A Memoir on Visits by Geoffrey Beale to Chulalongkorn University in Bangkok during 1977-1995, and on Collaboration with Sodsri Thaithong in Research on Malaria Parasites, with an Appendix Giving Some Suggestions for Future Work

First Visit to Bangkok Nov 1976-May 1977

In March 1975 I made a 2 weeks' holiday trip to Thailand (Bangkok, Phuket, Chiengmai) which was so enjoyable that I decided to try and organise a programme of collaborative research with Thai scientists, which would give me the justification for further visits to the country. During my 1975 stay I called in at the Hospital of the Faculty of Tropical Medicine of Mahidol University (Bangkok), which Cyril Garnham had recommended to me as a place where malaria research was going on (under Tranakjit Harinasuta and her husband Chamlong). At that place I talked to Dr Pramualmal Sucharit (in charge of protozoology) and Dr Tan Chongsuphajaisiddhi (who looked after malaria patients). They were very friendly and advised about interesting places for me to visit in Thailand.

When I got back to Edinburgh I immediately wrote to Dr Pramualmal on 26 Mar 1975 and enquired about the possibility of arranging a scientific visit to Thailand, in order to get some first-hand knowledge of human malaria in a country where it is a problem, and possibly do some research, assuming I could get a Visiting Professorship from the Royal Society. I also wrote to the Royal Society, and was informed that it would probably not be difficult for me to get such a professorship.

The response from Bangkok was at first not very encouraging. In fact Dr Pramualmal didn't answer my letter until sent a reminder on 6th May. On 12th May she did reply, merely informing me that she would have to refer the matter to the Dean of her Faculty. After that there was again a long silence, so I wrote to the British Council representative in Thailand (Mr Currie) on 24th July, asking whether he could help. He wrote back saying he had found out that a committee had been set up at Trop Med to consider my request, and that I should send an official application to this committee giving: (1) my c.v.; (2) a detailed statement of a proposed research programme, and (3) a list of equipment required. Moreover it was hinted that I would be expected to make a donation of 100,000 Baht (Thai currency, at that time equivalent to about £ 2300) towards one of the research programmes of the Institute. Finally, there was no possibility whatever that any financial help would be forthcoming from the Thai side. All this seemed very unhelpful and bureaucratic, and so I wrote again to Mr Currie on Aug 15 1975 asking whether he could suggest an alternative host institution for me in Thailand.

He replied on Sept 5 suggesting Chulalongkorn University, where there was an Institute of Health Research, the Director of which-Dr Charas Suwanwela-had expressed interest in my spending a period there. This seemed more promising and I immediately wrote to Dr Charas, who confirmed the invitation to me on Oct 9 1975. I then made a formal application to the Royal Society for a Visiting Professorship, which was rapidly approved. They even said that I would be able to retain the whole of my regular salary as a Royal Society Research Professor, while receiving the additional grant for the Visiting Professorship. The only condition was that the host institution should make some contribution towards my living and travel expenses in Thailand. This caused some embarrassment since one of the Royal Society staff (Mr Deverill) sent a rather unhelpful letter to Dr Charas on 26th July 1976, stating that'a condition of the award to me would be provision (by the Thais) of free accommodiation (such as a house), a car for local travel, and facilities suitable for a person of my seniorty'. The result of Mr Deverill's letter was another long silence from Bangkok. So I had to write again to the British Council representative, asking him to find out tactfully what had happened. He confirmed my suspicions that Mr Deverill's stipulations had been considered unacceptable. On March 22 1976 I wrote again to Dr Charas, rather apologetically stating that there was no need for Chula to provide any local expenses for me, and I would try and get additional support from some other source. Eventually the Wellcome Trust generously produced an additional £ 1000. So everything was smoothed over and it was agreed that I could go out to Bangkok on Oct 28 1976.

At the last minute however there was another hold-up, because just at that time - Oct 1976 - Thailand had one of its periodic political upheavals, and a lot of students at one of the universities - (Thamasart, not Chulalongkorn) - were shot at and ran away to join some "Communist Insurgents" in the mountains. All Universities and Schools were closed and a nightly curfew was imposed. But such occurrences are taken more calmly in Thailand than elsewhere - (there was another abortive coup in April 1977)-, and I was informed by Dr Charas that it would be quite safe for me to go out there as planned. So I was able to make the trip after all, and I stayed for 6 months.

I arrived in Bangkok by British Airways on Nov 1st 1976 and was met at the airport by one of Dr Charas' staff, who installed me in a room in the Institute of Health Research on the 7th floor of the modern "New Science" Building of Chulalongkorn University, which is situated on a pleasant campus in the middle of Bangkok. I had only the vaguest idea what I should be doing. The plan which I had previously put forward was : (1) to contribute to the work of a biological institute in a tropical country, and (2) to collect some first hand information on the spread of drug-resistant malaria parasites. Dr Charas had made some suggestions, which were: (1) to join a working group for research on infectious diseases at the Institute of Health Research and plan some research, especially on malaria and amoebiasia; (2) to give a series of lectures on non-chromosomal genetics to students in the Biology Department, and (3) to give some seminars on advanced parasitology and protozoology to students studying for masters' degrees. But he said that there was no need to follow this plan precisely. Moreover he thought that it would be desirable for me to have plenty of free time to travel about the country and become familiar with its health problems.

As it turned out, the only formal instruction I gave at Chula was: (1) one seminar on the Genetics of Malaria Parasites on Nov. 24 1976, at the Institute of Health Research, and (2) six lectures on Extranuclear Genetics (which was at that time my current interest) to Biology students. These were of course in English, and I was even asked to set some exam question on my lectures, which the students had to answer in English. I didn't insist on a very high literary (or scientific) standard. Thai students are very polite, and never ask awkward questions, so it was difficult to know whether they understood my lectures or not.

After my seminar on Genetics of Malaria Parasites, two members of the audience approached me and expressed interest in a possible research collaboration. These were: (1) Dr Tada Sueblinwong, a biochemistry lecturer in the Medical School of Chula, and (2) Asst. Prof. Sodsri Thaithong, a parasitologist in the Dept. of Biology of the Faculty of Science. Thus began a most valuable and pleasant collaboration which has continued till now (Dec 1995). On Dec 10th 1976 I had my first conference with Drs Tada and Sodsri. We planned to collect samples of malaria parasites from different regions of Thailand and do starch-gel electrophoresis with various enzymes, by the methods already worked out by Richard Carter and his

colleagues in Edinburgh. The aim was to establish the amount of genetic variation amongst parasite populations, and possible correlations between enzyme variation and other characters, especially drug resistance. Eventually we hoped to make comparisons between malaria parasites from Thailand with those from Africa and other parts of the world.

