Some Physiological Aspects and Consequences of Parasitism

Edited by William H. Cole

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FOREWORD

As a regular feature of its program of studies on protein metabolism, the Bureau of Biological Research at Rutgers University, the State University of New Jersey, has sponsored annual conferences on some aspect of that general theme since 1945. At each conference a small number of investigators, from four to eight, have been invited to present the results of their recent studies against the background of other contributions to the subject. Informality of presentation has been encouraged, and time has been allowed for free discussion of each report. The conferences have been open to all interested persons who registered for them. The average attendance has exceeded two hundred.

The first three conferences dealt with dietary proteins and protein derivatives, particularly therapeutic protein hydrolysates. The next four conferences considered caloric intake, certain amino acids, peptides, enzymes, vitamins, and minerals in relation to health and disease. The proceedings of the first seven conferences were distributed only to those who attended.

The next three conferences were concerned with protein metabolism, hormones, and growth; conjugated proteins; and serological approaches to protein structure and metabolism. The proceedings of those conferences have been published to make the material available to a larger audience than that actually in attendance. This policy is continued for the 1955 conference and is expected to be followed for future conferences.

During recent years particularly, it has become clear that the protein metabolism of animals is often modified by the presence of parasites. This is not surprising, of course, since the majority of parasites do not isolate themselves or their metabolism from the host. It is somewhat surprising however to discover that mild infestations, sometimes even subclinical ones, will alter the metabolic balance of the host. In other words experimental animals ordinarily considered "normal" may not give "normal" responses because of such minor interference.

Consequently, attention is being directed today to the problems of mutual modifications of each other's metabolism by host and parasite in experimental situations where they can be identified. Besides a knowledge of the host's metabolism unaffected by parasites, it is necessary to understand the parasite's metabolism unaffected by the host. This is made possible by the newer methods of culturing parasites on synthetic media outside of the host. Pioneering in this field of study is therefore now under way. Changes in metabolism of the host and of the parasite may then be determined and analyzed.

In order to provide a forum where a few workers might present their preliminary studies in the field, the Eleventh Conference on Protein Metabolism was devoted to the subject "Some Physiological Aspects and Consequences of Parasitism." It was hoped that progress in metabolic studies of host and parasite would be stimulated by the conference and that attention would be directed to the future work that needs to be done. Parasitic protozoa, both intracellular and extracellular, and helminths were discussed. Antibody formation and other host responses were also investigated.

Grateful acknowledgment is here expressed to Drs. Taliaferro, Trager, and Stauber for their help in arranging the program, to the Chemical Division of Merck & Co., Inc. for financial aid in conducting the conference, and to the authors for their assistance in preparing this book for publication.

WILLIAM H. COLE
Chairman
Conference Committee

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INTRODUCTORY REMARKS

Leslie A. Stauber, Rutgers University

Four recent events suggest that studies on the physiology of parasitism have begun to move from lag to log phase of activity. One was the first symposium on this topic in this country at the 1949 annual meetings of the American Society of Parasitologists in New York. The speakers on that occasion were Drs. Willard H. Wright, Theodor von Brand, James W. Moulder, Ernest Bueding, Quentin M. Geiman, and Ralph W. McKee. In 1951, there appeared a book edited by André Lwoff, called the *Biochemistry and Physiology of the Protozoa*, which contains a number of chapters dealing with the parasitic protozoa. In 1952, von Brand, long a leader in this field, published his book on the *Chemical Physiology of Endoparasitic Animals*, and in the same year, in recognition of a need, a new journal, *Experimental Parasitology*, published its first issue.

It is obvious that a parasite which synthesizes new parasite protoplasm and which reproduces in its host is exerting physiological and biochemical activity, some of which at least, by definition of the word parasite in the strict sense, will adversely affect its host. Such action of the parasite will elicit reaction on the part of the host. Some of this host reaction appears as the signs, symptoms, and pathology ordinarily associated with the infection. But the specific host-parasite interactions, the detailed causes and effects in these infections, except possibly for the stimulation of antibody formation, are all but entirely unknown. Even with respect to the antibodies mentioned, the parasite antigens involved are largely assumed and not yet characterized or identified, although they are results of the activities of the parasite. So the very fact that we seem to be entering the log phase of studies on the physiology of parasitism implies that an enormous amount of work is yet to be done.

