STUDIES ON THE EXTRACELLULAR CULTIVATION OF AN INTRACELLULAR PARASITE (AVIAN MALARIA)

I. Development of the Organisms in Erythrocyte Extracts, and the Favoring Effect of Adenosinetriphosphate

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Plates 19 to 22

(Received for publication, June 29, 1950)

All the viruses and rickettsiae, certain bacteria, and many of the protozoa in the class Sporozoa are obligate intracellular parasites. Although some of these agents have been grown in tissue culture or in suspensions of surviving host cells, none except a few of the bacteria has been observed to develop extracellularly *in vitro*. In a new approach to the problem of extracellular cultivation of an intracellular parasite, it has now been possible to obtain *in vitro* a considerable development of an avian malaria parasite freed from its host cells. This development was markedly favored by the addition to an already complex culture medium of adenosinetriphosphate and pyruvic acid.

Methods and Materials

The erythrocytic stages of *Plasmodium lophurae* (1), obtained from infected ducklings, served as the experimental organisms. The parasites were maintained by weekly passages in 2 to 3 week old ducklings. Parasitized blood was inoculated intravenously in an amount sufficient to introduce 12 to 25 million parasites per 100 gm. of body weight. Ducklings destined to serve as a source of parasites for the cultivation experiments were inoculated with 100 million parasites per 100 gm. of body weight and were utilized 4 days later. At this time they usually had 40 to 60 parasites per 100 erythrocytes, and most of the parasites were uninucleate trophozoites.

Removal of the Parasites from Their Host Cells.—The parasites were freed from their host red cells directly into a favorable medium by specific hemolysis, a method which has been used previously for early cultivation studies (2) and for physiological studies (3). About 3 ml. of blood from a donor duckling was drawn into 0.3 ml. of a solution of 27 mg. Connaught heparin in 100 ml. of 0.85 per cent sodium chloride solution. The blood was lightly centrifuged, the plasma was removed, and the red cells were made to a 20 per cent suspension in a special medium to be described further on, which contained a concentrated extract of duck erythrocytes. 1.6 ml. of the suspension was mixed in a 50 ml. Erlenmeyer flask with 2.0 ml. of additional erythrocyte extract, 0.07 ml. of guinea pig serum, and 0.4 ml. of an anti-duck erythrocyte serum from rabbits. The guinea pig serum, which furnished complement, was prepared in a relatively large batch and was distributed in 0.5 ml. amounts in small tubes which were capped and stored in a dry-ice chest. For use, one such tube was thawed in cold water. The

antiserum was prepared by the injection of rabbits with washed duck red cells from 2 to 3 month old ducks, using the schedule of injections recommended by Speck *et al.* (3). It was stored in a refrigerator for periods up to 2 weeks and in the frozen state for longer periods. The mixture of red cell extract, complement, antiserum, and parasitized cells—which showed immediate agglutination—was incubated at 37°C. for ½ hour on a vibrating shaking machine. This subjected the cells to rather violent agitation without splashing the contents of the flask against the cotton plug. The agitation was necessary to reduce to a minimum the formation of large clumps of agglutinated red cells which became only partly hemolyzed. Suspensions made in this way usually showed large numbers of entirely free parasites, most of which appeared normal as seen either in stained films (Fig. 9) or in the fresh condition with the phase microscope (see section on morphology). Other methods of preparation of the free parasites, as with saponin or hypotonic solution, or by specific hemolysis into media not containing concentrated red cell extract, yielded parasites which were already of abnormal appearance by phase microscopy. Immediately after preparation of the free parasite suspension small amounts of it (usually 0.3 ml.) were placed in the flasks containing the culture media to be tested. A thin film was prepared from the suspension and stained with Giemsa stain.

The Culture Media.—The basic culture medium has been an extract of duck erythrocytes (2, 4) prepared in a nutrient solution. The composition of the latter was varied in different experiments. It has been based in part on what is known concerning the apparent nutritional requirements of malaria parasites developing within their host erythrocytes (2, 4, 5), in part on data concerning the composition of red blood cells (6), and in part on general considerations. Table I shows the composition of the diluent which has given the best results thus far obtained. This solution has a pH of 5.5, but erythrocyte extracts prepared with it had the desired pH of about 6.8. Somewhat similar solutions but without gelatin had a pH of 7.1 and again gave extracts with a pH of about 6.8.

The medium shown in Table I was prepared by mixing aseptically the various stock solutions in amounts sufficient to make the desired quantity of final mixture. Generally about 100 ml. was prepared at a time. Since the stock solutions gave a total volume of 98.9 ml., the 1.1 ml. of water needed to give exactly 100 ml. was omitted. All the solutions were made up in water redistilled in a pyrex glass still. Most of the chemicals were reagent grade. The bovine plasma fraction V was obtained from Armour and Company and the gelatin from Wilson and Company, Inc. Stock solution 12 was sterilized by steaming for ½ hour on each of 3 successive days, and stock solutions 1, 2, 4, 5, 7, 10, 14, and 15 were sterilized by autoclaving. The other stock solutions were sterilized by filtration through either Selas 03 porcelain filters or ultrafine glass filters. All the stock solutions were stored for periods up to 3 months in a refrigerator, except solution 9, which was either freshly prepared on the day it was to be used or was stored in the frozen state for 1 or 2 days.

Stock solutions which required somewhat special procedures for their preparation were the following:

No. 8. 2.5 gm. of the magnesium salt of hexose diphosphate was dissolved in 30 ml. of water and the pH was adjusted to about 5.8 by the dropwise addition of 0.5 N KOH. The solution was diluted to 50 ml. A faint precipitate formed as the solution aged. Old solutions with a heavier precipitate were not used.

No. 10. The amino acid mixture was prepared by dissolving 20 mg. each of tryptophane and asparagine and 15 mg. each of glycine and histidine in 30 ml. of hot water. To this were added 35 mg. of cystine dissolved separately in 2 ml. of 6 N HCl and 2.8 ml. of parenamine. The pH was brought to 6.7 by the addition of 2 N NaOH and the solution was diluted to 100 ml. A slight fine precipitate formed on standing. It was resuspended when samples were removed.

No. 14. The biotin–folic acid mixture was prepared from two more concentrated solu-

tions. 5 mg. of each material was dissolved separately with the aid of a few drops of 0.1 N NaOH and gentle warming. Both solutions were brought to a pH of 6.7 and diluted to 100 ml. 1 ml. of the biotin solution and 2 ml. of the folic acid solution were mixed and diluted to 100 ml.

No. 15. 5 mg. each of adenine, guanine, xanthine, and uracil and 2 mg. of cytidylic acid were dissolved by heating in 2 ml. of 6 N HCl. The solution was diluted with a little water, adjusted to a pH of 6.7 with 2 N NaOH, and finally diluted to 100 ml.