Initially we had a lot of technical difficulties. There was no suitable equipment and the apparatus had to be constructed from such materials as were available locally. It was amazing that we got any results at all. On Jan 14th 1977, together with Sodsri, I visited SEATO (later called AFRIMS), the lavishly equipped U.S. Army Research Establishment in Thailand, and discussed our problems with a friend of Sodsri's - Dr Katcharinee Pavanand - one of the Thai workers employed by SEATO. Dr Katcharinee advised us to do electrophoresis with strains of malaria parasites which were available at SEATO, and which she said were held in culture there. But she thought it would be most convenient for us to do the research at Chulalongkorn Hospital, where Dr Tada worked. Later, however Dr Katcharinee informed us that we would need to write an official letter to the Head of SEATO, requesting permission for their collaboration. Obviously there would be a lot of red tape, and we didn't pursue the plan to collaborate with SEATO.

So then we decided to try and do the work ourselves at Chulalongkorn University in the Biology Dept. where Sodsri had a small lab for Parasitology. In order to collect the samples of malaria parasites, we made a series of about six excursions to Sriracha, a small town about 70 miles east of Bangkok, where there was a hospital receiving malaria patients throughout the year. At the same place there was also a Malaria Control Clinic (under the Thai Ministry of Public Health) to which the less severe cases of malaria went for diagnosis and treatment. Those in charge of both these departments were most helpful in allowing us to obtain samples of infected blood. In fact, we obtained about 70 samples of *P. falciparum* and *P. vivax* from Sriracha during the period of my stay at that time. At first we planned to do the research in a lab at the hospital at Sriracha, which is a very nice place on the seaside, but this turned out to be impracticable.

This preliminary work immediately brought to light certain practical problems. One related to the collection of parasite material. Usually we had to wait while patients were being examined and blood samples were taken in the hospital. When we received the blood we usually found that the parasitaemia was very low, and insufficient for determination of enzyme characteristics. Moreover the parasites in newly collected human blood are almost always at the ring stage, which yields very little enzyme material. There was also the problem of transporting the blood samples from Sriracha to the lab at Chulalongkorn in Bangkok. Apart from that, the electrophoresis technique at that time, as practiced by us in Bangkok, was not satisfactory. Bands of enzyme activity were sometimes seen, but the resolution was poor and the bands were faint. Often the gels were cracked, probably because of an unsatisfactory cooling system. So I suggested that Sodsri should make a visit to Edinburgh where she would be able to see how the work was done there. On April 15th 1977 I wrote to Dr Wernsdorfer at WHO in Geneva explaining the situation and asking for a grant to cover Sodsri's expenses for stay of one month in Edinburgh. This was approved very rapidly. At that time support from WHO was very helpful and expeditious, and Sodsri was able to pay a visit to our lab in Edinburgh in June 1977.

In spite of all these problems, we succeeded in making a start with research on characterization of malaria parasites in Thailand, a programme which has continued for many years, and yielded results of considerable value. Moreover Sodsri later became very well-known in Thailand and around the world for her work. As Dr Charas pointed out many years later, the most difficult moment in organising research is usually at the very beginning. We succeeded in overcoming that obstacle.

Apart from the little teaching and research which I did at Chulalongkorn during this 1976/7 stay in Thailand, I visited a considerable number of other departments and institutes in Bangkok, especially where there was some interest in malaria. I found out about these places from chance conversations with various people. It was a kind of chain reaction : one person just informed me about another who was doing related work. Apart from SEATO, the most important group in Bangkok working on malaria was

that at another university in Bangkok - Mahidol, especially the Biochemistry Department. This university had been founded with Rockefeller money, and was run more or less on American lines, all the instruction being in English and only graduate students being admitted. I was received there with much enthusiasm and invited to share the room of one of the staff (Dr Prapon), if I wanted to work there. The staff at Mahidol tended to regard their university as the only effective research institution in Thailand. Nearly all the people then at Mahidol had worked previously in the U.S.A. or U.K.

I also visited the HQ of the Malaria Control Section of the Thai Ministry of Public Health, which had an elaborate organization of Health Centres all over the country, at which people could be treated free of charge for malaria (and other disease). One such clinic was at Phrabuttabath, where I learnt how the clinics were run. On the whole the Thais have en effective system for keeping malaria under control in most of the areas where it occurs.

Apart from the malaria work, my hosts at the Institute of Health Research of Chulalongkorn University were keen for me to see something of their other activities, especially on drug addiction. I was taken up to a Hill Tribe (Meo) village (Ban Phui Nuea) in the mountains near Chiengmai in the north of the country. We were transported during the final stage of the journey by police helicopter as there was no proper road. I saw how the poppies were cultivated for opium production, and how the authorities were trying to encourage the villagers to change over from opium to cultivation of other crops - coffee, strawberries, rice etc., as part of a scheme initiated by the King of Thailand. At that time this didn't seem to be very successful, as opium was far more profitable than the other crops. Moreover it was used by the inhabitants themselves for "medical" purposes-i.e. to take away aches and pains. Sometimes on could see a cloud of opium-laden smoke rising through the roofs of the huts, before the inhabitants set out to work in the fields in the morning.

On another occasion I was taken to a temple (Wat Tam Kra Borg) at which drug addicts were being treated by a combination of herbal remedies (violent prugatives) and religious (Buddhist) instruction. I was told that the percentage of successful detoxifications achieved by this method was about the same (said to be 50%) as that produced by more orthodox medical treatments. I also joined with a group from the Institute of Health Research on a visit to a prison at Songkhla, where addicts were detained. I noticed that some of the inmates were chained to the floor.

I also visited provincial universities at Chiengmai (in the north), Songkhla (in the south) and Khon Khen (in the NE). These were all in pleasant modern buildings, but were mostly only teaching institutions. Obviously there is a considerable expansion of University education going on in Thailand.

I was able to see a lot of Thailand during this visit. I learnt a lot about the malaria problems in the country, the extent of occurrence of the disease, and the methods used for controlling it. It turned out that for my proposes Chulalongkorn University was the most suitable base for research. This was largely because of the presence there of Sodsri Thaithong, who willingly accepted my various suggestions about the work we might do together. This arrangement was only arrived at from the practical experience of spending some time there. In theory it might have seemed more sensible to plan the search at one of the other departments in Thailand, especially with the biochemical group at Mahidol University or the Trop Med Hospital Group. These had much better research facilities than Chulalongkorn, which is the oldest University in Thailand (founded 1917). It is a kind of 'Oxbridge' of Thailand, to which all the ambitious youngsters want to go, for prestige reasons. I never regretted the arrangements that I made with Sodsri, whose reputation has steadily increased over the years, and whose lab space, equipment and number of assistants have also steadily grown, as have the numbers of colleagues from other countries wishing to come and make use of the facilities and stocks of malaria parasites now available in her labs. It is interesting to note that this development did not arise as a result of a formal plan on my part, or as a result of a high-level decision by some International Agency like WHO, but started spontaneously and informally after a single seminar. After staying in Thailand for 6 months I returned to Edinburgh on May 3rd 1977.

After returning to Edinburgh I paid a short visit to WHO In Geneva (in May or June 1977), where I discussed the situation in Thailand with Wernsdorfer and others.