In this conference it might be anticipated that much might be said of protein nutrition or protein metabolism of parasites or infected hosts. As repeatedly pointed out by von Brand and Bueding, however, some progress has been made, especially by Moulder and Jack W. Daugherty in the United States, but studies in this field have lagged behind the studies of other physiological activities. The papers of this conference, therefore, represent selections of areas in which development is likely to occur and should be encouraged, rather than discussions of the protein metabolism of parasites or of the infected hosts. Since proteins are so basic to the activities of protoplasm, we cannot avoid the belief that we are but steps away from the direct references. It remains for a later symposium to deal more directly and more extensively with that subject.

In the series of papers to follow here, it will be noted that the topics selected include both the parasitic protozoa and the parasitic helminths, the cultivation and nutrition, the protein metabolism, and the specific nature of some enzymes of parasites. Host aspects to be considered deal with antibody formation, with the host as the environment or habitat of the parasite, and with such peculiarities as are incident to the site of the parasite in the host. The range of topics is very broad, but the program has unity within the bounds of the words parasite and host, and because physiological aspects of parasitism are stressed.

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STUDIES ON THE CULTIVATION OF MALARIA PARASITES

William Trager, Rockefeller Institute
for Medical Research

A variety of intracellular parasites may be propagated in tissue cultures or surviving suspensions of their host cells. From an analysis of such complex biological systems of host cell and parasite, much can be learned concerning the physiological relationships between intracellular parasites and the cells within which they develop. For example, it has been found that para-aminobenzoic acid (1) and other heat-stable factors (2) are required for the growth of psittacosis virus in chick embryos or in chick embryo tissue cultures. Certain metabolic analogues, such as oxythiamin (3), inhibit the growth of influenza virus in tissue culture, suggesting that the virus requires the corresponding metabolites. When malaria parasites are maintained in suspensions of their host erythrocytes, their survival and development are favored by the addition to the suspending medium of glutathione (4, 5), pantothenic acid (6), and para-aminobenzoic acid (7). Do these results mean that the virus of psittacosis and the malaria parasite utilize para-aminobenzoic acid, or that the influenza virus uses thiamin? Or is it that the thiamin and the para-aminobenzoic acid are in reality utilized by the host cell to elaborate other substances which in turn are essential for the development of the intracellular parasitic agent? The answers to these and many other basic questions will continue to elude us until we have succeeded in growing an intracellular parasite *in vitro* in the absence of its intact living host cell. Such extracellular cultivation of an intracellular parasite would also lead to information on the nature of the intracellular milieu, since one may assume that obligate intracellular parasites require physical conditions and chemical substances characteristic of the cells within which they live.

TECHNIQUES FOR THE CULTIVATION OF *PLASMODIUM LOPHURAE*

Some measure of success has attended experiments in the cultivation of an avian malaria parasite, *Plasmodium lophurae* (8, 9, 10, 11). The erythrocytic stages of this organism, like those of human malaria parasites, develop only within appropriate types of red blood cells. These stages may be propagated indefinitely by the subinoculation of blood from infected to fresh susceptible animals, of which the chicken and especially the duck are the most suitable. The entire development,

from a small infective merozoite through the large trophozoite to the segmenter dividing into 12 or so daughter merozoites, occurs within a single erythrocyte and requires about 40 hours. Only the merozoites are adapted to a very brief extracellular survival in the plasma between the time they break out from one cell and the time they invade new host cells.