In the preparation of duck erythrocyte extract, 45 to 50 ml. of blood was drawn aseptically from the right jugular vein of each of an appropriate number of ducks 7 to 11 weeks of age. The ducks used as blood donors were usually in apparently good condition and were bled only at intervals of 2 weeks. The blood from 2 ducks was run into a single 250 ml. Erlenmeyer flask equipped with glass beads, and was defibrinated by shaking for 35 minutes. Shorter periods of shaking did not always suffice to defibrinate duck blood. The defibrinated blood was placed in amounts of 25 ml. in 50 ml. centrifuge tubes and was centrifuged for 20 minutes in the cold. The serum was drawn off and its volume noted so as to permit an estimation of the volume of red cells left in the centrifuge tube. A portion of the serum was kept in the refrigerator for use in the preparation of the diluent. The centrifuge tubes containing the red cells were placed in a dry-ice alcohol bath, thus rapidly freezing the cells. They were then stored 1 to 3 days in a dry-ice chest. The cells were thawed by placing the tubes in cold water. The hemolyzed cell mass so obtained in each tube was suspended evenly in $1\frac{1}{3}$ its volume of the nutrient solution and was centrifuged for an hour at 2300 R.P.M. If the medium contained gelatin this centrifugation could not be done in the cold. With media devoid of gelatin, no advantage was noted if the centrifugation was carried out in the cold rather than at room temperature. The clear, very deep red supernatant liquid was then drawn off. The supernates from several centrifuge tubes were usually pooled, depending on the amount of medium required. For each culture experiment, red cell extract was prepared fresh on the initial day (0) of the experiment and daily thereafter. It was stored in a refrigerator except when in use, and was used before it was 24 hours old. The nutrient solution used as a diluent was prepared fresh on day 0 of the experiment in an amount sufficient for the preparation of red cell extract on both days 0 and 1. A further batch of nutrient solution was then mixed on day 2 for use in the preparation of extract on days 2 and 3 and, if necessary, on day 4.

Some materials were added as supplements to the fully prepared red cell extract. These included the following, all of which were stored in the frozen state in a dry-ice chest.

500 mg. of yeast adenylic acid was suspended in 5 ml. of water and dissolved by the dropwise addition of 2 N NaOH to a pH of about 5.5. The solution was diluted to 10 ml. and sterilized by filtration through an ultrafine glass filter. The solution was used at the rate of 0.1 ml. added to 10 ml. of complete red cell extract medium to give a concentration of 1.4 millimols per liter.

100 mg. of cozymase was dissolved in 20 ml. of water and the pH was adjusted to 5.5 by the addition of a few drops of 2 N NaOH. The solution was passed through an ultrafine glass filter. Either 0.1 ml. or 0.2 ml. was added to 10 ml. of red cell extract to give a concentration of 0.075 or 0.15 millimols per liter.

800 mg. of adenosinetriphosphate (free acid from the Schwartz laboratories) and 400 mg. of sodium pyruvate (prepared by neutralization of an alcoholic solution of pyruvic acid with alcoholic sodium hydroxide and one recrystallization) were dissolved in 12 ml. of water. The solution was brought to a pH of 5.6 by the addition of 0.5 N KOH and was diluted to 20 ml. It was sterilized by passage through an ultrafine glass filter. 0.1 ml. of this solution was added with each change of culture medium to each flask containing 3.3 ml. of fluid, to give final concentrations of approximately 2 millimols of ATP and 5 millimols of sodium pyruvate per liter. In some experiments the ATP and sodium pyruvate were prepared as separate solutions.

TABLE 1

Composition of Nutrient Solution Used in Preparing Duck Erythrocyte Extract

The solution was prepared by mixing appropriate amounts of sterile concentrated stock solutions. To each 100 ml. of the mixture was added 15 ml. of duck serum.

Stock solutions			Final mixture	
No.	Material	Concentration	Amount of stock solution	Concentration
		gm./liter	*ml./liter*	*mg./liter*
1	NaCl	66.00	50	3300.0
	KCl	88.00		4400.0
2	$NaH_2PO_4 \cdot H_2O$	5.52	25	138.0
	K_2HPO_4	62.72		1568.0
3	$NaHCO_3$	50.00	12	600.0
4	$CaCl_2$	2.61	15	39.0
5	$MnSO_4 \cdot 4H_2O$	2.20	20	44.0
6	$NaC_2H_3O_2 \cdot 3H_2O$	25.00	10	250.0
	Glycerol	25.00		250.0
7	Dextrose	50.00	43	2150.0
8	Hexose diphosphate (Mg salt)	50.00	14	700.0
9	Glutathione	20.00	50	1000.0
	Ascorbic acid	0.20		10.0
	Niacinamide	80.00		4000.0
10	Glycine	0.15	20	3.0
	Histidine	0.15		3.0
	Cystine	0.35		7.0
	Tryptophane	0.20		4.0
	Asparagine	0.20		4.0
	Acid hydrolysate of casein (parenamine)	4.20		84.0
11	Bovine plasma fraction V	20.00	300	6000.0
12	Gelatin	180.00	400	72,000.0
13	Riboflavin	0.05	10	0.5
	Thiamin hydrochloride	0.05		0.5
	Pyridoxine hydrochloride	0.05		0.5
	Pyridoxamine dihydrochloride	0.01		0.1
	Calcium pantothenate	1.00		10.0
	Choline chloride	0.10		1.0
	Inositol	0.10		1.0
	Para-aminobenzoic acid	0.10		1.0

TABLE I—*Concluded*

Stock solutions			Final mixture	
No.	Material	Concentration	Amount of stock solution	Concentration
		gm./liter	*ml./liter*	*mg./liter*
14	Biotin	0.0005	10	0.005
	Folic acid	0.001		0.01
15	Adeninesulfate	0.05	10	0.50
	Guanine hydrochloride	0.05		0.50
	Xanthine	0.05		0.50
	Uracil	0.05		0.50
	Cytidylic acid	0.02		0.20

Preparation of the Cultures.—The culture vessels were 50 ml. Erlenmeyer flasks equipped with a rubber stopper bearing small tubes plugged with cotton for the ingress and egress of gas. Each flask contained 3 to 4 ml. of culture medium. As quickly as possible after inoculation of the flasks with the free parasite material, they were placed on a rocker in an incubator at 39–40°C. The flasks were rocked slowly (16 full cycles per minute) and a mixture of 95 per cent air and 5 per cent carbon dioxide was passed through them at such a rate that at the outlet the bubbles came through at approximately one every 2 seconds. The gas was allowed to pass through not more than 3 flasks in a series. The culture fluid was changed usually after the first 18 hours and thereafter at intervals of 12 hours.

Further details of preparation of different types of cultures will be discussed together with the results which were obtained.

Criteria for Judging the Survival and Development of the Extracellular Parasites.—The condition of the free parasites in the cultures was followed by the daily removal of a small sample of material. This was used for the preparation of an ordinary coverslip mount, which was examined immediately by phase microscopy, and of a thin film which was dried, fixed with methyl alcohol, and stained with Giemsa stain. In the examination of both the wet mount and the stained slide, note was made of the appearance of the parasites as compared with those seen in preparations from infected ducks and from the freshly hemolyzed suspensions used to inoculate the culture flasks. On the basis of their morphology the parasites were classified as being all or mostly in excellent condition, some excellent with some degenerate, some fair with many degenerate, or mostly degenerate. From the stained slide, counts were made of the proportion of the various developmental stages and of the proportion of parasites which were definitely degenerate.

