FOLINIC ACID AND NON-DIALYZABLE MATERIALS IN THE NUTRITION OF MALARIA PARASITES

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Previous work has shown that the avian malaria parasite *Plasmodium lophurae* can be removed from its host erythrocytes and maintained alive and developing *in vitro* for 3 days (1, 2). The need of the parasites for materials present in red cell extract and for certain substances of known chemical nature has been demonstrated. It became apparent that two different equally important lines of work could be pursued. One would consist of attempting to improve the culture conditions so as to secure longer, and perhaps eventually continuous, cultivation. This would involve experiments of at least 4 days' duration, since with the culture conditions already developed over 90 per cent of the parasites present a normal morphology after 3 days *in vitro*. The other line of work would consist of trying to ascertain the nature of the materials in erythrocyte extract which are essential for even brief survival and development of the parasites. Here experiments lasting 1 day would suffice.

Some progress has been made along both of these lines. The experiments of long duration have shown a favorable effect of folinic acid. In connection with these experiments an observation has been made concerning the effect of folic acid on development of the human malaria parasite *P. falciparum* in suspensions of intact human erythrocytes. The experiments of short duration have shown the requirement of extracellular *P. lophurae* for non-dialyzable factors, other than hemoglobin, present in red cell extract and also in certain yeast protein preparations.

Materials and Methods

The maintenance of *P. lophurae* in ducklings and the methods used for freeing the parasites from their host erythrocytes and for the preparation of the culture media and the culture flasks were in general the same as previously described (2). The following points are sufficiently important to bear repetition.

The parasites were passed by blood inoculation once a week in accordance with a routine which had been found to produce 4 days later, in ducklings destined to serve as donors of infected blood for the culture experiments, a parasitemia of 50 to 100 parasites per 100 red blood cells with 90 per cent or more of the parasites in the young uninucleate trophozoite stage.

Inoculum for the 4 day experiments was obtained by mixing 2 ml. of a 20 per cent suspension of the red cells of the infected donor duckling in red cell extract (see below) with 1.6

ml. of red cell extract, 0.07 ml. of guinea pig serum and 0.4 ml. of rabbit anti duck–erythrocyte serum. Incubation at 37°C. for half an hour on a vibrator yielded a suspension containing free red cell nuclei, free parasites, and agglutinated, hemolyzed but unbroken (ghost) erythrocytes both with and without parasites. Parasites contained in such hemolyzed erythrocytes generally became free within the 1st day of incubation of the culture.

TABLE I

Composition of Solutions Used in Making the Culture Media

A-1. A solution approximately isotonic with erythrocytes. This was mixed directly with the frozen-thawed duck red blood cells.

A-2. A solution calculated to contain 1.25 times the main osmotically active ingredients of A-1. This was mixed with an equal volume of erythrocyte extract prepared in 0.05 M phosphate buffer.

A-3. A solution calculated to contain 1.7 times the main osmotically active ingredients of A-1. This was mixed with an equal volume of fractions derived from zone electrophoresis of erythrocyte extract resuspended in 0.05 M phosphate buffer and lacking the electrolytes of the erythrocytes.

Measurements of the freezing point depression of a series of 10 final media prepared by the appropriate use of solutions A-1, A-2, and A-3 indicated an osmotic pressure, expressed as molar concentration of NaCl, of 0.17 to 0.19 with an average of 0.18.

Stock solutions*			Final mixtures‡					
No.	Material	Concentration, gm./liter	Amount of stock solution, ml./liter			Concentration, mg./liter		
			A-1	A-2	A-3	A-1	A-2	A-3
1	NaCl	66.0	50.0	62.5	85	3300	4125	5610
	KCl	88.0				4400	5500	7480
2	$NaH_2PO_4 \cdot H_2O$	5.5	25.0	0§	0§	138	0	0
	K_2HPO_4	62.7				1568	0	0
3	$NaHCO_3$	50.0	18	22.5	31	900	1125	1550
4	$CaCl_2$	2.6	15	15	15	39	39	39
5	$MnSO_4 \cdot 4H_2O$	2.2	20	20	20	44	44	44
6	$NaC_2H_3O_2 \cdot 3H_2O$	25.0	10	12.5	17	250	312	425
	Glycerol	25.0				250	312	425
7	Dextrose	50.0	43	54	73	2150	2700	3650

* Stock solutions were prepared in water redistilled in a pyrex glass still. Solutions 1 to 7, 10, 13 *b*, 13 *d*, 14 B, 14 F, and 15 were sterilized by autoclaving, solutions 12 and 12-2 by steaming 20 minutes on each of 3 successive days. The other solutions were sterilized by filtration through either Selas 03 porcelain filters or ultrafine glass filters. The stock solutions were stored for periods up to 3 months in a refrigerator except for solution 13 which was kept in a deep freeze and solution 9 which was either freshly prepared or stored frozen for not over 2 days.

Only the following solutions require special comment: Solution 8, with the preparation of hexose diphosphate used in recent years, had a natural pH of 6, so that no adjustment was required as had been true in the earlier work. Solutions 10 and 15 were prepared exactly as previously described (3). Solutions 14 B and 14 F consisted of 5 mg. of biotin and folic acid respectively dissolved with the aid of 3 drops of 0.1 N NaOH and gentle warming, and then diluted to 100 ml.