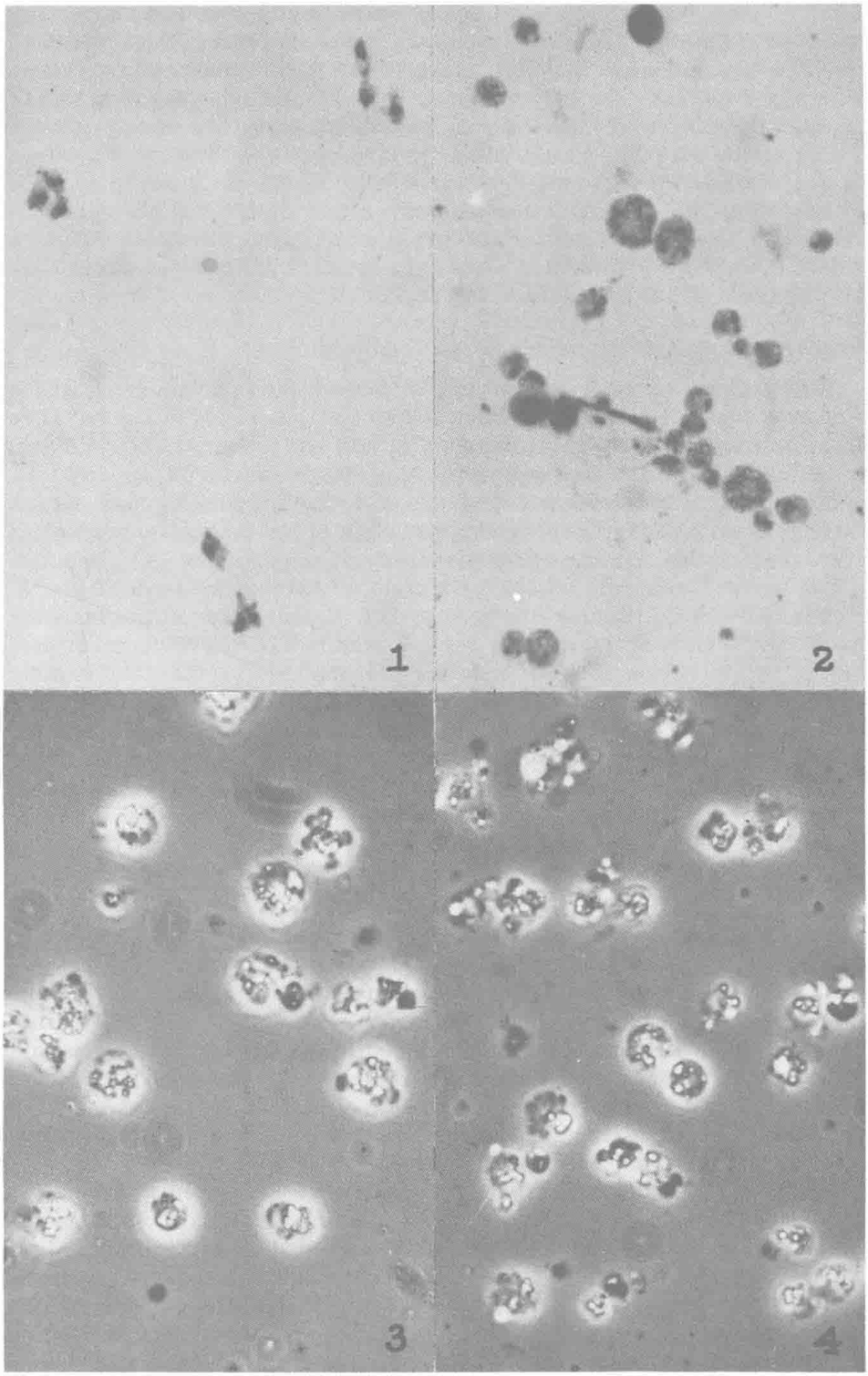
In parasites removed from their host cells and maintained extracellularly *in vitro*, it has proved possible to obtain a complete cycle of development and the beginning of a second cycle. Under the best conditions, 90 to 95 per cent of the parasites were of normal appearance after three days *in vitro*, and in some experiments a majority persisted through four days. The experiments were started with most of the parasites in the uninucleate trophozoite stage (Figure 1-1). Eighteen hours later, notable increases in the proportions of organisms with two to four nuclei and with over four nuclei were readily apparent (Figure 1-2). Moreover, estimates of the total number of parasites present in a culture initially and after 18 to 24 hours showed that this number either remained stationary or increased slightly, indicating that the increased proportion of multinucleate forms was a result of growth and not of differential mortality.

The avian parasites freed from their host cells directly into a suitable medium had cytoplasm with a characteristic, smooth, relatively bright appearance (Figure 1-3) (8). Under favorable culture conditions, this appearance was retained by most of the parasites for three days, and, in some experiments, by many of them for four days (Figure 1-4). Few retained this appearance on the fifth day, even under the most favorable conditions tried. It is evident that the parasites have important requirements concerning which we have no knowledge whatever. This is not surprising. More significant is the fact that cultural conditions have been devised which support for several days the initial extracellular development *in vitro* of an intracellular parasite and permit a study of some of its nutritional requirements.