The need for research on malaria parasites

Malaria is still a serious problem in many tropical countries, including Thailand, though the situation there is less serious than in some neighbouring countries, such as Burma, Cambodia and Viet Nam, as well as many African and S. American countries. It has to be admitted that at the present time (1995) no solution to this problem of malaria is in sight. This unhappy situation has been ascribed to three main factors: (1) development of drug-resistant malaria parasites; (2) insufficient or ineffective anti-mosquito measures, and (3) disturbed political or economic situations. We have concentrated on the first of these factors: drug resistance. There is need for research on malaria parasites because our present knowledge is insufficient to solve the medical problems which exist. Our research aims to increase our basic biological knowledge of the parasites. Such knowledge should help to make clear why current clinical procedures are sometimes unsuccessful, and suggest new control measures. It is important that such work should be done in a country containing areas where malaria is endemic, since an abundant supply of parasites can be obtained only from such areas. Working in Bangkok it is possible to collect samples of blood from malaria patients coming from various endemic regions of Thailand, and immediately set up laboratory cultures of the parasites. Another advantage of Thailand for this work is the high university standards which exist there, thus making it possible to carry out relatively sophisticated laboratory procedures. Thailand is almost unique in being a country where malaria is a serious problems in some regions, and which has good facilities and people capable of studying it.

It should be pointed out however, that the work which we have done had basically **scientific** rather than **medical** objectives. The knowledge obtained will, we hope, extend our knowledge of an interesting group of organisms, namely that of the parasitic protozoa.

My Collaboration with Sodsri Thaithong during the years 1977-1995

Introduction

Between 1977 and 1995 I paid visits to Bangkok every year, each usually lasting 2-4 months, during which I collaborated with Sodsri in her work on malaria parasites. In the early years she had only a single lab for teaching and research in parasitology, but as time went on she was able to expand into neigbouring rooms, acquiring a considerable amount of equipment, and employing increasing numbers of assistants (all Thai women). She received substantial grants from WHO and other bodies. In fact her work seemed to me to become one of the main research components of the biology department of Chulalongkorn University, which is primarily a teaching institution. Sodsri also paid several short visits to our lab at Edinburgh University, both in Thailand and in other countries. I was certainly very lucky to make contact with her, almost by accident, so it seemed, during my first visit to Thailand in 1977. In August 1990 she was awarded the prestigious 'Outstanding Scientist' Award by the Thai Foundation for the Promotion of Science and Technology, under the Patronage of H.M. the King of Thailand. She asked me to make a special trip out to Bangkok so that I could be present when she received this award. In 1992 Sodsri and I wrote a little book entitled *"Malaria Parasites"*, summarizing our work to that time This was published by the Chulalongkorn University Press, and I believe had a good sale in Thailand, even though it was written in English. Unfortunately it does not appear to have been sold much in any other country.

My role in this work was largely advisory: I suggested the kind of research that might be done, helped with the writing of reports and grant applications to WHO and other bodies, and also helped in the writing of papers for scientific publications. Sodsri's knowledge of English, though quite adequate for reading and conversation, was not such as to enable her to write idiomatically in English. During earlier years, she had spent time studying in Denmark and France, but had never worked in an English-speaking country. However, her knowledge of English was a thousand times greater than mine of Thai. Occasionally I did some actual bench work, particularly when I was devising a method for preparation of clones of parasites by micromanipulation. But in general all the lab work was done by Sodsri and her assistants,

not by me. I was kindly provided me with a small room in which I could read and write, and coffee was served to me frequently in the main lab adjoining my room. Thais are very polite, especially to the elderly. Certainly they gave me the impression that I was a welcome visitor; in one of her letters Sodsri even suggested that I should live in Bangkok permanently, and make occasional visits to Edinburgh, instead of the reverse, which was the actual situation!

The work which we did during my early visits was of course very crude according to present standards. During the succeeding 15 years many new techniques have developed, especially in molecular biology. Nevertheless without the results from the earlier crude experiments, the knowledge and materials necessary for the later work would not have been available. The methods which we used in the early days for characterising malaria parasites (e.g. starch gel electrophoresis) would not be used if we were starting the work now. Probably we would extract DNA, amplify it by the PCR technique, and do Southern blots and agarose gels etc. However none of those techniques were available in 1977. The plan which we made at that time was to arrange for the collection of samples of malaria parasites from different endemic areas of Thailand, have them brought to the lab in Bangkok, and study their biological characteristics, especially by 'enzyme typing'. This involved setting up cultures and growing the parasites in the lab. When we started no one knew how to do the culturing, and equipment for cryopreservation was not available.

Fortunately, in April-May 1978, WHO arranged for a workshop to be held at Mahidol University in Bangkok, at which W. Trager demonstrated his well-known 'candle-jar' method of culturing malaria parasites in human red blood cells. Sodsri was invited to be a participant in this workshop. As a result she became very expert at the technique. She set up in her lab at Chulalongkorn University the necessary equipment for doing it, and in fact her lab was the only one in Thailand where this has been done. It requires a great deal of skill and patience and attention to small details. Only Sodsri was prepared to take the trouble to keep the cultures going for long periods without death of the parasites, or contamination. It was quite irritating how often she was bothered by people from other departments or universities wanting to obtain cultures from her. Very often such cultures which were sent away to other places soon died because other workers were not prepared to take the trouble to look after them properly.

Collection of parasites

During the period 1977-1989 Sodsri organized many expeditions to various provincial areas, usually to the Malaria Clinics of the Thai Ministry of Public Health. She was very good at maintaining good relations with the staff as these Centres. This is very important, since such staff do not usually respond well to invasion by outsiders, especially from foreign scientists having vastly greater financial resources than the local people, and simply wanting to collect material for their own investigations. To collect material, we usually set out from Bangkok very early in the morning in Sodsri's Toyota Station Wagon, in order to avoid the frightful Bangkok traffic, and to arrive at one of the provincial Centres at a convenient time. The main places we visited were Sriracha (which is relatively near to Bangkok), Chantaburi, Kanchanaburi (near the 'bridge over the river Kwai'), Tak (Mae Sod) (on the Burmese frontier), and Borai (near to the Cambodia border). Usually we stayed a night at a hotel and went to the Malaria Clinic the next morning. At the clinic near Chantaburi the best day for collection was market day, when the local villagers would come into town and bring anyone they thought might have malaria. It was always uncertain how many malaria patients would turn up. Sometimes there were none. They would be seen first by the local staff of the clinic and have a finger-prick sample of blood would be taken and examined under the microscope. If it was positive for *Plasmodium falciparum* and confirmed as suitable by Sodsri, (i.e. showing reasonably high parasitaemia), the patient would be passed over to her, and - if the patient gave his or her consent - a five ml sample of blood was taken from a vein in the arm. This was usually done by Tada who was medically qualified. In 1993 it was found that better (and quicker) results were obtained in subsequent culturing work when only finger-prick samples of blood were taken, instead of the five ml samples, and so the whole technique then became simpler. The blood samples were put in sterile tubes, together with RPMI medium, and heparin to stop clotting, - and taken back to Bangkok the same day. After arriving

there quite late in the evening, Sodsri and her assistants would go immediately to the lab and carry out the procedure for setting up the samples by the 'candle-jar' system devised by Trager and Jensen. If and when the cultures were seen to be growing satisfactorily (after several days or weeks), they were studied by the enzyme-typing and other characterization methods (see below). Eventually samples would be cryopreserved in liquid nitrogen containers.

