COMMENTS ON CULTIVATION OF EXOERYTHROCYTIC STAGES OF PLASMODIA*

R. L. INGRAM†

I am grateful for the opportunity to open the discussion of Dr. Huff's paper. First of all, I think we should recognize that Dr. Huff and his colleagues are the ones to prepare such a paper; they are unquestionably the leaders in this area of malarial research.

The paper is both interesting and stimulating. It covers a multitude of scientific disciplines and technologies. Consequently, I think it quite likely that there will be a lively discussion. There may be temptations to stray into biochemical, physiological, embryological, and other considerations beyond the scope of the present workshop. However, should this happen, it is a problem for the moderator and not for me.

Now, with Dr. Huff's indulgence, I should like to make a few comments about the paper; hopefully I shall be able to cover some of the highlights of this interesting work.

Dr. Huff did not go into the history of *in vitro* culture of malarial parasites. For this, he refers us to a couple of excellent review papers. I might just say in this regard that apparently the first attempts to grow tissue stages of these organisms was made by Drs. Huff and Bloom in 1935. So you see, as of now, he is the beginning and the end of this kind of endeavor.

The authors mention a number of factors which contributed significantly to their success in growing the parasites. One of these was that they were able to get three species of avian malaria parasites to undergo a kind of laboratory adaptation by successively passing the organisms through chick embryos—the source of their host cells for the *in vitro* cultivation of the parasites. How much information one loses about the true nature of the organisms that have undergone this kind of laboratory adaptation is questionable. It probably varies from organism to organism and probably is dependent somewhat on the extent of change necessary for adaptation. Also, it might be simply a selection process whereby certain members of the population are favored while others are not until the population becomes more homogenous.

An early point made by Dr. Huff which I think has wide application is the fact that his studies demonstrated that the morphology of the exoerythrocytic stages of three species of malarial organisms was essentially as seen in fixed stained preparations. Histologists, pathologists, and others have contemplated and worried about the amount of distortion of cells, tissue, organisms and pathological processes seen in material which was prepared for conventional microscopic examination.

I am not going to say much about the heading *Culture and Observation Chambers*. Much of this aspect of the work is unique and many of the advances along these lines have come out of Dr. Huff's laboratory. As a parting remark on the culture chambers, I am pleased that we got to see these. No mention was made in the manuscript about figures, slides, etc. I think these chambers have to be seen to be appreciated, especially by those who may not have had a great deal of exposure to cell culture methods.

I think it is interesting that parasites were maintained in cultures for as long as 7 months and that they were still reproducing at this time. In mammalian malarias, scientists have speculated as to whether or not there are succeeding generations after the first crop. Of certain simian malarial parasites with which we have had some experience,‡ the tissue stage seems to disappear after the first generations of parasites. However, the host may subsequently show a malarial relapse, thereby indicating that apparently a few exoerythrocytic (E. E.) forms persist. We wondered whether or not some of the E. E. stages have an exceptionally slow growth rate;

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† National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland.

‡ Work referred to was done while the author was at the Laboratory of Parasite Chemotherapy, National Institutes of Allergy and Infectious Diseases.

if some of the merozoites from the first crop of parasites might have been destined to invade tissue cells instead of erythrocytes; or if the parasites from blood stages might, under certain circumstances, invade tissue cells. Dr. Bray has made some observations along these lines and I am sure he will want to comment on this point.

I believe most of the cultivation of parasites was done with embryonic tissue infected with the organisms. However, several times reference was made to sporozoite-inoculated cultures. I think there are two points here which should be explored. First, how are the microflora eliminated from the sporozoite suspension? We found, for instance, that a combination of penicillin and streptomycin in sufficient concentrations to control microflora contamination was quite detrimental to the survival of the sporozoites. As you know, these antibiotics seem to be the most common ones used for maintaining sterility in tissue culture systems. The next point I wish to raise is that unless we can study the early phase of the E. E. forms, *e.g.*, the invasion of the host cell by the sporozoite and its subsequent transformation into a merozoite, then we have probably missed an important segment of the life cycle of the organism.

The media employed by Dr. Huff's group in the work reported here consist of the ingredients commonly reported for cell cultures, especially those of an embryonic origin. Someone may wish to know why pyruvate was added to some of the media. I think the media are so undefined that it is questionable whether we should speak of them at all as defined media. All of them contain serum and/or egg ultrafiltrate. I doubt that the added amino acids and other ingredients played an important role in the survival of the cells or the propagation of the parasites. I shall have additional remarks to make about the media later.