‡ Each 100 ml. of mixture received 15 ml. of duck serum (from ducks 6 to 11 weeks old).

§ No phosphate was included in A-2 and A-3 since these solutions were mixed with erythrocyte extract or fractions from it prepared in 0.05 M potassium phosphate buffer.

TABLE I—*Concluded*

Stock solutions*			Final mixtures†					
No.	Material	Concentration, *ml./liter*	Amount of stock solution *ml./liter*			Concentration, *mg./liter*		
			A-1	A-2	A-3	A-1	A-2	A-3
8	Hexose diphosphate (Mg salt)	50.0	14	17.5	24	700	875	1200
9	Glutathione	20.0	50	100	100	1000	2000	2000
	Ascorbic acid	0.2				10	20	20
	Nicotinamide	80.0				4000	8000	8000
10	Glycine	0.15	20	25	34	3	3.8	5
	Histidine	0.15				3	3.8	5
	Cystine	0.35				7	8.8	12
	Tryptophane	0.20				4	5	6.8
	Asparagine	0.20				4	5	6.8
	Acid hydrolysate of casein (parenamine)	4.20				84	105	141
11	Bovine plasma fraction V	20.0	300	0	0	6000	—	—
11 *b*	Bovine plasma fraction V	40.0	0	220	200	—	8800	8000
12	Gelatin	180.0	400	0	0	72,000	—	—
12-2	Gelatin	360.0	0	400	350	—	144,000	126,000
13 *g*	Riboflavin	0.05	10	20	20	0.5	1.0	1.0
	Thiamin-HCl	0.05				0.5	1.0	1.0
	Pyridoxin-HCl	0.05				0.5	1.0	1.0
	Pyridoxamine di-HCl	0.01				0.1	0.2	0.2
	Pyridoxal-HCl	0.01				0.1	0.2	0.2
	Inositol	0.10				1.0	2.0	2.0
	Choline-Cl	0.10				1.0	2.0	2.0
13 *b*	Calcium pantothenate	2.0	5	10	10	10.0	20.0	20.0
13 *d*	*p*-aminobenzoic acid	1.0	1	2	2	1.0	2.0	2.0
14 B	Biotin	0.05	0.1	0.2	0.2	0.005	0.01	0.01
14 F	Folic acid	0.05	0.2	0.4	0.4	0.01	0.02	0.02
15	Adenine sulfate	0.05	10	20	20	0.5	1.0	1.0
	Guanine-HCl	0.05				0.5	1.0	1.0
	Xanthine	0.05				0.5	1.0	1.0
	Uracil	0.05				0.5	1.0	1.0
	Cytidylic acid	0.02				0.2	0.4	0.4

For the 1 day experiments 6.3 ml. of the 20 per cent suspension of infected blood was mixed with 0.13 ml. of guinea pig serum and 0.7 ml. of the rabbit anti serum. The mixture was incubated on a rocker at 40°C. The large clumped masses which formed were broken up by sucking the material in and out of a 5 ml. pipet several times. The suspension was then transferred to a centrifuge tube and centrifuged 45 to 60 seconds at approximately 400 R.P.M. The agglutinated, hemolyzed, but unbroken erythrocytes were sedimented, leaving a supernatant

containing only free red cell nuclei and free parasites. Such suspensions are designated purified parasite suspensions.

Erythrocyte Extract.—This was prepared under aseptic conditions from frozen-thawed duck erythrocytes by methods previously described (3, 4). For experiments of long duration, concerned only with the effect of adding known substances to the complete red cell extract, the hemolyzed cells were extracted directly in the isotonic nutrient solution. For experiments in which fractionation of the extract was attempted, the hemolyzed cells were extracted in water or dilute buffer. The extract or fractions prepared from it were then mixed, just before use, with an equal volume of a modified nutrient solution having an appropriate hypertonicity, so as to restore isotonicity in the final medium. The composition of the various diluents used is shown in Table I.

The final media were adjusted with 0.1 N HCl or KOH to a pH of 7.0. To each was then added, at the rate of 0.2 ml. per 10 ml. of medium, a solution containing malic acid 6.0 mM, yeast adenylic acid 1.4 mM, and cozymase (Schwarz 85 per cent) 0.15 mM (1). The following supplements were added to the culture flasks just before inoculation and, for the long experiments, with each change of culture medium:

1. Adenosinetriphosphate (ATP)-Pyruvate.—One gm. sodium pyruvate (prepared by neutralization of an alcoholic solution of pyruvic acid with alcoholic sodium hydroxide and one recrystallization) and 2 gm. ATP (Pabst, potassium salt) were dissolved in 10 ml. redistilled water. The solution was brought to pH 5.8 with 0.5 N KOH and diluted to 25 ml. It was sterilized by filtration through an ultrafine glass filter and stored frozen in a dry-ice box. Each culture flask received 0.05 ml. from a freshly thawed tube of this solution.

2. Coenzyme A (Co A).—0.5 gm. Armour's liver coenzyme concentrate, with a Co A activity of 15 Lipmann units per mg., was ground in a mortar and dissolved in 14 ml. redistilled water plus 1.9 ml. stock solution 2 (Table I). The pH was brought to 6.5 with 0.5 N KOH and the solution diluted to 57 ml. It was sterilized by filtration through a Selas 03 porcelain filter and stored in a deep freeze. 0.2 ml. of this solution was added to each culture flask.