Preparation of the Extracellular Parasites

The parasites were freed from their host red blood cells in the following way (8). Red cells from a duckling heavily infected with *P. lophurae* were prepared as a 10 or 20 per cent suspension in a special medium containing a concentrated extract of duck erythrocytes. The cells were exposed to the action of guinea pig complement and a hemolytic antiserum prepared in rabbits. There resulted a suspension con-

Figure 1. 1 – Three clusters of uni-nucleate trophozoites of *Plasmodium lophurae*, as seen in a Giemsa-stained film of a freshly prepared, purified suspension of extracellular parasites used to inoculate culture flasks. x1242. 2 – Extracellular *Plasmodium lophurae* after one day in culture, as seen in a Giemsa-stained film prepared from a flask which had been inoculated with the suspension illustrated in 1. Note the forms with more than one nucleus. x1242. 3 – Extracellular *Plasmodium lophurae* after two days in culture, as seen in a fresh preparation with phase contrast. x1242. 4 — Extracellular *Plasmodium lophurae* after four days in culture, as seen in a fresh preparation with phase contrast. Note a cluster of merozoites adherent to a residual body. x1242.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taining clumps of agglutinated, hemolyzed red cells, free red cell nuclei, and free parasites. For most experiments this suspension was inoculated into the experimental flasks. Most of the parasites retained within hemolyzed but unbroken erythrocytes came free during the first day of incubation. For some experiments it was necessary to use as inoculum a suspension containing only fully freed parasites. This was obtained by subjecting a 20 per cent hemolyzed suspension to about 45 seconds of centrifugation in which a maximum speed of 400 to 500 rpm was reached. The agglutinated ghost erythrocytes and parasites retained within them were sedimented, leaving a faintly cloudy supernatant containing free parasites and free red cell nuclei.

Preparation and Maintenance of the Cultures

The culture vessels were 50 ml. Erlenmeyer flasks equipped with a rubber stopper bearing gas inlet and outlet tubes. The flasks were lined internally with a thin plasma clot (10) and received about 3.5 ml. of culture medium. They were rocked gently in an incubator at 40° C. and a slow current of 95 per cent air with 5 per cent CO_2 was passed through them. Under these conditions much of the inoculum, whether it consisted of the mixed hemolyzed suspension or of the purified free parasites and red cell nuclei, accumulated during the first 18 hours' incubation in a fine scum, adherent to the plasma layer at the margins made by the fluid surface as it rocked back and forth. This scum formation made it possible to draw off the old culture fluid and rapidly replace it with fresh warm medium, with a minimum of disturbance to the parasites. Otherwise, it would have been necessary to change the medium by centrifugation, a procedure which injures the free parasites (9). The culture medium was renewed after the first 18 hours and at approximately 12 hour intervals thereafter. No advantage was found in more frequent renewal of the culture medium.

Determination of Parasite Survival and Development

The survival and development of the parasites were followed primarily by morphological methods (8, 9). At the beginning of the experiment and daily throughout its course, material was taken from each flask for the preparation of a wet mount and a Giemsa-stained film. The wet mounts were examined immediately with a phase contrast microscope. They served to give a qualitative impression of the general condition of the free parasites. The Giemsa-stained films were used to determine the proportions of degenerate parasites and of normal parasites in different stages of development.

In those experiments in which the flasks were inoculated with purified parasite suspensions, an estimate of the concentration of parasites initially present was obtained by making a direct count of the parasites, using a Petroff-Hauser bacterial counting chamber and phase contrast optics. For the count after one day of incubation, the parasites were removed from the scum and returned to reasonably even suspension by the following procedure. Each flask received 0.2 ml. of a solution of trypsin (200 mg. twice recrystallized trypsin with magnesium sulfate,

kindly supplied by Dr. M. Kunitz, in 20 ml. 0.003 N HCL) sterilized by filtration. The flask was then returned to proper culture conditions (rocking machine, 40°C., 95 per cent air with 5 per cent CO_2) for three to three and one-half hours. The plasma clot was digested by this treatment, and the scum was loosened. The flask was swirled to suspend the scum, and was then vibrated on a Boerner shaker for one-half hour at 37° C. The resulting suspension contained individual parasites and clumps which were generally small enough to permit counting. A sample was taken for counting in the Petroff-Hauser chamber within five minutes or less after removal of the flask from the shaker. The chief error in this method derives from the difficulty of breaking up some of the large clumps of parasites accumulated in the scum. This and other sources of error would tend to lower the counts obtained after one day as compared to the initial count.