The manner in which it was necessary to maintain the extracellular parasites precluded the determination of changes in their absolute number per milliliter of culture fluid. Infectivity for ducklings could not be expected to be a useful criterion of the extent of survival, since stages other than those about to segment would have little chance of survival in the animal. It was hoped that in fresh preparations held on a warm stage the parasites might be sufficiently motile to permit the use of motility as a criterion of survival. This proved not to be the case. Only very occasionally could slight streaming movements be observed either in free parasites or in intracellular parasites contained in a drop of whole infected duck blood freshly removed from the animal and examined immediately on a warm stage. Since it was necessary therefore to rely exclusively on the morphological criteria, a brief description of the normal and abnormal morphology of *P. lophurae* follows. It is worthy of note that in spite of the some-

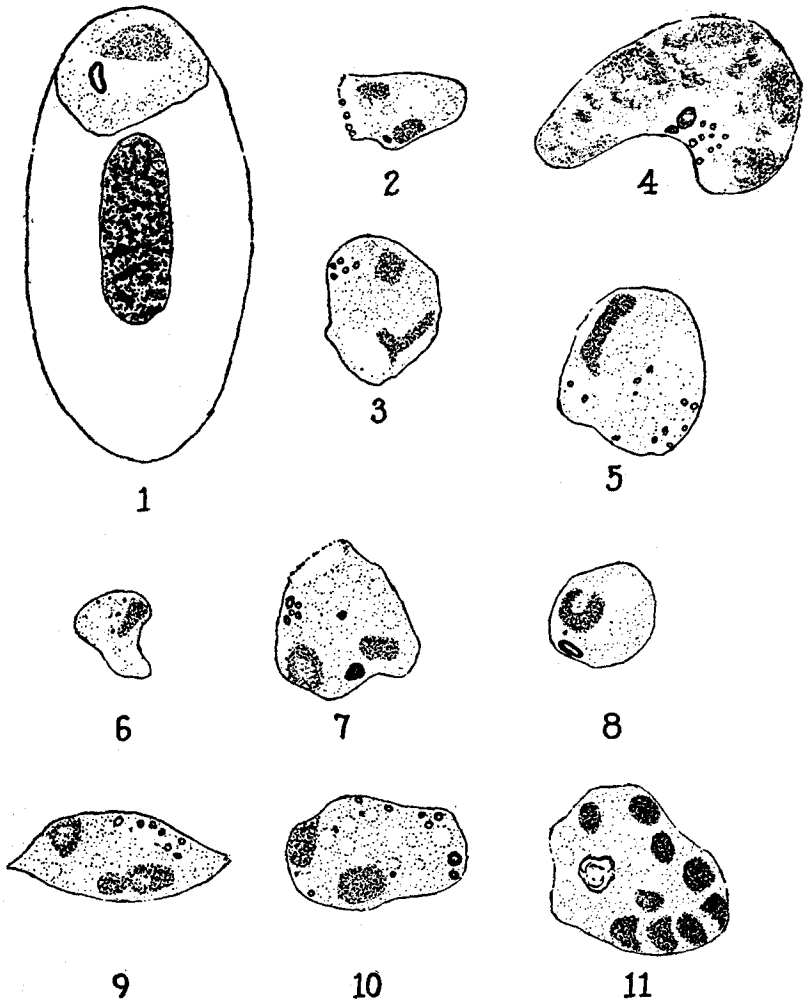
what too subjective nature of the morphological criteria, their use has permitted the development of progressively better culture conditions for the extracellular parasites.

The Morphology of P. lophurae in the Fresh State by Phase Microscopy and in Stained Films.—The morphology of the erythrocytic stages of *P. lophurae* in stained films from infected ducks has been described and figured by Hewitt (7).

The very young parasites are small, fairly compact, somewhat ovoid or pear-shaped organisms with a relatively large purplish red nucleus and blue cytoplasm. The growing trophozoites vary considerably in shape as well as size. They may have a single large vacuole as well as several small ones which appear entirely colorless. The pigment usually occurs as small almost round granules most of which are in one region of the cell. Occasionally it exists as a single large mass, as illustrated in Text-fig. A, 1 and 8, or it may be placed in granules along the border of a large vacuole (Text-fig. A, 2). In older parasites with several nuclei there are sometimes one large pigment mass and a number of small granules (Text-fig. A, 4). The nuclei of the developing trophozoites are stained a purplish red, are highly irregular in form, and often have a vague outline (Text-fig. A, 1–4). A conspicuous feature of the cytoplasm, which was noted by Hewitt (7), is the presence of a number of rather uniform circular vacuole-like bodies which stain a pale pink with Giemsa stain. A small proportion of trophozoites contain 1 or 2 granules stained a very dark purple (Text-fig. A, 2). Mature parasites approaching segmentation show more compact, more darkly stained nuclei than the developing trophozoites, and have most of the pink vacuoles accumulated around the clumped pigment mass (Text-fig. A, 11). Mature gametocytes are larger than the asexual stages and may completely surround the nucleus of the red cell. Both sexes have numerous pink vacuoles and large, elongate pigment granules. The female gametocyte has blue cytoplasm and a distinct reddish nucleus, while the male has pale, sometimes pinkish cytoplasm with diffusely scattered pink nuclear material.

Extracellular parasites freshly prepared by the method already described have the same morphology in stained preparations as parasites within the erythrocytes of infected ducks (Fig. 9).

During maintenance *in vitro* under favorable conditions, many parasites presented a normal morphology in stained smears even on the 3rd or 4th day of cultivation (Text-fig. A, 5–11, and Fig. 15), and this was true of almost all of them on the 1st and 2nd days of cultivation (Figs. 10, 11, and 13). They tended to be less elongate, suggesting that the elongate shape of some large trophozoites may be partly a result of the shape of the cytoplasmic portion of the host red cell. Mature segmenters during the first 2 days of cultivation had the usual number of merozoites, but in segmenters present on the 3rd and 4th days this was reduced (Text-fig. A, 11). Degenerate parasites had either a pale pinkish stain or a dark purplish cast to the cytoplasm. Parasites with distinctly blue cytoplasm and reddish nuclei were considered in good condition even though they might show certain deviations from the usual morphology. The



TEXT-FIG. A. Drawings of various stages of *P. lophurae* as seen in stained films. 1–4: parasites in duck erythrocytes in freshly drawn infected duck blood before hemolysis. The erythrocytes have been omitted from the drawings except in 1. 5–11: free parasites which have developed extracellularly in a gelatin–red cell extract medium containing adenosine triphosphate and pyruvic acid; 5–9 from a culture on the 4th day, 10 and 11 from a culture on the 3rd day.

chief deviation of this type encountered in parasites otherwise apparently in excellent condition has been the presence of one to several large granules stained a deep purple (Text-fig. A, 7). Gametocytes which have been just released from the erythrocyte, or have developed in culture, show the typical morphology of the intracellular forms. Although when freed from the restraint of the ellipsoidal host cell they have a tend-

ency to round up somewhat, most of them retain to a considerable degree their characteristic elongate form (Fig. 14).

Living malaria parasites examined with the ordinary light microscope appear as little more than clear spaces within the red cells, with the pigment granules their only conspicuous structure. Young parasites devoid of pigment are difficult to see at all. But when the parasites are examined under an oil immersion lens with dark contrast phase illumination they have a very definite body and structure, and even the smallest stages are readily seen (Figs. 1 and 2). The pigment granules are highly refractile, but they are not any more conspicuous than the sharply outlined circular bodies which correspond to the pink vacuoles of the stained preparations. A single tiny circular dot of this nature may be seen in very young parasites and at the tip in pear-shaped merozoites. Nuclear material cannot be seen except in very late segmenters in which the nuclei are barely visible as dark shadows against the lighter cytoplasm. The most striking feature of the parasites is the smooth bright appearance of the ground cytoplasm as contrasted with the darker appearance of the cytoplasm of the erythrocytes. If pressure is applied on the coverslip over a thin mount of infected duck blood, some of the red cells are hemolyzed and the contained parasites may or may not be squeezed out of the cell. In either case the parasite at first retains its bright appearance. In Fig. 2, one such large parasite is shown lying beside the hemolyzed cell from which it was extruded. But very soon the parasites, whether still within the hemolyzed cell or free in the serum, acquire a dark appearance. Several such parasites may be seen in Fig. 2. Even ripe merozoites, which appear fairly bright when they burst out from the host cell (though not as bright as larger forms), become dark after a few minutes in the serum. This darkening of the cytoplasm as seen with phase microscopy is a very early degenerative phenomenon and is not necessarily accompanied by any degenerative changes visible in dried, fixed, and stained films. Free parasites prepared by specific hemolysis into red cell extract medium retain the characteristic bright appearance, as do the parasites maintained extracellularly under appropriate conditions for 1 to several days (Figs. 3–8). The relative brightness of the cytoplasm as seen with phase microscopy has been a valuable criterion of the condition of extracellular parasites cultured *in vitro*. Parasites presenting a dense, dark appearance were judged to be degenerate, as were those parasites which were exceptionally bright and refractile or which had a patchy appearance to the ground cytoplasm. Parasites containing unusually large or numerous pink vacuoles were considered as beginning to degenerate. The dark purple granules of the stained forms appeared in living parasites as smooth, very bright, refractile bodies quite similar to the pigment. Gametocytes could be recognized readily by their large size, somewhat elongate form, and scattered large pigment granules (Fig. 7). Clusters of merozoites which had developed extracellularly usually showed a typical rosette arrangement (Fig 8).