3. Folinic Acid.—An ampoule of Lederle leucovorin (3 mg. calcium salt of formyltetrahydropteroylglutamic acid in 1 ml.) was added aseptically to 4 ml. sterile redistilled water. Alternatively, 12 mg. crystalline leucovorin, obtained through the kindness of Dr. T. H. Jukes of the Lederle Laboratories, was dissolved in 20 ml. redistilled water and the solution sterilized by filtration through an ultrafine glass filter. In either case the solution was stored in a deep freeze. When used, it was added usually in the amount of 0.04 ml. per flask.

Preparation and Handling of the Cultures.—The methods used in the more recent experiments have already been described in sufficient detail (2).

Assessment of the Effects of Culture Conditions.—For the long experiments, reliance was placed mainly on differences in the proportions of parasites judged to be degenerate as counted in Giemsa-stained films made after 4 days of incubation at 40°C. (the day of preparation of the cultures being counted as day 0), and on the over-all appearance of the parasites in fresh preparations examined by phase contrast microscopy. For the 1 day experiments an additional criterion was used—the proportion of parasites having more than one nucleus. The use of this criterion was made possible by the relatively high synchronicity of the infection in the donor ducklings. The proportion of parasites having 2 to 4 nuclei was ordinarily very small on day 0 and much greater after 1 day's incubation at 40–40.5°C. in a favorable medium. The extent of this increase in multinucleate forms (never so great as that which would occur in the infected duckling) gave a rough measure of the favorableness of the medium. It was less the less concentrated the erythrocyte extract.

Zone Electrophoresis on Starch.—The methods were essentially those of Kunkel (5). It is a

pleasure to acknowledge here the helpful advice of Dr. H. G. Kunkel and also of Dr. S. Granick and Dr. M. Kunitz of The Rockefeller Institute. The potato starch was thoroughly washed, allowed to dry, and stored in large closed bottles. For preparation of the block 900 gm. of starch was suspended in enough (750 to 950 ml.) 0.05 M phosphate buffer, pH 7.3–7.5, (in redistilled water) to give a slurry of appropriate consistency. This was poured on polyethylene sheeting held on a glass plate 35 × 46 cm. and formed into a trough by wooden supports on each side and a clean folded towel at each end. When the starch had dried sufficiently a slit was made down the center of the block with a stainless steel spatula. Ten ml. of red cell extract (see below) was applied drop-wise along the slit in as uniform a way as possible. This usually spread to give a red band about 2 cm. wide. The block was covered, except at each end, with a sheet of polyethylene. Towels soaked in the 0.05 M phosphate buffer established contact at each end of the block with a trough of buffer solution, which in turn was connected by a U tube to a battery jar filled with the buffer solution and holding a carbon electrode. Current from a powerstat unit was set at 90 to 100 ma. with a voltage of 250 to 300 volts. The electrophoresis was set up in a large refrigerator at a temperature of 1°C. and was allowed to run overnight. The starch block was then freed from excess fluid by the application of a clean dry towel to each end, the upper sheeting was removed, and desired measured portions of the block were cut out and suspended in 0.05 M buffer. The starch was removed by filtration through hardened paper on a Buchner funnel and was washed once with buffer. The filtrate was sterilized by passage through a Selas 03 porcelain filter and was stored in a refrigerator. Concentration of protein was determined colorimetrically, using the Folin-Ciocalteu phenol reagent (6).

Preparation of Culture Media for Tests of Fractions Obtained by Electrophoresis.—Red cell extract to be used for electrophoresis was prepared in the following way. Blood obtained aseptically from ducks (not over 3 months old) and defibrinated by shaking with glass beads was centrifuged, in amounts of 20 to 25 ml. per tube, for 20 minutes in the cold at 2000 R.P.M. The serum was removed and the cells in each tube resuspended in 10 to 12 ml. of a cold solution of the following composition: 84 ml. sterile redistilled water, 5 ml. stock solution 1 (see Table I), 2.5 ml. solution 2, 1.8 ml. solution 3, 5.0 ml. solution 7, 1.5 ml. solution 4, 2.0 ml. solution 5. The suspensions were again centrifuged in the cold and the supernatants discarded. The cells were frozen by immersion of the tube in a dry ice–ethanol mixture and thawed by placing the tubes in ice water. The hemolyzed cells were then suspended evenly in 1⅓ times their volume of sterile 0.05 M potassium phosphate buffer of desired pH (7.4–7.5 for most of the work). The mixtures were now centrifuged 1 hour in the cold at 3000 R.P.M. The deep red supernatants were drawn off and pooled. Portions were used for application to the starch block and other portions stored in the refrigerator (1–2°C.) for preparation of the positive control medium.

The positive control medium was freshly mixed on the day the culture experiment was set up. It consisted of equal volumes of the erythrocyte extract in buffer and medium A-2 (Table I). The pH was adjusted to 7.0 by the addition of 0.1 N KOH. This gave a medium containing red cell extract at half the concentration obtained when frozen-thawed cells were extracted directly in isotonic nutrient medium (A-1). The latter concentration is designated as full strength (1 ×), the former as half strength (0.5 ×) and other concentrations accordingly. Free parasites were suspended in either 1 × or 0.5 × extract, so that when they were introduced into flasks containing less than 0.5 × concentration of extract the final concentration of extract was raised somewhat. This change in concentration was calculated and taken into account in all the tables presented below. Hence the lowest concentration of red cell extract (RCE) in any flask was 0.06 ×, obtained when 0.5 ml. of parasite suspension made in 0.5 × extract was added to 3.5 cc. of a solution containing no red cell extract.