In this way Sodsri gradually built up a large collection (>1,000 by 1995) of samples of malaria parasites, with then became available for study by our groups in Edinburgh and Bangkok, and by others elsewhere. Some of our isolates (e.g. those denoted K1, T9 etc.) were used quite a lot in research by people in UK, USA, Australia etc. The collection formed the basis of the WHO Collaborating Centre on the biological Characterization of Malaria Parasites at Chulalongkorn University, which was officially set up in 1984. The number of samples obtained from any one place varied greatly. At one time Borai was one of the best places for getting material, because men used to cross the border near that place and go into Cambodia to dig in the ground for gems, e.g. rubies. This was illegal and was eventually stopped when the border was closed for political reasons. Another 'good' area was Mae Sod, where large numbers of Karen tribe refugees came over from Burma as a result of attacks by Burmese government troops. Most other regions of Thailand became gradully free of malaria, due to the measures taken by the Thai anti-malaria organization, as well as other developments, such as deforestation. So our success in collecting samples was very much tied up with the political and economic situation in Thailand and neighbouring countries. Fortunately Bangkok was not in a region where malaria was transmitted, due apparently to the absence of the *Anopheles* vectors, so I never had to take any anti-malarial pills while working there.

Characterization - Enzyme Typing

It had been one of my original objectives to establish a system of biological characterization of malaria parasites collected from nature. This seemed to be desirable for its own sake, especially for measurements of drug susceptibility, and it would also make possible the establishment of genetic markers by which any strain of parasites could be identified then or later. This seemed very important to me because it was always possible that during culturing work in the lab, or when sending cultures around the world to other labs, that different strains of parasite might get mixed up. In fact several clear cases of such mix-ups occurred in our labs both in Bangkok and Edinburgh, - and also by other workers elsewhere, - though this was rarely admitted by other groups.

The first characterization technique which we used was that of starch-gel electrophoresis of enzymes. This technique had previously been used with rodent malaria parasites by Richard Carter when he was one of my Ph.D. students in Edinburgh. Andy Tait, another Edinburgh Ph.D. student, - who like Richard had also been an undergraduate at the Biochemistry Department at Edinburgh, where I used to give a few lectures on genetics-, had developed the enzyme technique in his work on genetics of *Paramecium,* and Richard applied the technique to rodent malaria parasites with great success. This work had been the main cause of getting our malaria work in Edinburgh well known, and it seemed reasonable to hope that the method would be equally successful with the **human** species of malaria parasites, such as *P. falciparum*. As it turned out, however, these hopes were not fulfilled as well as we expected, due to the relatively small amount of variation displayed in by the enzymes which we studied in human parasites. Nevertheless Sodsri's extensive work along these lines proved to be of great value. Her first WHO grant, applied for in June 1977 and awarded in 1978, was entitled "Enzyme typing of malaria parasites". In the early work we used starch for the gels, and I have a record that in October 1979 I took supplies of starch of suitable quality, and chemicals for staining enzyme bands, to Bangkok. We later changed over to the more convenient cellulose acetate gel electrophoresis technique, (though Andy Tait remained loyal to starch for many years, specially in his later work with Trypanosomes).

Sodsri and her assistants typed all the isolates of *P. falciparum* which she collected, by enzyme electrophoresis. In our little book "Malaria Parasites", which was published by Chulalongkorn University Press in 1992, we have a Table on p. 17 classifying some hundreds of isolates of *P. falciparum* for variation

of four enzymes: GPI, ADA, PEP, LDH, though of these only GPI showed much variation. It occurred in three forms: GPI-1, GPI-2 and GPI-3, but GPI-3 was very rare in Thailand. (This variant was also found by our Edinburgh colleague Alison Creasey amongst parasites collected in Zimbabwe in Africa). As regards LDH, we found only one form of this enzyme (LDH-1) amongst our collections of wild isolates, but were surprised to observe a single occurrence of a second form (LDH-2) in some mutagen-treated material which we were studying for mutation to drug-resistance. This LDH-2 type occurs quite frequently in Africa, but had never been present previously in any material we had in our Bangkok lab; so its appearance there was a mystery which was never cleared up. Perhaps it was a chance mutation, though that seems very unlikely.

In spite of the disappointing lack of variation, Sodsri developed the routine of passing nearly all the isolates which were kept in out collection of cryopreserved stocks over to her assistants to by typed by the enzyme method. In later years I thought this was not revealing any important new information, though of course it was useful to have some sort of marker attached to all the isolates which were kept in the collection of cryopreserved stocks; and on several occasions the enzyme method was used to re-check the purity and identity of certain stocks which had been sent away, and had later been suspected of being mislabelled or contaminated. I was constantly urging people, in Edinburgh and elsewhere (usually without success), to check that their stocks really corresponded to the label on the ampoule containing them. The enzyme technique was also exploited in our early work on the production of clones. (See below)

Nowadays, we see that it would have been better if we had been able to use, for identification and classification of our stocks, characters that showed more variation than the enzymes; but initially we had only the enzyme methods. As happens so often in research, the methods used in earlier work were later seen to be less effective than other methods which were developed later; but at any given time one can only use the methods which are available at that time.

A number of isolates of *P. falciparum* contained two variants of one or more enzymes (e.g. GPI-1 + GPI-2, or ADA-1 + ADA-2, or PEP-1 + PEP-2). This was shown particularly by our isolate T9 from Mae Sod near Tak, which was studied both by Sodsri in Thailand, and also by her and Virgilio Rosario in Edinburgh. This clearly demonstrated the heterogeneity of individual isolates of malaria parasites in a single patient, i.e. - the presence in a single person at one time of more than a single genetic type of parasite, - a finding which I stressed early in our work, but which was at first little appreciated by others. Later on this led to controversies about 'clonality', as will be discussed below.

Other characterization methods

After we found that relatively little variation in *P. falciparum* was revealed by the enzyme typing method, I asked Andy Tait in Edinburgh to try to devise a more sensitive method. As a result he developed the 2D (2-dimensional polyacrilamide gel electrophoresis, or PAGE) method for use with malaria parasites. This was certainly much more successful than the enzyme typing method in revealing differences between different stocks of parasite, but had various disadvantages: it required a lot of technical skill, used of radioactive chemicals, and above all tremendous patience and concentrated attention by the researcher in interpreting the results, which were produced in the form of immensely detailed patterns showing the varying positions of hundreds of peptide spots on a chart. The method was most used in Edinburgh by Alison Walker and Brian Fenton, and in Bangkok by Chutaphan Pinswasdi. We used the method in certain studies involving detailed comparison of particular clones of parasites, in the work on mutations to drug resistance (see below), and in the work on 'recrudescence' which Sodsri did in collaboration with Dr Webster of AFRIMS in Bangkok. However we eventually abandoned the PAGE method when the MSP method became available.

