1,4,5)P3 and DAG,(the PI hydrolysis products) as the secondary messengers involved in signal transduction, a condition which suggests that in *P. falciparum*, the process of gametogenesis may be a redundant system, involving both second messengers.

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