The negative control medium was a mixture of equal parts of medium A-3 and the same buffer used for preparation of the red cell extract (Table I). This was adjusted to a pH of 7.0 with 0.1 N KOH.

A third control medium with an intermediate concentration of red cell extract was usually included and was prepared by mixing equal volumes of the positive and negative control media.

The fractions eluted from the starch block were of necessity considerably more dilute than the original RCE. They were concentrated in the following way. On the day before the culture experiment was to be begun, the previously sterilized fraction was distributed in 15 to 25 ml. amounts in sterile cellophane bags prepared from 1 inch cellophane tubing, a 13 cm. length being attached to a glass tube 2 cm. in diameter and 11 cm. in length bearing a gauze and cotton plug. The tube with its plug had been previously wrapped in paper and dry-sterilized. The cellophane was attached tightly with fine thread, and at the lower end was tied off to form a bag 8 cm. long. These preparations were then sterilized by overnight exposure in a cabinet to an ultraviolet sterilamp. Each bag containing the fluid to be concentrated was suspended immersed in a previously prepared and chilled 20 per cent solution of polyvinylpyrrolidone (PVP)[1] held in a beaker. For each 20 ml. of fluid in the bag, 40 gm. of PVP dissolved in 160 ml. 0.05 M potassium phosphate buffer was used. The dialysis and concentration were allowed to proceed overnight at 1–2°C. A twenty ml. volume of fluid was usually reduced to a volume of 2 to 3 ml. It must be noted, of course, that this procedure concentrated only non-dialyzable materials. The liquid remaining in the bags was removed for measurement of its volume to sterile graduated tubes, diluted if necessary with 0.05 M buffer and finally mixed with an equal volume of medium A-3 (Table I). The pH was adjusted to 7.0. It will be apparent that such a mixture corresponds to the negative control medium except for non-dialyzable substances which it may contain.

After the control and experimental media were prepared, each medium received an appropriate volume of the malate-adenylate-cozymase supplement. Three ml. of each medium were placed in each of two flasks (previously lined in the usual way with a thin layer of clotted duck plasma). The ATP-pyruvate and Co A supplements were then added, and finally 0.5 ml. of the purified suspension of free parasites.

Methods having a bearing only on particular experiments will be considered together with the results of these experiments.

RESULTS

1. The Effect of Folinic Acid on Extracellular Survival of Plasmodium lophurae.—The addition of synthetic leucovorin at relatively high concentrations to flasks containing the fully supplemented erythrocyte extract and inoculated with a crude hemolyzed parasite suspension had no effect on the development of multinucleate parasites during the 1st day of incubation. It likewise had no effect on the small proportion of degenerate parasites seen on the 3rd day. But on the 4th day some interesting differences appeared.

Representative results taken from 7 out of a series of 12 such experiments are shown in Table II. In some experiments, as in Experiments A and C of the table, only half as many degenerate parasites were counted in stained films

[1] PVP, polyvinylpyrrolidone.

TABLE II

Effect of Added Folinic Acid (as Leucovorin) on Survival in Vitro of Extracellular P. lophurae

All flasks contained the standard full strength duck erythrocyte extract and were supplemented at each change of medium with ATP, pyruvate, and coenzyme A.

Experiment	Flask	Added leucovorin, µg./ml	Per cent degenerate parasites on 4th day (from stained films)	Experiment	Flask	Added leucovorin, µg./ml.	Per cent degenerate parasites on 4th day (from stained films)
A	1	0	26	E	1	0	33
	2		21		2		37
	3	4	12		3	6	32
	4		14		4		31
B	1	0	48		5*	0	40
	2		39		6*		48
	3	4	19		7*	6	30
	4		41		8*		22
	5	8	33	F	1	0	42
	6		36		2		46
	7	16	14		3	6	36
	8		31		4		37
C	1	0	40	G	1	0	22
	2		42		2		21
	3*	6	22		3‖	0	19
	4*		21		4‖		19
D	1	0	21, 26‡		5	15	15
	2		36, 27				
					7‖	7.5	14
	3§	0	22, 21		8‖		14
	4§		25, 23				
	6	6	19, 16				

* These flasks (Experiments C and E) also contained added cocarboxylase (12 µg./ml. and uridine triphosphate (70 µg./ml.).

‡ Counts were made on two separate films prepared from each flask.

§ These flasks (Experiment D) contained additional folic acid (1.2 µg./ml.). (The standard medium contained throughout 0.005 µg./ml.).

‖ In Experiment G, additional folic acid was contained in flasks 3 and 4 (0.8 µg./ml.) and in flasks 7 and 8 (0.4 µg./ml.).

from cultures with added leucovorin as in those from cultures without it. It will be remembered that all the flasks contained added folic acid at a low concentration (0.005 μg. per ml.). In other experiments, as in B and D, the results were much more variable, but always the best cultures would be among the flasks with added leucovorin. This favorable effect of leucovorin was apparent not only in the stained films but also in fresh preparations examined by phase contrast. Most of the parasites would show evenly bright cytoplasm characteristic of these organisms when they are in good condition (3). Equally good preparations have not been seen from flasks lacking the added leucovorin.