Preparation of the Culture Medium

The mode of preparation of the medium, which is a concentrated extract of duck erythrocytes, is indicated briefly in Table I. It is important to note that the duck blood is obtained and handled aseptically. Aseptic precautions are likewise used in the preparation of the medium,

TABLE I

COMPOSITION OF THE ERYTHROCYTE EXTRACT CULTURE MEDIUM*

A. Nutrient solution used to prepare the extract:

High K^+ content.
Also the following ions; Na^+, Ca^{++}, Mg^{++}, Mn^{++}, Cl^-, $PO_4^{\equiv}$, HCO_3^-, $SO_4^{=}$.
Acetate, glycerol, glucose, hexose-diphosphate.
Acid hydrolysate of casein, glycine, histidine, cystine, tryptophane, asparagine.
Glutathione.
Water-soluble vitamins.
Adenine, guanine, xanthine, uracil, cytidylic acid.
Bovine plasma fraction V.
Gelatin.
Duck serum.

B. Red cell extract:

One volume frozen-thawed duck erythrocytes in 1-1/3 volumes of A, centrifuged 50 minutes at about 2,000 G.
Clear, deep red supernatant constitutes the extract. pH 7.0-7.1.

C. Special additions of known value:

1. To each 10 ml. of extract added 0.2 ml. of a solution giving concentrations of added yeast adenylic acid 1.4, L-malic acid 6.0, and cozymase 0.15 mM per l. pH 6.8-6.9.

2. Originally and with each change of culture medium added to each flask supplementary solutions giving final concentrations of added:

adenosine triphosphate (ATP)	2 mM per l.
sodium pyruvate	5 mM per l.
coenzyme A (CoA)	0.02 mM per l.
pH 6.8	

* For details of preparation see 8 and 9.

the culture flasks, and the parasite suspension. Hence, the medium does not have to be subjected to any sterilizing treatment (such as filtration), and there is no need for antibiotics in the culture vessels. The composition of the diluent (Table I-A.) was arrived at gradually from a number of considerations. Early experiments (5) on the survival of *P. lophurae in vitro* demonstrated the favorable effect of a salt solution of high potassium content as compared to the usual balanced salt solution with much sodium and little potassium. These early experiments, for the most part concerned with dilute suspensions of infected chicken or duck red cells, also showed that the survival of the parasites was favored by aeration (but not by a high oxygen tension) and by appropriate concentrations of glucose, glutathione, serum, and calcium pantothenate (5, 6). Liver extract and embryo extract also exerted some favorable effects (5), suggesting a need for water soluble vitamins. The acetate, glycerol, and casein hydrolysate of the diluent are based on the medium of Anfinsen *et al.* (7), found suitable for the intraerythrocytic development of a monkey malaria parasite in concentrated suspensions of monkey erythrocytes. The favorable effects of gelatin were first noted in a rather striking manner in experiments with the extracellular parasites (8). The remaining ingredients of the diluent are included because of their known beneficial effects in the cultivation of various cells and organisms, because they are assumed to be harmless, and because they help to provide a basal medium which makes it less likely that future fractionation work with the red cell extract would be devoted to the isolation of an already known metabolite. A favoring effect on the extracellular parasites has been demonstrated for each of the materials which is added to the completed red cell extract (Table I-C.).

EFFECTS OF ALTERING THE RED CELL EXTRACT

Addition of Metabolites of Known Chemical Nature

Yeast adenylic acid and cozymase were first observed to improve the extracellular survival of *P. lophurae* in experiments in which the parasites were imbedded in a soft agar gel (8). These two substances have not been studied in the liquid gelatin-containing medium used at present, but they are regularly included in this medium.

Good extracellular survival of the parasites beyond 24 hours was first noted in flasks which were supplemented with both adenosinetriphosphate and sodium pyruvate (8). Adenosinetriphosphate alone had but slight effect (Table II), and pyruvate alone had no effect, but together they permitted virtually 100 per cent survival of the extracellular parasites after two days *in vitro*. This effect was evident in both fresh and stained preparations and in the presence as well as in the absence of malate.

When a mixture of fumarate, succinate, and malate was added to the medium supplemented with adenosinetriphosphate and pyruvate, no effect was observed. When malate alone was added, fewer degenerate parasites were seen in fresh preparations made after three days of incuba-