The last three topics deal with (1) advances yet to be attained, (2) attempts to cultivate exoerythrocytic stages of *Plasmodium cynomolgi*, and (3) possibilities for the use of tissue culture. With your indulgence, I should like to comment on the first and last of these headings together because I think they are closely related. They constitute, to a great extent, the stimulating part of the paper. There is much food for thought here which I am sure will provoke a lot of comments. I think Dr. Huff has spelled out very well the goals which they hope to attain. He points out that their great need is to obtain established avian cell lines with which to work. I was intrigued by the statement that, "If more success is not attained in these efforts it may conceivably be possible to grow the avian parasites in established lines of mammalian cells." I think this is not likely even though the parasite, unlike mammalian malarial organisms, invades a wide variety of cell types. You will recall that another goal of Dr. Huff's group is to determine the capability of differing cell types for serving as host to the E. E. stages. They state that one possibility in determining what cells are invaded by the E. E. forms is the presence of an anatomical barrier in certain cells of the host. Another possibility would be that the parasite knows what cells have the material or can be induced to synthesize the material necessary for its survival and propagation. We know that some of the worms and other forms of parasites selectively choose their site of invasion. How far this selectivity extends down the evolutionary tree is an interesting question.

I think another goal might well be to formulate a chemically defined medium in which to grow the organisms. From a biochemical and a pharmacological standpoint much could be accomplished if this could be done. For instance, one could study metabolic pathways, utilization of various substrates, mechanism of action of various drugs on the organism and/or the host cells and much more.

This leads us to possibilities of tissue culture in the study of malaria. I think these have been spelled out quite well. One point is made which I think is especially worth reemphasizing. That is the possibility of using tissue culture methods in the area of chemotherapeutics. The number of compounds which could be tested at various dosage levels *in vitro* would be tremendous. Also, think of all other possibilities and advantages of studying the effect of drugs *in vitro*. There are possibilities too numerous to mention. Dr. Huff and his colleagues have already opened the way for many of these studies. I should like to quote from their paper: "Our technique already permits us to deal with exoerythrocytic stages in the complete absence of erythrocytic stages, admittedly an important tool in dealing with differing types of chemotherapeutic agents which appear to differ in their actions against

erythrocytic and exoerythrocytic stages." This is indeed a fine contribution to malariology.

In summary, I think the successful cultivation of E. E. stages of avian malarial parasites *in vitro* as presented here today has contributed immeasurably to a better understanding of the morphology and biology of these organisms. Advances in many technicological fields, microscopy and the like, have contributed much in making this possible. I think Dr. Huff's work has brought us to the point where we can extend our horizons of *in vitro* culture of E. E. stages of malarial parasites in areas of: a) immunological and immunochemical studies, b) host-parasite relationships, c) species susceptibility, d) pathological aspects of the organisms, e) biochemical considerations, and f) effect of chemotherapeutic agents against the parasite and/or the host cell. These are just some of the areas in which *in vitro* studies of the tissue stages offer considerable promise.

Now, I should like to say a few words about the cultivation of exoerythrocytic stages of mammalian malaria *in vitro*. Our experiences with simian malaria agree well with what Dr. Huff has said about his experience with *P. cynomolgi*. However, we have had limited success in this area. Before we attempted to grow the parasite *in vitro*, we tried to grow liver parenchymal cells and to maintain organized adult monkey liver tissue *in vitro*. We felt that this must be done if there were to be any success with the *in vitro* cultivation of tissue forms. By the same token, it was our belief that if hepatic cells could be cultured or maintained *in vitro*, the parasite could be cultured. Details of the liver maintenance studies have been published in *Experimental Cell Research*. Infected liver tissue removed from a monkey 6 days after sporozoite inoculation and maintained in culture for 2 or 3 days showed almost as much development as did comparable parasites which continued their development *in vivo*. Liver tissue removed from a monkey 7 days after sporozoite inoculation and kept *in vitro* for 2 additional days was injected into a "clean" monkey. This animal subsequently developed a parasitemia. Also, we have set up liver tissue from an animal 4 days after sporozoite inoculation. Two days later the tissue was examined for parasites. Parasites were demonstrated which compared favorably with those seen in tissue removed from the animal 6 days after sporozoite inoculation. With an extension of this work one might be able to culture E. E. stages of simian malaria through a complete generation.