TABLE III

Effect of High Folic Acid on the Development of Plasmodium falciparum in Suspensions of Human Erythrocytes

Each flask contained originally about 70 parasites per 10,000 red blood cells. After 1 day the count was about 60 per 10,000 red cells with large multinucleate parasites predominating.

Flask	Folic acid	Parasites per 10,000 red blood cells after 2 days at 38°				
		Rings	1 nucleate trophozoites	Trophozoites with 2 or more nuclei	Total parasites of normal appearance	Degenerate parasites*
	μg. per ml.					
1	0.01	33‡	2	1	36	44
2	0.01	40‡	1	2	43	21
3	1.7	60	6	3	69	8
4	1.7	51	6	0	57	6

* In parasites counted as degenerate the chromatin and cytoplasm did not stain differentially.

‡ Most of these parasites were not true rings but were small solid structures, quite unlike the typical rings which predominated in flasks 3 and 4.

No similar favorable effect was observed in three experiments in which folic acid was added at the relatively high concentration of 0.8 to 1.2 μg. per ml. (Table II, Experiments D and G).

2. The Effect of High Folic Acid on the Development of Plasmodium falciparum in Suspensions of Human Erythrocytes.—In the course of other work, it was possible to obtain a result of considerable interest in relation to the folic acid metabolism of malaria parasites.

Through the kind cooperation of Dr. Martin D. Young, heparinized blood was obtained from a patient at Columbia, South Carolina, with a severe infection of *P. falciparum*, Panama strain. The blood was drawn aseptically at 5 p.m. and shipped by air in a thermos jar packed in ice. It reached New York on the following morning, at which time the blood was still cold and both the blood cells and parasites appeared microscopically in good condition. Four flasks (50 ml. Erlenmeyer) were prepared each containing 4.5 ml. of a nutrient solution

modified (7) from that used by Geiman *et al.* (8) for maintenance of parasitized monkey erythrocytes, 1.0 ml. of freshly drawn heparinized normal human blood and 0.5 ml. of the blood infected with *P. falciparum*. Each of two of the flasks received a supplementary 0.2 ml. of stock solution 14 F (Table I), so that they contained a much higher concentration of folic acid than the other two. The flasks were incubated at 37.5–38°C. on a rocker (16 cycles per minute) and received a slow current of air with 5 per cent CO_2. The normal blood used for preparing the flasks had 4.9 million red cells per c.mm. and the infected blood 4.3 million, so that the flasks had a red cell concentration of 1.2 million per c.mm. There were present initially in the infected blood about 25 parasites (all in the ring stage) per 1000 red cells, or roughly 100,000 parasites per c.mm. Hence the initial density of parasites in the flasks was about 9000 per c.mm. Blood films stained with Giemsa were prepared from each flask initially and after 1, 2, and 3 days' incubation, and at the same time fresh mounts were examined with the phase contrast microscope.

The red cells appeared in excellent condition even after 2 days *in vitro*, few of them being crenated. At this time the red cell counts for the four flasks were 1.1, 1.2, 1.2, and 1.0 million per c.mm., so that little red cell destruction had occurred. In the wet mounts parasites with characteristic bright cytoplasm were seen after 2 but not after 3 days *in vitro*. Whereas the stained films made at 0 time showed chiefly rings, those made at 1 day showed 35 to 40 per cent segmenting parasites (with over four nuclei, see Fig. 1). No difference was noted between the flasks with low and high folic acid. In the films made after 2 days, however, a marked difference appeared. In all flasks reinvasion of erythrocytes by young parasites occurred, but only in the flasks with high folic acid were most of the parasites typical ring forms (Figs. 2 *a* to 2 *c*) and only in these flasks were there few degenerate forms (Table III). In none of the flasks did an over-all increase in parasites occur, the extent of reinvasion being only enough to maintain nearly the original parasite density.

3. Activity for Extracellular P. lophurae of a Non-Dialyzable Material from Yeast.—In these experiments, as in those dealing with fractionation of the red cell extract (section 4), the culture flasks were inoculated with purified free parasite suspensions and the results were based primarily on the relative extent of increase in proportion of multinucleate parasites (chiefly forms with 2 to 4 nuclei) which occurred during an incubation period of 1 day (18 to 21 hours). The reliability of this increase in proportion of multinucleate parasites (the total number of parasites meanwhile remaining constant or increasing slightly (4)) as a measure of the activity of the red cell extract is attested by the results of numerous control flasks. Compare, for example, the results of flasks 1 and 2 with 5 and 6 in Experiment 2 (Table IV), those of 1 and 2 with 11 and 12 in Experiment 8 (Table V), those of 1 and 2, 9 and 10, and 11 and 12 in each of Experiments 11 and 12 (Table VII).