This MSP method involves comparisons of the merozoite surface proteins denoted MSP-1 and MSP-2. Initially the comparisons were made by an immunological technique, using fluorescence microscopy, as developed by Jana Mcbride and others in the Zoology Dept at Edinburgh. This method revealed a large amount of variation amongst our stocks of malaria parasites, but again required a good deal of skill, and also some specialized equipment, e.g. a fluorescence microscope and a set of specific monoclonal antibodies.

Subsequently however these MSP proteins could be compared more easily by a totally different method, which involved isolating DNA of the appropriate gene, amplifying it by the now widely used PCR technique, and comparing the amplified DNA samples by electrophoresis on agarose gels. At the time of writing (1995), the MSP technique is the one which seems most convenient for identification of particular isolates and clones of malaria parasites. One great advantage lies in the requirement of only a very small amount of parasite material - even a single schizont or gametocyte can be used, as we have shown, using single parasites isolated by my micromanipulation technique. The MSP technique was developed in Edinburgh mainly by Lisa Ranford-Cartwright who went out to Bangkok in 1994 and demonstrated it to Sodsri's assistants. So now we have a considerable choice of methods for the characterization of our parasite material, though Sodsri continues to characterize her isolates by the enzyme-typing method, which has become a routine in her lab at Chulalongkorn. Drug susceptibility is also a character which can be used for identification purposes, and is also of importance for itself, as discussed below.

Drug susceptibility

Thailand is one of the countries in which drug-resistant strains of malaria were first discovered. By 1972 resistance to chloroquine had become so widespread that use of that drug was officially discontinued for treatment of *falciparum* malaria, and Fansidar (pyrimethamine + sulphadoxine) became the drug of choice. By 1985 resistance to Fansidar however had risen to such a level that it also became virtually useless for treatment of *falciparum* malaria in Thailand. After that, mefloquine (or 'Fansimef' = mefloquine + Fansidar), or quinine + tetracycline, was used; but resistance to these substances also soon became a problem, so that eventually there was virtually no satisfactory prophylactic drug which could be recommended for use against malaria in Thailand.

In view of this rise in drug resistance, we considered that it was important to make careful surveys of the reaction of malaria parasites to drugs by *in vitro* tests at different times and places. Sodsri therefore developed a method for doing this. Other workers also used various other methods, some being very sophisticated, such as measurement of the incorporation of radioactive hypoxanthine into DNA in drug-treated parasites, and expression of the results on computers; but Sodsri continues to use her original, and relatively simple, visual, method. This involves growing samples of parasites in wells of microtiter plates in media containing known concentrations of a drug for certain specified times (usually 72 hrs), and observing under the microscope whether or not any parasites remain alive. The results are expressed in terms of the 'MIC' (minimum inhibition concentration) of the given drug. Over the years Sodsri has accumulated a mass of data on the susceptibility of *P. falciparum* to various drugs, especially pyrimethamine, chloroquine, and quinine. In general she found, as expected, that there was a gradual rise in resistance to all the drugs which have been used to treat malaria patients in Thailand, though it was interesting to note that some individual **clones** of sensitive parasites remained in some drug resistant populations of parasites. We published a number of papers giving our results of this work.

Mutation of genes affecting drug resistance

Malaria workers have assumed that drug resistance of malaria parasites arises as a result of gene mutation and selection in nature under the influence of the drugs used to treat the disease; but actual proof of such mutation under controlled lab conditions has rarely if ever been convincingly demonstrated. Some work was done by an American group under Inselburg, but there was evidence that his alleged 'mutants' could have been stray contaminants from drug-resistant parasites which were present in the initial strains used. I thought that very careful attention should be given to starting with pure clones of parasite used in these experiments, and checking that no contaminants got in during the process of selection of mutants. This would involve use of genetically marked clones at the beginning and end of the experiments.

Several papers have also appeared in the literature comparing wild (i.e. uncloned) isolates of malaria parasites for susceptibility to pyrimethamine, and at the same time analysing the molecular structure of the enzyme dihydrofolate reductase (DHFR) in drug-sensitive and drug-resistant parasites. The authors of these papers have found that one particular amino acid at the site '108' in the gene for DHFR was

often involved. But neither I nor John Scaife was convinced that this was the whole story. He was one more Edinburgh colleague that I think I persuaded to work on malaria, and he became a very close friend. Sadly, he passed away in 1991.

Sodsri and I started an experiment using various of her pyrimethamine-sensitive clones (or supposed clones), treating them with a chemical mutagen (nitrosoguanidine), and selecting the treated parasites by growth in medium containing a lethal concentration of pyrimethamine. In due course some parasites having increased resistance to pyrimethamine were obtained, and these were cloned by me by micromanipulation, and studied by others for chemical changes in DHFR. Unfortunately this work had to be done in collaboration with quite a large group of people, some in Sodsri's lab, but others in the Biochemistry Dept of Mahidol University, and some in the Molecular Biology Dept in Edinburgh, at first under John Scaife, and later under Rob Ridley. I acted as a general coordinator of all these people and finally wrote up the work for publication (see *Molec. Biochem. Parasitol.* 1992: **52,** 149.) The results were very interesting, since it appeared that there were several different mechanisms controlling the change from pyrimethamine-sensitivity to pyrimethamine-resistance. This was published in due course but our results did not seem to have much impact on the molecular biological community interested in malaria parasites, and I am hoping that the problem can be pursued further, assuming that Sodsri can get some younger people in her lab capable of doing this kind of molecular work. (see Appendix, p. 15 : running pages 177)