EXPERIMENTAL RESULTS

The Agar Gel Type of Culture.—For cultures of this type the red cell extract was made in a diluent which differed from that shown in Table I in the following respects: It contained no gelatin, hexose diphosphate, or manganese sulfate,

but did have $MgSO_4 \cdot 7H_2O$ at 300 mg. per liter. The following materials were present at the concentrations indicated (in mg. per liter) in place of the concentrations given in Table I: $NaH_2PO_4 \cdot H_2O$ 350; K_2HPO_4 1300; $NaHCO_3$ 400; $CaCl_2$ 65; dextrose 2500; bovine plasma fraction V 10,000; niacinamide 2.

The flasks were prepared in the following manner:—

0.9 ml. of red cell extract medium was mixed with 0.3 ml. of the suspension of free parasites and hemolyzed red cells. To this was added 0.6 ml. of a melted 6 per cent agar solution containing 0.3 per cent NaCl and 0.4 per cent KCl and brought to a temperature of 44°C. The contents of the flask were mixed rapidly and allowed to gel. Then 3 ml. of liquid red cell extract medium was added. The flasks were aerated and rocked in the usual manner. At intervals of 12 to 18 hours the liquid portion of the medium was drawn off and replaced with the same volume of fresh culture fluid. Samples of the agar could be readily obtained with a fine tipped capillary pipette and either crushed under a coverslip for examination in the fresh state or broken up with a glass slide and spread for the preparation of a stained film. Although the layer of agar in the flask usually broke up into small pieces, this type of preparation permitted replacement of at least a part of the culture fluid without disturbing the parasites.

Earlier work had shown the value of frequently changing the medium (2). But the agar gel had some beneficial effect in itself. Even after the first 18 hours of incubation, parasites embedded in the agar gel medium appeared in better condition than those suspended in the same red cell extract medium but without agar.

The agar type cultures always showed degenerate forms after 1 day, but some parasites in excellent condition were also present. After 2 days' incubation the majority of the parasites were degenerate, but a number in good condition could be found. By the 3rd day few or no parasites were in good condition. When yeast adenylic acid and cozymase were added to the medium, the proportion of parasites in good condition on the 1st and 2nd days was always greater than in control flasks containing the same red cell extract but without the supplements. Even on the 3rd day a small number of free parasites of normal appearance could be found in stained films from the flasks which had received the yeast adenylic acid and cozymase. These two substances were therefore included in all the subsequent experiments. Such preparations were the first which gave some indication that a small proportion of the parasites might be undergoing development. On the 1st and 2nd days clusters of very young trophozoites were noted in both fresh and stained preparations. These were disposed around a pigment mass, like merozoites, but were somewhat larger, and occasionally 1 or 2 in the cluster would contain a very small pigment granule. The appearance suggested that some few merozoites, formed by segmentation which had occurred extracellularly and possibly held together by the agar gel, had begun development extracellularly.

Slightly better survival on the 2nd day was obtained when a part of the glucose in the nutrient solution was replaced by hexose diphosphate. The

further addition to red cell extract medium, made with either the glucose diluent or the diluent containing hexose diphosphate, of a chick embryo liver extract likewise consistently gave a small beneficial effect evident on the 2nd day of cultivation.

Chick embryo liver extract was prepared by removing aseptically most of the liver (avoiding the gall bladder) from each of a number of 14 to 17 day old chick embryos. Each liver was ground in a glass grinder in 2 ml. of the same nutrient solution used for making red cell extract. The suspension was lightly centrifuged for 5 minutes. The evenly turbid pale yellow supernatant fluid constituted the extract. The operations were carried out and the extract was stored in the cold as much as possible.

The results obtained with the agar gel cultures were duplicated in cultures in which the agar gel was replaced by a plasma clot.

These clots were prepared by mixing 0.9 ml. of red cell extract, 0.3 ml. of the free parasite suspension, and 0.6 ml. of plasma from the blood of a young duckling drawn without any anticoagulant and centrifuged immediately. After a clot had formed, 3 or 4 ml. of the appropriate red cell extract medium was added. In these preparations the clot retracted and floated loosely in the fluid portion of the medium. In order to obtain a sample it was necessary to tear off a bit of the clot with fine forceps.

The Gelatin Type of Culture.—Although both the agar gel and plasma clot cultures indicated that extracellular development of *P. lophurae* was possible, and although both types could be used to study the effects on this development of changes in the composition of the culture medium, nevertheless both left much to be desired. The percentage of survival even on the 2nd day was not high and the extent of development was limited. In an attempt to find more favorable physical conditions, a medium containing gelatin was tried.

This medium differed from the one used for the agar gel cultures only in that the diluent for preparing the red cell extract contained 0.6 per cent bovine plasma fraction V and 6 or 7 per cent gelatin. Yeast adenylic acid and cozymase were always added to the completed red cell extract. This medium remained entirely liquid at the incubator temperature of 40°C. and difficulty was anticipated in replacing the old culture fluid with fresh medium, since the free parasites could not withstand drastic treatments such as separation by centrifugation. However, it was found that after a mixture of 0.3 ml. of free parasite suspension and 3 ml. of a medium with 6 or 7 per cent gelatin had been rocked in the incubator for a period of 18 hours, the free parasites and the remains of the hemolyzed red cells had accumulated in a small scum on the inner surface of the flask at the boundary formed by the liquid as it rocked to and fro. The parasites were then held in position while the liquid medium washed over them. The clear liquid could be easily drawn off and replaced with fresh culture medium, which was always first warmed to 40°C. After the first 18 hour period, the medium was changed at intervals of 12 hours, a procedure which invariably gave better results than those obtained in experiments in which fresh medium was supplied only every 24 hours.

Small samples taken from the scum with a fine tipped pipette revealed that most of the free parasites presented a normal morphology after 1 day *in vitro* and a great many of them after 2 days *in vitro*. Normal parasites were

readily apparent after 3 days (Figs. 7 and 8) and some could be found after 4 or even 5 days of incubation. If the cultures were begun with parasites chiefly in the early uninucleate trophozoite stage, there were present 18 hours later chiefly large parasites and early segmenting forms. At 42 hours there appeared numerous fully segmented parasites, merozoites, and some developing young trophozoites (Fig. 12). The relatively large accumulation of segmenters and especially of merozoites showed that, although conditions were sufficiently favorable to support the extracellular maturation and segmentation of most of the trophozoites originally present, only a small proportion of the newly formed merozoites were able to develop further. It is interesting to note that while the numerous merozoites and very young trophozoites present on the 2nd day appeared quite normal in stained films, they presented a somewhat dark appearance when examined in the fresh state by phase microscopy. However, a number of half-grown to large trophozoites of excellent appearance by phase microscopy could be seen in samples from 4 day old gelatin type cultures.