A 1 gm. sample of a particular lot (here designated A) of crude yeast hexokinase type II obtained from the Sigma Co. and having an activity of 48,500 Kunitz-McDonald units (9) per gm. was dissolved in 10 ml. redistilled water with 100 mg. glucose. The solution was steri-

TABLE IV

*Partial Replacement for P. lophurae of Red Cell Extract (RCE) by Yeast Protein Fractions**

Experiment	Flask	Medium ‡	Yeast protein		Per cent multinucleate parasites on day	
			Preparation	Concentration, µg./ml.	0 (inoculum)	1
1	1	0.3 × RCE	—	0	3	9
	2					9
	3	0.3 × RCE	A	2.5		14
	4					16
	5	0.3 × RCE	A	7.5		24
	6					23
	7	1 × RCE	—	0		20
	8					24
	9	0.3 × RCE	A	2.5		22
	10					25
2	1	0.3 × RCE	—	0	4	14
	2					14
	3	0.3 × RCE	A	7.5		25
	4					30
	5	1 × RCE	—	0		24
	6					33
	7	0.3 × RCE, pH 7.0	—	0		23
	8					17
	9	0.3 × RCE, pH 7.0	A	7.5		30
	10					29
3	1	0.3 × RCE, pH 7.0	—	0	4	19
	2					16
	5	0.3 × RCE, pH 7.0	A	7.5		26
	6					32
	9	1 × RCE	—	0		23
	10					24

* Flasks inoculated with purified parasite suspension. Counts made on inoculum (day 0) and after 1 day at 40°C.

‡ The medium contained the supplements of known activity and had an initial pH of 6.8 unless otherwise indicated.

TABLE IV—*Concluded*

Experiment	Flask	Medium‡	Yeast protein		Per cent multinucleate parasites on day	
			Preparation	Concentration, μg./ml.	0 (inoculum)	1
4	1	0.15 × RCE pH 6.9	—	0	6	22
	2					20
	3	0.15 × RCE pH 6.9	B	7.5		33
	4					37
	5	0.15 × RCE pH 6.9	C	0.75		33
	6					39
	11	0.6 × RCE	—	0		37
	12					43
5	1	0.25 × RCE pH 7.1	—	0	8	21
	2					24
	3					22
	4	0.25 × RCE pH 7.1	B	7.5		31
	5					27
	6	0.26 × RCE pH 7.1	C	0.75		28
	7					32
	8					29
	9	1 × RCE	—	0		25
	10					38
	11	1 × RCE, lower NaCl, KCl§	—	0		36
	12					38

§ The NaCl and KCl were reduced (33 ml. stock solution 1 of Table I per liter) so as to give a solution of slightly lower osmotic pressure.

lized by filtration through a Selas 03 porcelain candle and was stored in a deep freeze. When it was added to flasks containing ⅓ strength red cell extract the parasites in such flasks showed as much development as those in control flasks with full strength RCE[2] (Table IV, Experiments 1 to 3).

The presence of the added hexokinase brought about a marked drop in pH, presumably as a result of glucose phosphorylation, since glucose and ATP were both present in the culture medium. Whereas the control flasks with ⅓ strength RCE had a pH of 6.7 after a day's incubation those with added hexokinase had a pH of 6.4. Even if the initial pH was raised to 7.1 instead of 6.8

[2] RCE, red cell extract.

TABLE V

*Effect of Temperature on Activities of Yeast Protein Fractions for P. lophurae**

Experiment	Flask	Medium‡	Yeast protein			Final pH	Per cent parasites	
			Prep.	Conc.	Treatment		Multinucleate	Degenerate
				mg./ml.				
6	Inoculum						7	0
	1	0.25 × RCE	—	0	—	6.82	20	14
	2	pH 7.0				6.74	28	12
	3	0.25 × RCE	C	0.75	None	6.57	32	17
	4	pH 7.0				6.53	38	18
	5	0.25 × RCE	B	7.5	18 hrs. at	6.72	38	3
	6	pH 7.0			27°C.	6.70	31	6
	7	0.25 × RCE	B	7.5	18 hrs. at	6.67	31	6
	8	pH 7.0			38°C.	6.64	31	6
	9	0.25 × RCE	B	7.5	None	6.51	29	15
	10	pH 7.0				6.50	31	13
	11	1 × RCE	—	0	—	6.89	30	1
	12					6.85	40	1
7	Inoculum						3	0
	1	0.25 × RCE	—	0	—	6.82	24	1
	2	pH 7.0				6.86	18	1
	3	0.25 × RCE	B	7.5	18 hrs. at	6.71	24	2
	4	pH 7.0			27°C.	6.78	25	2
	5	0.25 × RCE	B	7.5	None	6.62	28	0
	6	pH 7.0				6.59	24	1
	7	0.25 × RCE	C	0.75	18 hrs. at	6.63	23	14
	8	pH 7.0			27°C.	6.63	24	12
	9	0.25 × RCE	C	0.75	None	6.61	23	6
	10	pH 7.0				6.63	31	4
	11	1 × RCE	—	0	—	6.91	39	0
	12					6.92	26	0

* Flasks inoculated with purified parasite suspension. Counts made on inoculum (day 0) and after 1 day at 40°C.

‡ The medium contained the supplements of known activity and had an initial pH of 6.8 unless otherwise indicated.

TABLE V—*Concluded*

Experiment	Flask	Medium‡	Yeast protein			Final pH	Per cent parasites	
			Prep.	Conc.	Treatment		Multinucleate	Degenerate
				mg./ml.				
8	Inoculum						5	0
	1	0.25 × RCE	—	0	—	6.82	13	10
	2	pH 7.1				6.82	18	7
	3	0.25 × RCE	B	7.5	18 hrs. at	6.77	20	10
	4	pH 7.1			27°C.	6.77	20	5
	5	0.25 × RCE	B	7.5	None	6.65	26	7
	6	pH 7.1				6.67	22	10
	7	0.25 × RCE	C	0.75	18 hrs. at	6.70	16	25
	8	pH 7.1			27°C.	6.67	17	25
	9	0.25 × RCE	C	0.75	None	6.66	22	21
	10	pH 7.1				6.68	22	21
	11	1 × RCE	—	0	—	6.88	26	3
	12					6.90	38	2

the final pH in flasks with added hexokinase was about 6.5. Despite the development of this acidity, relatively few of the parasites appeared degenerate.