Sodsri decided to take up this work again during my annual visit to Bangkok in Jan/Feb 1995. In our previous mutation work we had been much confused because some contaminating parasites had crept into our experimental material during the process of selection for drug resistance, as shown by the existence of some changes in the genetic markers (enzymes and MSP characters) which were recorded among the supposed 'mutations' which were obtained. Strictly speaking this should have resulted in our discarding the whole experiment, but by careful study of the pedigreees of the various lines in the experiment, I concluded that there was evidence for the existence of 'spontaneous' (i.e. not chemically - induced) mutations in some of the lines which had been selected in presence of pyrimethamine, but which had not been contaminated. So I was very pleased when Sodsri decided to go on with this work. One of the important experimental details was that the selection of parasites should be done in the wells of microtiter plates, rather than in Petri dishes, and also that the initial treatment with pyrimethamine-containing medium should be long enough to ensure that **all** the non-mutant parasites were killed - a process taking a surprisingly long time (7 days or more). On this basis Sodsri resumed the mutation work in Jan 1995, and in due course obtained some supposed drug-resistant 'mutations'. Unfortunately however we **once again** ran into the problem of 'contamination' or 'impurity' of the starting material, as revealed by study of genetic markers in some clones which I prepared by micromanipulation from this starting material. This was only revealed after about 4 months' through study of these clones which Sodsri and her assistants did in the spring of 1995, after my return to Edinburgh. Apparently one of the 'clones' used in this work was one called T9/94 which had been produced by Virgilio and Sodsri many years previously by the 'dilution' method, before I had developed the micromanipulation method for cloning the parasites. It was shown by careful analysis of 'sub-clones' which I made by micromanipulation in Jan/Feb 1995, that one sample of T9/94 was not a pure clone of pyrimethamine-sensitive parasites, but contained a proportion of more resistant cells, which were selected out during the mutation experiment. Whether these resistant parasites had been present all the time in the original T9/94, or had crept in by contamination during the long period since its original production, could not be decided; but obviously the whole experiment was ruined. Sodsri fortunately is a very patient person, and says she will start the experiment yet once more, using thoroughly checked starting material. All this shows once again how important it is to do this checking for purity of the material at all stages in these experiments. Such checks are hardly ever done by other workers, so I am very distrustful of any mutation work done by others working malaria parasites.

Additional work on spontaneous mutation to pyrimethamine resistance, using recloned material, was done in the summer and autumn of 1995. This was done with new clones (so-called "reclones" of T9/94, TM4 and G112), which were treated in Petri dishes or microtitre wells with concentrations of

pyrimethamine equivalent to the MIC for each clone, for varying periods from 6 to 21 days. Any survivors were allowed to recover in normal medium and then tested for susceptibility to pyrimethamine. No mutations were found with any of these clones, though if the period of exposure to pyrimethamine was relatively short (e.g. only for 6 days), some "unstable" increases in resistance occasionally occurred, but reverted to the original MIC level on being allowed to grown in Pyrimethamine-free medium. This study will be continued in future work, especially with regard to the possibility of getting mutations by treatment with 'sub-lethal' doses of pyrimethamine. (see Appendix p.16: running pages 178)

The question of 'Clonality'

From very early on in my work with malaria I had been interested in the question of whether the 'strains' of parasite were genetically homogeneous or not, even in samples obtained from one infected person (or one rodent) at one time. This was first studied by Richard Carter (then a Ph.D. student in Edinburgh) using a collection of freeze-dried specimens of P. falciparum kindly presented to me by Ian McGregor, which I brought back from the Gambia after a visit there in 1972. Some of these samples were shown by Richard to contain mixtures of two or more enzyme types. A more detailed study was later made with a Thai isolate called T9 which Sodsri obtained from a patient at Mae Sod near Tak in 1980. This was taken to Bangkok and eventually to Edinburgh where Virgilio Rosario (who was also a postgraduate student at that time) developed the method of cloning by dilution. He showed that the mixed enzyme types present in the original isolate T9 could be separated by preparing individual clones containing only one type. Sodsri later came to Edinburgh to learn this dilution technique, and prepared a lot more 'clones' from the isolate T9. Eventually some ten of these 'clones' were typed by the enzyme method, and also by various other methods (e.g. 2D-PAGE, drug resistance, antigens etc.). This was a joint effort by some seven people, mostly in our Edinburgh group. The result was that out of these ten 'clones' at least seven appeared to be genetically different from each other. Unfortunately, one of the people involved in this work was another Ph.D. student (Alison Walker), who thought that some of these 'clones' had been 'contaminated', or mixed up in the lab. (It was she who first demonstrated how easy it is to mix up cultures of these parasites in the lab, though she was very reluctant to reveal that information to others). It was impossible to check her suspicions except by starting the work all over again and making more clones from the original stock of T9, which itself might also have become contaminated by then. So I reluctantly decided to publish the results as they stood, (Thaithong et al., 1984) without comment, knowing that in any case there was evidence from other cloned isolates that mixtures of genotypes were often present in single wild isolates. This was notably so in an isolate called CH 150, which was obtained from parasite material from patient at Chantaburi, and which we were able to demonstrate by the 2D technique contained six genetically distinct clones of parasite. This matter was clearly settled so far as I was concerned, but others seem to disagree, notably a French worker named Tibayrenc, who claimed that populations of *Plasmodium* and other parasitic protozoa are basically monoclonal. This hypothesis, which seems rather absurd to me, has been much discussed, but I leave these disputes to others.

Cloning by micromanipulation

My main contribution to the actual lab work done at Chulalongkorn University involved developing a method for the production of pure clones of parasites by micromanipulation. Various cloning methods had previously been described by others. As already mentioned, Virgilio Rosario had worked out a method of cloning by dilution, but in my opinion this method had certain defects, the main one being that one could never be certain that the 'clones' produced by this method were completely homogeneous. Micromanipulation had been attempted by others (e.g. by Odeola and Trager), but I was never able to get successful results using their methods. So I spent a lot of time - in fact several years, though only two or three months each year, - during my annual visits to Thailand, at this work, with the aid of Sodsri's very concientious assistant Napaporn Siripoon. Eventually, after many apparently trivial difficulties had been overcome, the method was successful and became quite easy and rapid, so far as I was concerned. I wrote a brief description of it in a short paper in the *Trans. R. Soc. Trop. Med. Hyg.* in 1991, (though I had

some trouble persuading the editors of different scientific journals that the work was worth publishing); and I again described the method in our little book which was published by Chulalongkorn University in 1992. After this Sodsri seemed to consider that only I could do this kind of work in Bangkok. Later on, in collaboration with Lisa Ranford-Cartwright in Edinburgh we also succeeded in isolated single sporozoites by this method. This was really my only contribution to the experimental side of our malaria work in Bangkok, or in Edinburgh, for that matter!

In the APPENDIX (see below), I summarise the present situation with regard to the causes of the origin of pyrimethamine resistance in *Plasmodium falciparum* and in other species, and make some suggestions for future work on that subject.

Publications by S. Thaithong and G.H. Beale *et al.*

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1981 S. Thaithong & G.H. Beale. Resistance of ten isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. *Trans. Roy. Soc. Trop. Med. Hyg.* **75,** 271-273.

1983 S. Thaithong, G.H. Beale & M. Chutmongkonkul. Susceptibility of *Plasmodium falciparum* to five drugs: an *in vitro* study of isolates mainly from Thailand. *Trans. Roy. Soc. Trop. Med. Hyg.* **77,** 229-231.

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1987 C. Pinswasdi, S. Thaithong, G.H. Beale, B. Fenton, H.K. Webster & K. Pavanand. Polymorphism of proteins in malaria parasites following mefloquine treatment. *Mol. Biochem. Parasitol.* **23,** 159-164.