The Effect of Adenosinetriphosphate and Pyruvic Acid.—The beneficial effects of yeast adenylic acid, cozymase, and hexose diphosphate, together with slight favorable effects which were observed in some but not all experiments upon the further addition of manganese sulfate, suggested that cofactors involved in glycolysis might be essential to the extracellular survival of malaria parasites. Accordingly, the composition of the nutrient solution used for making the red cell extract was modified so as to include manganese sulfate, the magnesium salt of hexose diphosphate, and a very high concentration of niacinamide (Table I). The last was used to inhibit the possible enzymatic breakdown of cozymase (8). Gelatin type cultures made with red cell extract prepared in this modified diluent and containing 0.0014 M yeast adenylic acid and 0.00015 M cozymase gave results little better than those obtained with the diluent previously employed. However, when both adenosinetriphosphate at 0.002 M and sodium pyruvate at 0.005 M were included in the medium, strikingly better survival and development of the extracellular parasites were seen. Since the adenosinetriphosphate and the pyruvate might be expected to be readily decomposed in the presence of the enzymes of red cell extract, they were added to each culture flask from a concentrated stock solution at the start of the experiment and again with each addition of fresh culture medium. Gelatin type cultures containing the modified red cell extract medium with adenosinetriphosphate and sodium pyruvate showed practically all the parasites in excellent condition on the 2nd as well as on the 1st day of incubation. The free parasites developing extracellularly on these days presented a truly remarkable picture, whether seen in the living condition by phase microscopy (Figs. 5 and 6) or in stained films (Figs. 10, 11, 13, and 14). On the 3rd day a large majority of the free parasites were still of normal appearance (Fig. 15). By the 4th day considerable degeneration had set in but numerous developing parasites of

TABLE II

The Effect of Adenosinetriphosphate and Pyruvic Acid on the Extracellular Development of P. lophurae as Seen in Stained Films

200 parasites in successive microscopic fields were counted and classified.

Experiment	Preparation	Time *in vitro*	Supplements	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Larger, 1 nucleus	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
A	Original	0	—	4	182	13	1	0	200	0
	Flask 1	1	None	0	139	36	14	0	189	11
	2		Phospholipid	13	134	34	12	2	195	5
	3		ATP and pyruvic acid	1	154	38	6	0	199	1
	4		Phospholipid, ATP, and pyruvic acid	0	143	53	4	0	200	0
	Flask 1	2	None	44	86	22	16	0	168	32
	2		Phospholipid	32	107	17	12	0	168	32
	3		ATP and pyruvic acid	0	152	38	6	0	196	4
	4		Phospholipid, ATP, and pyruvic acid	1	128	56	1	1	187	13
B	Original	0	—	5	175	16	1	0	197	3
	Flask 1	1	None	20	153	17	4	1	195	5
	2		None	11	154	25	4	0	194	6
	3		ATP	4	137	47	9	0	197	3
	4		ATP	11	147	29	10	1	198	2
	5		ATP and pyruvic acid	5	151	35	9	0	200	0
	6		ATP and pyruvic acid	8	149	43	0	0	200	0
	Flask 1	2	None	34	100	35	12	0	181	19
	2		None	12	126	31	9	1	179	21
	3		ATP	6	116	47	17	1	187	13
	4		ATP	2	130	41	15	1	189	11
	5		ATP and pyruvic acid	8	161	22	5	0	196	4
	6		ATP and pyruvic acid	7	153	33	2	1	196	4
	Flask 1	3	None	14	97	16	5	0	132	68
	2		None	1	72	13	4	0	90	110
	3		ATP	2	122	11	1	0	136	64
	4		ATP	1	105	19	2	1	128	72
	5		ATP and pyruvic acid	1	151	10	1	0	163	37
	6		ATP and pyruvic acid	8	132	34	1	1	176	24

TABLE II—*Concluded*

Experiment	Preparation	Time *in vitro*	Supplements	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Larger, 1 nucleus	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
C	Original	0	—	0	180	15	4	0	199	1
	Flask 1	1	None	5	149	35	5	0	194	6
	2		None	4	139	44	11	1	199	1
	3		ATP and pyruvic acid	2	133	56	7	1	199	1
	4		ATP and pyruvic acid	3	141	53	2	0	199	1
	Flask 1	2	None	66	69	31	10	1	177	23
	2		None	46	72	36	9	0	163	37
	3		ATP and pyruvic acid	0	144	47	3	0	194	6
	4		ATP and pyruvic acid	4	144	40	5	1	194	6

normal morphology were present. Few normal parasites could be found on the 5th day, and most of the experiments were carried only through a period of 4 days. It was noted that during the first 2 days the scum containing the parasites had a soft consistency. Small samples could be easily sucked up into the fine tip of the capillary pipette. By the 3rd or 4th day the scum had a firmer consistency and the material tended to occlude the opening of the pipette without being sucked up into it. There were indications that the change in consistency might be associated with the onset of generally unfavorable conditions, but no method has yet been found for preventing or controlling the change.

Differential counts from the stained films showed in a clear and consistent manner the favorable influence of the addition of both adenosinetriphosphate and sodium pyruvate (Table II). The differences between cultures with and without these substances were apparent on the 1st day but showed up especially well on the 2nd day. In Experiment A (Table II) the two flasks without adenosinetriphosphate and pyruvate both showed on the 2nd day much larger numbers of degenerate parasites and of very young forms (chiefly free merozoites), regardless of whether they contained a supplement of phospholipid (prepared from egg yolk and added so as to give a concentration of 0.4 mg. per ml. of medium). In Experiment B, on the 2nd day the flasks with adenosinetriphosphate alone and with adenosinetriphosphate plus pyruvate had fewer very young forms than the unsupplemented ones. The flasks with both supplements showed the fewest degenerate parasites, those with adenosinetriphos-

phate alone somewhat more, and those without either supplement still more. These differences were more accentuated on the 3rd day, when the proportions of degenerate parasites in the flasks with both adenosinetriphosphate and pyruvate were not much greater than those of the unsupplemented flasks on the 2nd day. Results of a similar nature are illustrated in Experiment C.

It is evident that in the cultures supplemented with adenosinetriphosphate and pyruvate at the concentrations indicated above, the newly formed merozoites did not accumulate and degenerate but instead developed further extracellularly. Tripling the concentrations of both the adenosinetriphosphate and the sodium pyruvate had a deleterious effect.

The pH of the fluid drawn off from the cultures at 18 and 12 hour intervals has been measured with a glass electrode and found to lie between 6.72 and 6.82, usually around 6.74. Preliminary measurements of the reducing sugar in the culture medium and in the fluid drawn off after incubation indicate that the glucose consumption during the second as well as during the first 24 hours of cultivation, when calculated on an hourly basis, was as high as that of parasites in a parasitized red cell suspension freshly removed from the host animal (9, 10).

DISCUSSION

The nutritional and cultural requirements of malaria parasites have been studied previously only with parasites living within their host erythrocytes. It was found that the survival of *P. lophurae* in dilute suspensions of chicken or duck erythrocytes maintained *in vitro* was favored by a number of factors including aeration, an optimal density of parasitized cells per cubic millimeter, frequent renewal of the suspending medium, concentrated red cell extract, and appropriate concentrations of glucose, glutathione, and calcium pantothenate (2, 4). During the first 2 days of incubation of such preparations a limited multiplication of the parasites with the invasion of previously uninfected red cells occurred. Much better multiplication and reinvasion of red cells were later obtained in experiments with *P. knowlesi*, using concentrated suspensions of monkey erythrocytes maintained *in vitro* (5). It could be shown that para-aminobenzoic acid and methionine (11), among many other less well defined factors, were essential for the development of this parasite within its host cells. Similar results have since been observed with *P. lophurae* in concentrated suspensions of duck erythrocytes (12). In all of the experiments growth and multiplication of the malaria parasites occurred exclusively within living host erythrocytes not more than 1 or 2 days removed from the intact animal. The prime requisites for successful cultivation were conditions which would maintain the integrity of the red blood cells and would favor their reinvasion by merozoites. Such studies, although extremely important in other respects, could be expected to be of only limited value in the problem of the physiology of intracellular parasitism.