When attempts were made to repeat the results of Experiments 1 to 3 with samples from other lots of Sigma hexokinase or with hexokinase concentrates from other sources no favorable effect could be found; on the contrary all of these preparations were markedly toxic, as shown by large numbers of degenerate parasites after 1 day *in vitro*. Since no more of the initial lot was available, the work was held in abeyance until a preparation could be made in as nearly as possible the same manner as had been used for making the lot which had given favorable results.

Through the cooperation of the Sigma Co. two such yeast protein preparations were obtained (here designated as B and C respectively): Lot 27-632-2, a crude concentrate like the original favorable batch; Lot 27-621, a more highly purified yeast enzyme concentrate. One gm. of the less pure preparation B was dissolved in 10 ml. 0.1 per cent glucose solution and filtered through a Selas 03 candle. 100 mg. of preparation C was dissolved in 10 ml. of 0.1 per cent glucose and filtered through an ultrafine glass filter. Both of these solutions showed no toxicity and a favorable effect on the increase in multinucleate forms (Table IV, Experiments 4 and 5). The final pH in the presence of the yeast enzymes was again 6.4 to 6.6, as compared to 6.8 to 6.9 in their absence. If preparation B was allowed to stand overnight at 27° or 38°C. its acid-forming property (presumably the hexokinase) was lost, but the favorable

TABLE VI

*Development of P. lophurae in Duck Erythrocyte Extracts Prepared in Different Ways**

Experiment	Flask	Medium‡	Per cent parasites	
			Multi-nucleate	Degenerate
9	Inoculum		11	0
	1	0.6 × standard RCE	34	2
	2		40	1
	3	Aqueous RCE made to 0.6 ×	41	4
	4		40	2
	5	Same as for 3, 4 but kept 1 day at 1°C.	40	2
	6		42	1
	7	Aqueous RCE, dialyzed 18 hrs. at 1°C.	41	1
	8	against 20 per cent PVP and made to 1 ×	38	0
	9	Standard RCE (1 ×)	43	1
	10		38	2
10	Inoculum		10	0
	1	Aqueous RCE kept 5 days at 1°C.	31	2
	2	Made to 0.6 ×	29	0
	3	Fresh aqueous RCE made to 0.6 ×	33	3
	4		37	1
	5	Aqueous RCE kept 5 days at −20°C.	37	2
	6	Made to 0.6 ×	29	2
	7	Aqueous RCE kept 5 days at +27°C.	22	12
	8	Made to 0.6 ×	26	8
	9	0.6 × standard RCE	25	3
	10		29	2

* Flasks inoculated with purified parasite suspension. Counts made on inoculum (day 0) and after 1 day at 40°C.

‡ The medium contained the supplements of known activity and had an initial pH of 6.8 unless otherwise indicated.

effect on the parasites was retained (Table V, Experiments 6 to 8) and could in general be more readily demonstrated because of the absence of low pH toward the end of the incubation period. Keeping preparation C at 27°C. did not remove its acid-forming property but tended to remove the favorable effect on the parasites (Table V, Experiments 7 and 8). In this preparation a fine white precipitate appeared on standing at 27°C. These results suggest that the activity of the yeast protein preparations in partially replacing red cell extract does not depend on their hexokinase content.

4. Activity for Extracellular P. lophurae of a Non-Dialyzable Fraction from Erythrocyte Extract.—Preliminary experiments showed that the development of purified free parasites during 1 day *in vitro* was not adversely affected by the procedures used in electrophoresis of red cell extract, namely dilution of the extract in water or buffer, subsequent mixing with other ingredients of the medium, dialysis, and concentration against 20 per cent PVP, and storage at −20° or 1°C. for periods up to 5 days (Table VI). Storage at 27°C., however, did have a deleterious effect (Table VI, Experiment 10). In 1 day experiments 0.6 strength extract usually gave about the same result as full strength extract, but this was not true in experiments of longer duration.

When duck erythrocyte extract was subjected to electrophoresis on starch in buffer of pH 7.3 the hemoglobins migrated toward the cathode and some activity for *P. lophurae* was found associated with the hemoglobin fraction. At a pH of 7.5 three colored bands formed on the cathode side of the origin. Farthest from the origin (about 12 cm. away) was a narrow brownish band 1 cm. or less wide. This was followed by a broad, bright red band about 6 cm. wide and then by a pale red band about 3 cm. wide which often trailed somewhat toward the origin. No color ever appeared on the anode side of the origin.

The hemoglobin fraction obtained at pH 7.5 regularly was toxic to the malaria parasites, (perhaps because of some methemoglobin formed at this pH) but fractions taken from the part of the starch block extending from the origin 5 to 7 cm. toward the anode favored the development of multinucleate *P. lophurae* (Table VII). The smallest band containing activity was 3.5 cm. wide (Experiment 13, Table VII). Whereas original red cell extracts contained about 14 per cent protein, the fractions from the starch block showing activity contained, after concentration against PVP, only about 0.05 per cent protein. The activity of these fractions was approximately half that of the original extract (corresponding closely to that of 0.3 × RCE); therefore, relative to protein the activity had been concentrated about 140 times.