1988 S. Thaithong, G.H.Beale & M. Chutmongkonkul. Variability in drug susceptibility amongst clones and isolates of *Plasmodium falciparum. Trans. Roy. Soc. Trop. Med. Hyg.* **82,** 33-36.

1988 S. Thaithong, L. Suebsaeng, W. Rooney & G.H. Beale. Evidence of increased chloroquine sensitivity in Thai isolates of *Plasmodium falciparum. Trans. Roy. Soc. Trop. Med. Hyg.* **82,** 209-211.

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1990 S. Thaithong. Suegorn, A., Siripoon, N. and Beale, G.H. Drug susceptibility of malaria parasites *(Plasmodium falciparum)* in Thailand. *J. Sci. Res. Chula. Univ.* **15,** 73-80.

1991 G.H. Beale, S. Thaithong & N. Siripool. Isolation of clones of *Plasmodium falciparum* by micromanipulation. *Trans. Roy. Soc. Trop. Med. Hyg.* **85,** 37.

1992 S. Thaithong, S-W. Chan, S. Songsomboon, P. Wilairat, N. Seesod, T. Sueblinwong, M. Goman, R. Ridley & Beale. Pyrimethamine resistant mutations in *Plasmodium falciparum. Mol. Biochem. Parasitol.* **52,** 149-158.

1994 S. Thaithong, A. Seugorn & G.H. Beale. Culturing *Plasmodium falciparum* from finger-prick samples of infected blood. *Trans. Roy Soc. Trop. Med. Hyg.* **88,** 490.

Book - Sodsri Thaithong and Geoffrey Beale. "Malaria Parasites". (Chulalongkorn University Research Report Series No. 1) pp. 1-100 (1992).

APPENDIX

Review of previous work on pyrimethamine resistance of malaria parasites, and proposals for further work at Chula after December 1995, based on a re-examination of previous literature by other workers, and on our work at Chulalongkorn University.

1. Introduction : The genetic basis of pyrimethamine - resistance

(i) Evidence from comparisons between isolates and clones of *P. falciparum*

Many studies by other workers have shown that pyrimethamine-resistance is associated with sequence changes in the DHFR gene, especially at position 108, where presence of the amino acid asparagine (asn), as opposed to serine or theonine (ser/thr) is correlated with increased resistance. This difference between differently-located isolates is often referred to as a 'mutation' (incorrectly, in my opinion). It is implied, but not proved, that these differences between the sequences of DHFR of different isolates, have previously arisen by mutation at this locus in nature.

(ii) Evidence from the genetic cross between HB3 (resistant) X 3D7 (sensitive) clones of *P. falciparum*

This cross was made by Peterson, Walliker and Wellems (1988, P.N.A.S. 85, 9114-8), and analysis of the progeny clones showed that those which were resistant had an RFLP like HB3, while those which were sensitive had an RFLP like 3D7. This confirms that the change from sensitivity to resistance is linked with a change in a particular chromosome (no.4) of *P. falciparum*. However, actual sequencing of the DHFR gene in these progeny clones had never been done.

(iii) Other evidence for role of asn at position 108

Worachat Sirawarapon *et al.* (1990, *Biochemistry* **29,** 10779-83) transferred the DHFR gene of *P. falciparum* to *E. coli* and made a whole series of mutations in the gene in *E. coli,* and showed that asn at position 108 affected inhibition of DHFR by pyrimethamine.

(iv) Spontaneous mutation of pyrimethamine-sensitivity to resistance of *P. falciparum*

This has **never** been conclusively demonstrated in pure clones of lab cultures of *P. falciparum.*, and this will be the object of our future work.

2. Previous attempts to demonstrate mutations affecting DHFR

(i) Work of Inselburg *et al.* on *P. falciparum*

This group claimed to have obtained both spontaneous and mutagen-induced mutations affecting pyrimethamine resistance in *P. falciparum* (Banyal & Inselburg 1986, *Exp. Parasitol.* **62,** 61-70), but these claims cannot be accepted since the original culture (FCR 3), which was sensitive to pyrimethamine, and was used in this work, was later shown not to be a pure clone. In addition, contamination by the pyrimethamine-resistant isolate Honduras I was thought to have occurred during the experiment. These objections were later accepted in a paper by Tanaka, Gu, Bzik, Li & Inselburg (1990, *MBP* 39, 127-134), and are also mentioned by others, e.g. Peterson *et al.* (1988, *PNAS* **85,** 9114-9118); Zolg *et al.* (1989, MBP *36,* 253-262); Hyde (1990, *Pharmac. Ther.* **48,** 45-59). Therefore, the work of Inselburg *et al.* on mutation of DHFR must be disregarded.

(ii) Work of Thaithong *et al.* on *P. falciparum* (see also p. 11 : running pages 174)

In recent (1995) work, many attempts to get spontaneous mutation from pyrimethamine-sensitivity to pyrimethamine-resistance in carefully cloned material of *P. falciparum* have failed. this work was done by exposing large numbers of parasites in wells or Petri dishes to concentrations of pyrimethamine equivalent to the MIC of the original clones, for periods of 6-21 days, and subsequently transferring the surviving parasites (if any), to normal medium lacking pyrimethamine, but unfortunately no stable changes in MIC were obtained in these experiments. The total numbers of parasites studied were as follows:

Clone T9/94/RC17 - 10^9 parasites (in 7 experiments)
Clone TM4/C8.2 - 3×10^8 parasites (in 4 experiments)
Clone G112/CB 1-3 - 3×10^8 parasites (in 4 experiments)

It is possible that these numbers of parasites are too low to make possible the detection of any spontaneous mutations. Alternatively the mass of dead parasites produced by pyrimethamine treatment of a dense culture in a Petri dish or well may inhibit growth of a few resistant parasites which might be present.

Occasionally, when the selecting dose of pyrimethamine was applied for eight or fewer days at the MIC concentration, a few parasites survived and subsequently grew in drug-free medium in wells, but these parasites did not show any permanent increase in MIC when tested subsequently. However, in one experiment, after the clone T9/94 was exposed to pyrimethamine at the MIC dose (5×10^{-8}) M for only six days, a few parasites survived, and after recovery the MIC was found to have changed to 10^{-6}. After a further three weeks in normal medium, however, the MIC reverted to 5×10^{-8} M, (i.e. the same value as that of the original T9/94). Either there had been a mutation to an unstable form, or there had been mutation to a form with increased resistance, and subsequent overgrowth by unmutated sensitive parasites in a well containing both sensitives and resistants. This needs to be re-investigated.

(iii) Evidence of spontaneous mutation to pyrimethamine resistance in rodent *Plasmodium* species.

This has been successfully demonstrated in several species, after inoculation of clones of parasites into mice, and selection with a lethal dose of pyrimethamine.