In a different approach to the latter problem, studies have been made on the metabolism of malaria parasites separated from their host cells (13, 3, 9). These studies, chiefly of the Warburg respirometer type, involved very short term observations of the enzymatic activities of parasites which must have been mostly dead or dying. Almost every type of enzymic reaction discovered in the cells of higher animals was found in malaria parasites, leading Moulder (14) to conclude that we cannot at present explain why the rapidly metabolizing blood stages of malaria parasities live and grow only within erythrocytes, which have much lower metabolic rates.

The results of the present paper put the matter in a quite different light. The malaria parasite *P. lophurae* can develop and multiply extracellularly *in vitro*, at least for a period of several days, but only if it is provided with a very special set of environmental conditions. It seems reasonable to hope that an analysis of these conditions may ultimately answer the question as to why malaria parasites in nature develop only intracellularly. Thus there are indications that the physical state of the medium may be of importance. Perhaps the beneficial effects of agar and of gelatin can be accounted for on this basis. The role of the red cell extract is of special interest, since it may provide relatively labile enzyme systems which are utilized by the parasite. It is interesting to note that red cell extract, which was first observed to favor the survival of the erythrocytic stages of *P. lophurae* in dilute suspensions of erythrocytes (4), also prolongs the survival *in vitro* of the sporozoites of *P. gallinaceum* (15). The salt solution of high potassium content which has been used for making red cell extract is not unlike a salt solution which has been found to be least deleterious when injected into the cytoplasm of amebae or slime molds, and has therefore been considered to represent an approach to the inorganic composition of the intracellular fluid (16, 17). The red cell extract–gelatin medium, which supports growth of an intracellular parasite, must represent a closer approach to the essential composition of the intracellular matrix. It is pertinent to note that a basal salt solution high in potassium, rather than one high in sodium, has been found to favor the survival of rickettsiae (18), which represent another type of intracellular parasite.

The experiments with adenosinetriphosphate indicate that *P. lophurae* requires but is incapable of synthesizing this compound. This synthetic deficiency alone would be sufficient to account for the obligate intracellular parasitism of this organism, since adenosinetriphosphate, a compound occupying a prominent position in the energy transformations of the cell (19, 20), is a labile material which in nature exists chiefly within living cells. The favorable effect of pyruvic acid may be related to its demonstrated role in the oxidation reactions which in red blood cells are coupled with the esterification of inorganic phosphate (21). It is interesting to note that adenosinetriphosphate previously has been found to stimulate the respiration of surviving preparations of free malaria parasites but not of parasites within their host cells (3, 9). Monkey

erythrocytes parasitized by *P. knowlesi* have been shown to contain a somewhat higher concentration of adenosinetriphosphate (among other compounds) than unparasitized erythrocytes (10). This may represent an accumulation rather than a synthesis of adenosinetriphosphate, just as some yeasts which require biotin are able to accumulate it in higher concentration than that of the surrounding medium (22). There are some indications that adenosinetriphosphate functions in the synthesis of bacteriophage within the bacterial cell (23). Perhaps many of the obligate intracellular parasites have lost the capacity to synthesize adenosinetriphosphate.

SUMMARY

The erythrocytic stages of *Plasmodium lophurae* were freed from their host red cells by specific hemolysis directly into a favorable medium containing an extract of duck erythrocytes. Extracellular survival and development of the parasite *in vitro* occurred in culture media consisting essentially of a very concentrated extract of duck red cells prepared in a special nutrient solution. Omission or dilution of the red cell extract resulted in rapid degeneration of the parasites. Their survival and development were favored by the presence in the erythrocyte extract of gelatin, yeast adenylic acid, and cozymase, and especially by the further addition of adenosinetriphosphate and sodium pyruvate. Under the best conditions yet tested, all the free parasites continued their development extracellularly during the first two days of cultivation. Merozoites formed by the extracellular segmentation of the free parasites originally present developed further into trophozoites. On the third day a majority of the free parasites were still of normal appearance, but by the fourth day more were degenerate, and very few normal parasites remained on the fifth day.

BIBLIOGRAPHY

1. Coggeshall, L. T., *Am. J. Hyg.*, 1938, **27**, 615.
2. Trager, W., *J. Exp. Med.*, 1941, **74**, 441.
3. Speck, J. F., Moulder, J. W., and Evans, E. A., Jr., *J. Biol. Chem.*, 1946, **164**, 119.
4. Trager, W., *J. Exp. Med.*, 1943, **77**, 411.
5. Anfinsen, C. B., Geiman, Q. M., McKee, R. W., Ormsbee, R. A., and Ball, E. G., *J. Exp. Med.*, 1946, **84**, 607.
6. Ponder, E., Hemolysis and Related Phenomena, New York, Grune and Stratton, 1948.
7. Hewitt, R., *Am. J. Hyg.*, 1942, **36**, 6.
8. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, 1941, **35**, 502.
9. Bovarnick, M. R., Lindsay, A., and Hellerman, L., *J. Biol. Chem.*, 1946, **163**, 523, 535.
10. Ball, E. G., McKee, R. W., Anfinsen, C. B., Cruz, W. O., and Geiman, Q. M., *J. Biol. Chem.*, 1948, **175**, 547.
11. McKee, R. W., and Geiman, Q. M., *Fed. Proc.*, 1948, **7**, 172.

12. Trager, W., *J. Parasitol.*, 1947, **33**, 345.
13. Christophers, S. R., and Fulton, J. D., *Ann. Trop. Med. and Parasitol.*, 1938, **32**, 43.
14. Moulder, J. W., *Ann. Rev. Microbiol.*, 1948, **2**, 101.
15. Whitman, L., *J. Immunol.*, 1948, **59**, 285.
16. Chambers, R., *in* Frontiers in Cytochemistry, (N. L. Hoerr, editor), Lancaster, Jacques Cattell Press, 1943, 91.
17. Kassel, R., and Kopac, M. J., *Anat. Rec.*, 1949, **105**, 106.
18. Bovarnick, M. R., Miller, J. C., and Snyder, J. C., *J. Bact.*, 1950, **59**, 509.
19. Baldwin, E., Dynamic Aspects of Biochemistry, Cambridge, Cambridge University Press, 1947.
20. Lipmann, F., and Kaplan, H. O., *Ann. Rev. Biochem.*, 1949, **18**, 267.
21. Dische, Z., *Enzymologia*, 1936–37, **1**, 288.
22. Chang, W., and Peterson, W. H., *J. Bact.*, 1949, **58**, 33.
23. Price, W., *J. Gen. Physiol.*, 1947–48, **31**, 135.

EXPLANATION OF PLATES

PLATE 19

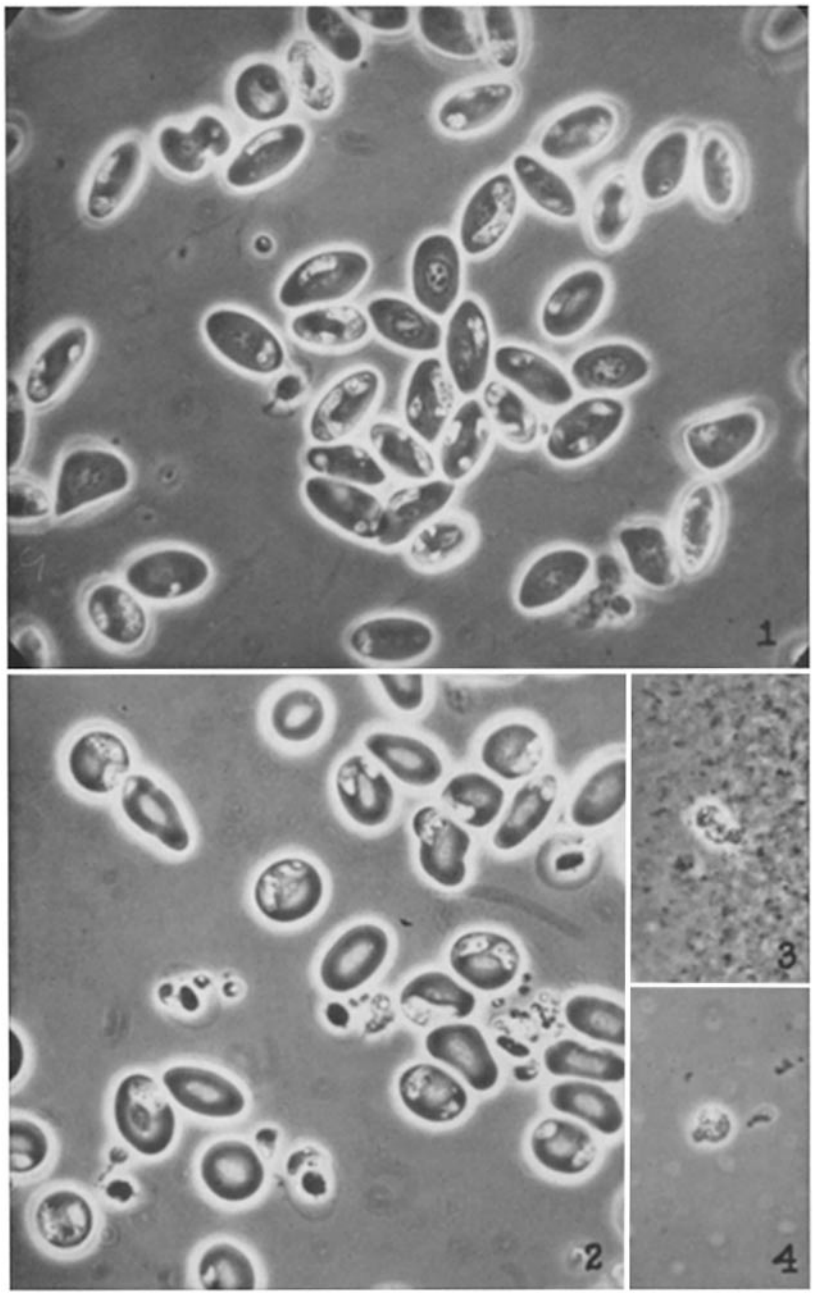
Photomicrographs of fresh preparation of *P. lophurae*. Phase contrast. × 776.

FIG. 1. A mount of whole blood from a heavily infected duckling.

FIG. 2. A preparation similar to that of Fig. 1 but thinner and with some of the red cells hemolyzed by pressure on the coverslip.

FIG. 3. Extracellular trophozoites after 1 day in a culture of the agar gel type.

FIG. 4. A large free trophozoite after 1 day in a culture of the gelatin type.



(Trager: Extracellular cultivation of intracellular parasite)

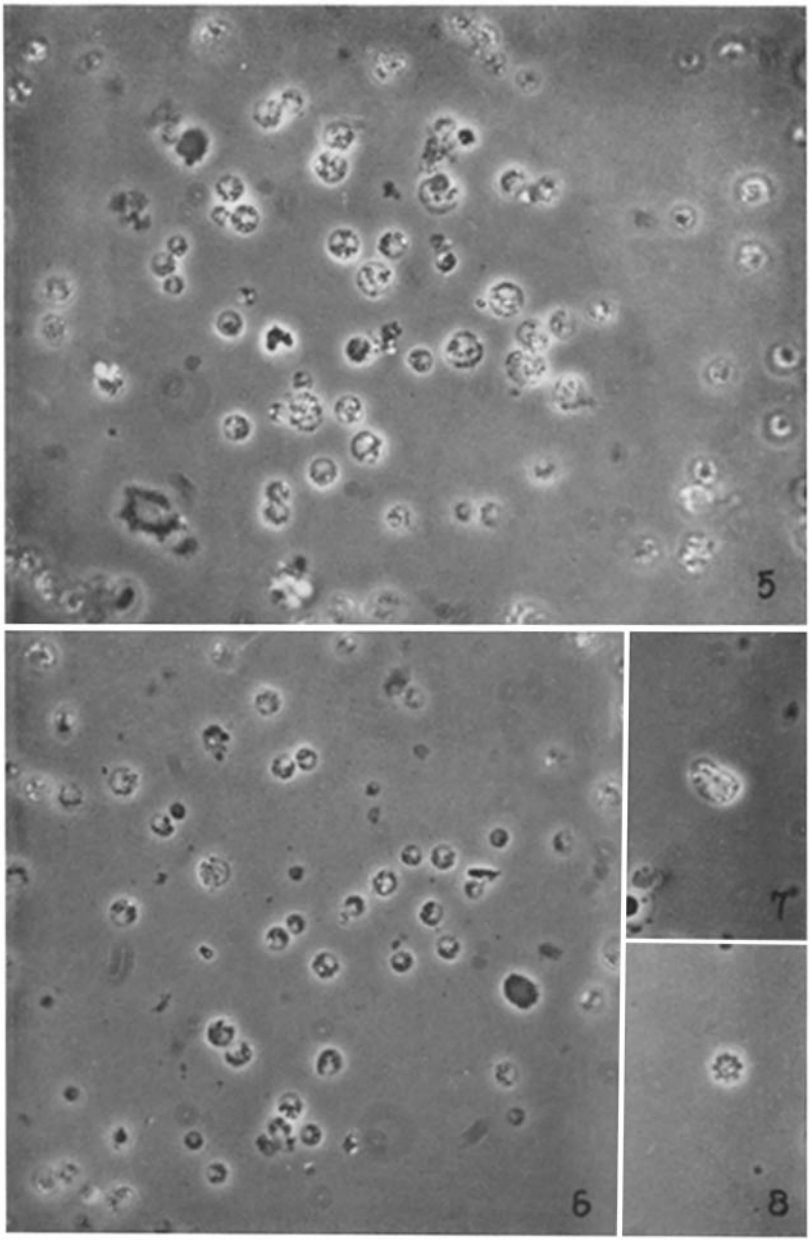
PLATE 20

Photomicrographs of fresh preparations of *P. lophurae* developing extracellularly. Phase contrast. × 776.

FIGS. 5 and 6. Trophozoites after 1 day in a culture of the gelatin type with adenosinetriphosphate and pyruvic acid.

FIG. 7. A gametocyte after 2½ days in a culture of the gelatin type.

FIG. 8. A cluster of merozoites after 3 days in a culture of the gelatin type.