In *P. bergei,* Diggens *et al.* (1970, *Nature* **228,** 579-580) exposed mice containing sensitive parasites to pyrimethamine, and obtained one out of seventy mice containing pyrimethamine-resistant parasites. Furthermore, van Dijk *et al.* (1994, *MBP,* **68,** 167-171) found that a 'large proportion' of 75 mice showed increased parasitaemia after treatment with pyrimethamine, and from two mice parasites were obtained which grew in pyrimethamine-containing mice at the same rate as control (sensitive) parasites in undrugged mice. These workers also obtained resistant clones showing the change in DHFR from ser to asn at position 110, and one clone with an additional, (i.e. a second), change (serto phe) at position 177.

In *P. yoelii* Morgan (1974, Ph.D. Thesis, Edinburgh University) estimated that the mutation rate from sensitive to resistant parasites was $1/10^{10}$; Chang & Saul (1994, MBP **65,** 361-363) examined some mutants of this species obtained from D. Walliker, and found that one mutant had a change from ser to asn at position 106, and one had a 115 bp deletion. It is possible however that there may have been more than a single clone in the original material used in this work.

In *P. chabaudi,* Cowman & Lew (1990, *MBP,* **42,** 21-30) obtained a pyrimethamine-resistant mutant showing the change ser to asn at position 106 (which is analogous to position 108 in *P. falciparum*), by selection of a clone of parasites growing in mice first with a sublethal dose of pyrimethamine, followed by a lethal dose. These authors also obtained some *unstable* changes in pyrimethamine resistance caused by chromosome duplication, following treatment with sub-lethal pyrimethamine treatment.

Hence, there is no doubt that spontaneous mutations to pyrimethamine resistance can be obtained by selection of clones of **rodent** plasmodia in mice in presence of pyrimethamine. It follows that spontaneous mutation should be detectible also in lab cultures of *P. falciparum.*

(iv) Chemically induced mutation in *P. falciparum.*

Inselburg *et al.* have claimed that they could obtain mutations to increased pyrimethamine-resistance in isolate FCR-3 following treatment with the chemical mutagen MNNG (nitroso-guanidine). These results must however be disregarded for the reasons given above, concerning the heterogeneity of the original parasites treated, as already stated above (p. 14 : running pages 177)

As mentioned on p. 10, the group of Thaithong *et al.* obtained clones of parasites showing increased pyrimethamine resistance following treatment with MNNG (Thaithong *et al.* 1992, *MBP,* **52,** 149-158). There clones have been analysed for sequence differences in DHFR by colleagues at Mahidol University, Bangkok, and for other variations affecting DHFR by Shu-Wan and other colleagues at Edinburgh University:-

Mutant TM4/8.2/4.1 showed the change ser to asn at position 108 in DHFR;

Mutant T9/94 M1-1/b3 showed no change in the coding sequence of DHFR, but increased quantity of the enzyme DHFR, possibly due to an increase in expression of the DHFR gene;

Mutant T9/94/300.300 was obtained following treatment with a second mutagen-EMS, -and this mutant (which was not cloned) showed a change in DHFR at position 164, from ile to met.

Many other mutant clones were isolated by micromanipulation from material treated with MNNG by Thaithong *et al.*, but they have not yet been sequenced. It is much to be hoped that this will be done in the near future. These may be summarised as follows-

Mutant T9/95/M1-1 "a" series-14 clones

Mutant T9/94/M1-1 "b" series-25 clones

Mutant TM4/8.2/4.1 -21 clones

Some of these clones vary in their MIC values, and in general the "a" series of T9/94/M1-1 have a slightly lower MIC than the "b" series. There is therefore possibly some variation in the DHFR bases which might be changed in this material.

All these mutants are thought to have been produced from pure clones of T9/94 or TM4, as shown by comparisons fo mutants and original non-mutant clones in regard to other characters such as enzymes and 2D-PAGE variant, which are controlled by other genes. These 'background characters' were found to be the same in the original non-mutant clones and in the mutants. Unfortunately, however, at the time that these mutation experiments were being done (about five years ago), some contamination was found to have occurred in some parallel cultures growing at the same time as the mutants. (See Sodsri's 'Saga' diagram). It would be desirable therefore to recheck some of these mutants, which will be sequenced in future work, by studying their MSP 1 and MSP 2 variants and comparing them with similar variants in the original clones. These are now the most convenient and sensitive 'background characters' available for checking the possible occurrence of contaminations.

3. Summary of suggested future work

(i) Spontaneous mutation to increased pyrimethamine-resistance

In spite of previous failures, it is very important to continue with this part of the work. Only by analysis of such mutants will it be possible to find out exactly how much of the increase in resistance is due to changes in particular parts of the DHFR gene. The method of selection with a sub-lethal dose of pyrimethamine, as used by Cowman and Lew with *P. chabaudi,* could be explored with *P. falciparum.* Culture of parasites to which non-lethal, but inhibitory, doses of pyrimethamine could be set up, and sampled at monthly intervals, when samples could be tested for changes in MIC, and immediately cryopreserved for future study, and then carried on for further growth in presence of a non-lethal dose of pyrimethamine. The monthly samples could be tested for growth in medium containing pyrimethamine at the MIC dose and allowed to continue growing in this concentration for at least 10 days, to eliminate all non-mutant parasites. If a permanent change in MIC is found, the culture should be cloned by micromanipulation as quickly as possible, to avoid accumulation of unmutated or back-mutated cells from unstable mutants and over-growth of stable mutants by unmutated or unstable cells.

Another approach might be to treat cells at the MIC dose of pyrimethamine for less than 8 days, and to recover with care any surviving cells, as described above in para **2** (ii). If 'unstable' variants are found, they might be tested for duplications of chromosomes or other abnormalities as found by Cowman and Lew and others with rodent *Plasmodium* species. (Cowman has volunteered to do this for us)

(ii) MNNG-induced mutation

(a) The previously obtained mutant clones of T9/94, kept in liquid nitrogen, could be sequenced, as suggested above, to find out which amino acids are affected, and whether there are more mutations like T9/94/M1-1/b3, lacking any change in the coding sequence of DHFR.

(b) The upstream (promoter?) region of mutant T9/94 M1-1 (b3) by comparison with that of the original clone T9/94, could be investigated. (This is already being done in by Chutaphan).
(c) The cryopreserved material of T9/94 which was previously treated with MNNG, could be re-examined to check for presence of mutations by the current technique of growing parasites in wells for 10 days with MIC concentrations of pyrimethamine.
(d) The original T9/94 clone which was used for the experiment described in (c) above, could be examined with respect to the MSP-1 and MSP-2 markers, to check that this clone has not become contaminated.

Conclusion

The general objective of this work is to study the genetic and molecular basis of pyrimethamine resistance in *Plasmodium falciparum*. This is the only drug-resistance character at present available for the study of mutation at the level of sequence changes in DNA. Many people assume that these mutations occur frequently in nature, but this has not yet been proved. It is also widely believed that the change at position 108 in DHFR is the main cause of the occurrence of pyrimethamine-resistance, and this is a best an over-simplification of the drug resistance story.