(Trager: Extracellular cultivation of intracellular parasite)

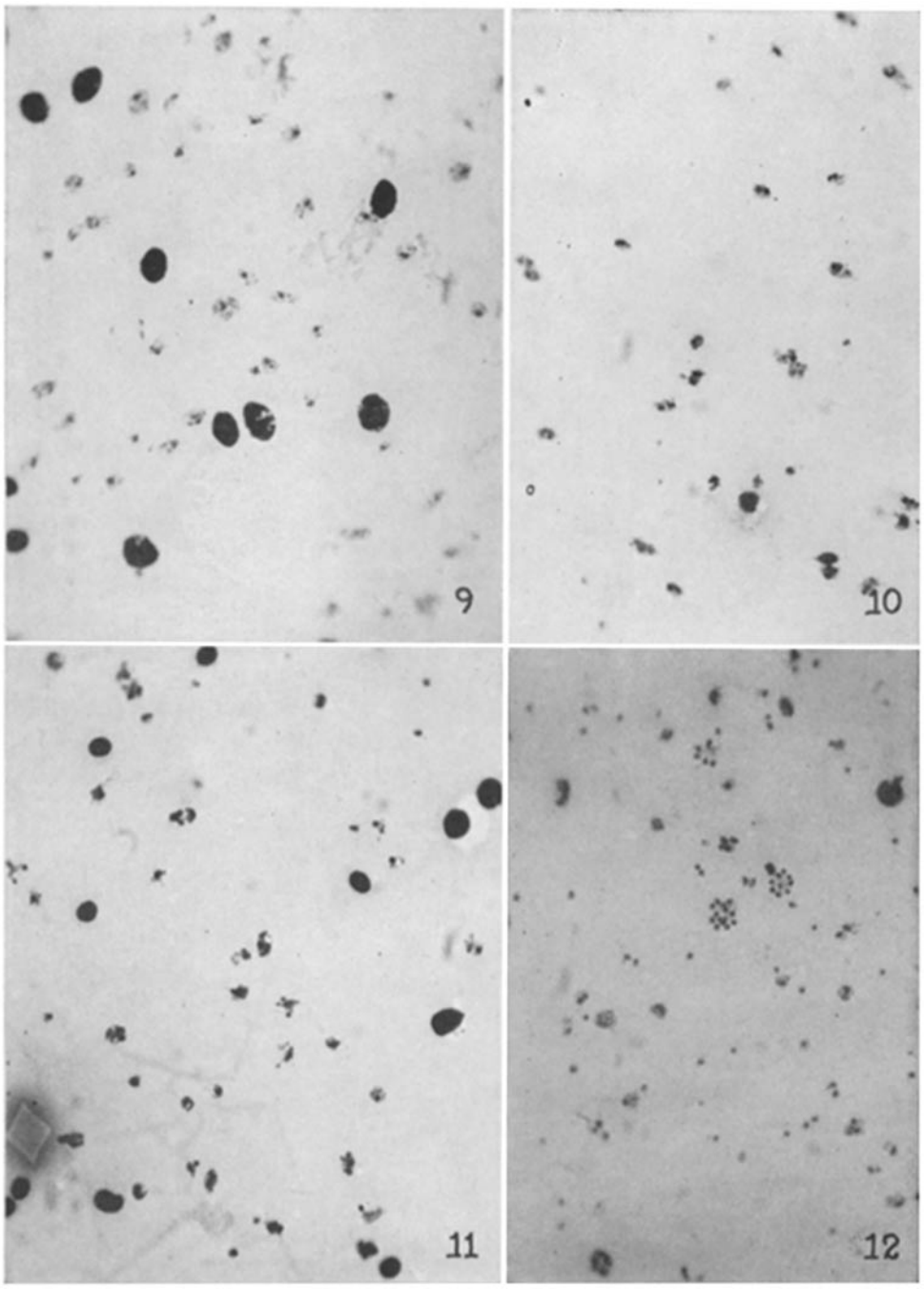
PLATE 21

Photomicrographs of extracellular *P. lophurae* from stained films. × 776.

FIG. 9. Parasites in a freshly hemolyzed suspension, such as was used to inoculate the culture flasks. Note the nuclei of hemolyzed red cells.

FIGS. 10 and 11. Parasites after 2 days in two different cultures of the gelatin type with adenosinetriphosphate and pyruvic acid.

FIG. 12. Parasites after 2 days in a culture of the gelatin type.



(Trager: Extracellular cultivation of intracellular parasite)

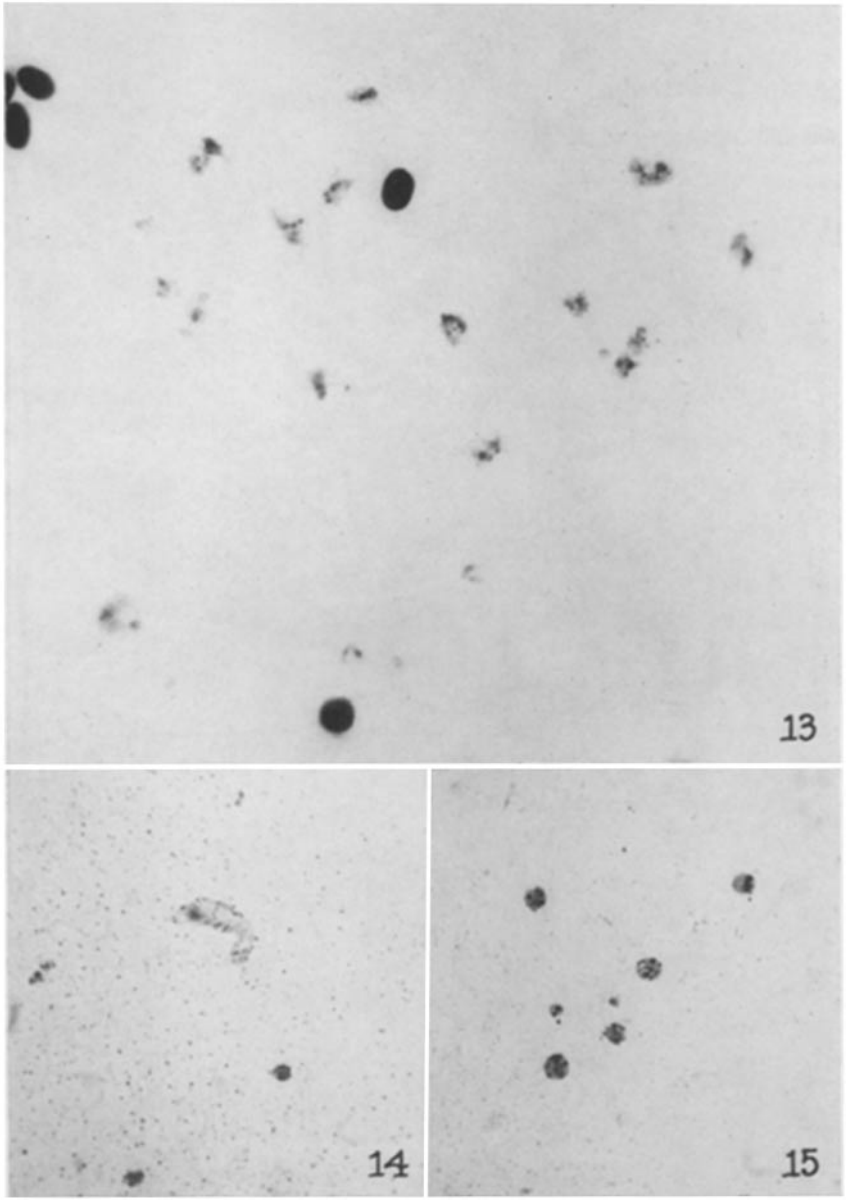
PLATE 22

Photomicrographs of extracellular *P. lophurae* from stained films made from cultures of the gelatin type with adenosinetriphosphate and pyruvic acid.

FIG. 13. After 2 days *in vitro*. × 1388.

FIG. 14. A female gametocyte. 2 days *in vitro*. × 776.

FIG. 15. After 3 days *in vitro*. × 776.



(Trager: Extracellular cultivation of intracellular parasite)