DISCUSSION

The importance of folic acid and related compounds in the metabolism of malaria parasites has been indicated by several facts: the powerful antimalarial effect of substances such as pyrimethamine, which seem to act by interfering with the formation of folinic acid (10); the favoring effect of *para*-aminobenzoic

TABLE VII

*Activity for P. lophurae of Fractions Separated from Duck Erythrocyte Extract by Electrophoresis on Starch**

Experiment	Flask	Medium‡	Fraction		Per cent parasites	
			Zone	Per cent protein	Multinucleate	Degenerate
11	Inoculum				11	0
	1	0.06 × RCE	—	—	28	7
	2				29	17
	3	0.06 × RCE	4.5 to 11.5 cm.§ left of origin (toward cathode)	8.4	12	44
	4				11	62
	5	0.06 × RCE	0.5 cm. left to 6.5 cm. right	0.06	40	16
	6				38	13
	7	0.06 × RCE	7 to 14 cm. right	0.05	27	23
	8				25	23
	9	0.3 × RCE	—	—	45	6
	10				46	9
	11	0.6 × RCE	—	—	58	3
	12				59	5
12	Inoculum				7	0
	1	0.06 × RCE	—	—	26	19
	2				22	21
	3	0.06 × RCE	1 cm. left to 4 cm. right	0.05	30	7
	4				30	6
	5	0.06 × RCE	4 to 9 cm. right	0.05	26	15
	6				21	14
	7	0.06 × RCE	9 to 14 cm. right	0.05	20	9
	8				20	9
	9	0.3 × RCE	—	—	34	5
	10				32	4
	11	0.6 × RCE	—	—	41	3
	12				37	3

TABLE VII—*Concluded*

Experiment	Flask	Medium‡	Fraction		Per cent parasites	
			Zone	Per cent protein	Multinucleate	Degenerate
13	Inoculum				6	0
	1	0.06 × RCE	—	—	13	22
	2				14	24
	3	0.06 × RCE	0 to 3.5 cm. right	0.021	18	11
	4				23	8
	5	0.06 × RCE	3.5 to 7 cm. right	0.026	12	28
	6				11	34
	7	0.06 × RCE	Above fractions combined	0.036	15	15
	8				21	13
	9	0.3 × RCE	—	—	25	6
	10				22	14
	11	0.6 × RCE	—	—	31	1
	12				29	1

* Flasks inoculated with purified parasite suspension. Counts made on inoculum (day 0) and after 1 day at 40°C.

‡ The medium contained the supplements of known activity and had an initial pH of 6.8 unless otherwise indicated.

§ This fraction included both bands of hemoglobin.

acid (8) and folic acid (11) on the development of malaria parasites growing intracellularly in suspensions of intact erythrocytes *in vitro*; and the greatly enhanced content of folic and folinic acids in erythrocytes of ducks infected with *P. lophurae* as compared to the erythrocytes of uninfected ducks ((12) and data in press by Trager). An experiment reported in the present paper shows the favorable effect of a high concentration of folic acid on yet another species of malaria parasite, *P. falciparum*, when developing intracellularly.

In experiments with *P. lophurae* maintained extracellularly, however, no effect of a high concentration of folic acid could be shown. On the other hand, folinic acid at about 5 μg. per ml. had a distinct favoring effect on survival of the extracellular parasites on the 4th day of incubation *in vitro*. It may be that although *para*-aminobenzoic acid and folic acid influence the growth of the malaria parasite within a living red cell, the parasite itself requires folinic acid or some other coenzyme form of folic acid (13). Such a situation would be analogous to that already shown for pantothenate and coenzyme A. Whereas

the combination host red cell plus malaria parasite required pantothenate for the development of the parasite, the parasite itself could not utilize pantothenate but required the complete Co A (1, 14).

The partial replacement of erythrocyte extract, in the early extracellular development of *P. lophurae*, by certain yeast protein concentrates and by a non-dialyzable fraction from red cell extract, other than hemoglobin, indicates a requirement by the parasite for growth factors of high molecular weight normally obtained from the host cell. Since the parasites are intracellular phagotrophs (15) and engulf large portions of the cytoplasm of their host cell, they may in this way obtain not only hemoglobin as their bulk source of nitrogen but also proteins and other high molecular weight compounds required as growth factors in trace amounts.

Few growth factors of high molecular weight are known. The ciliate *Paramecium* requires a non-dialyzable factor from yeast, which is evidently a complex of lipide, protein, and carbohydrate material (16). The parasitic nematode *Neoaplectana glaseri*, as well as the free-living nematode *Caenorhabditis briggsae*, requires a material of high molecular weight present in suitably prepared liver extract (17, 18). A partially purified protein, obtained from certain mouse tumors, greatly stimulates the outgrowth of new fibers from the ganglia of chick embryos (19). All of these materials, and not least among them the malarial growth factor from red cells, must play important roles in cellular physiology.

SUMMARY

The extracellular survival of the malaria parasite *Plasmodium lophurae* was favored on the 4th day of incubation *in vitro* by the presence in the medium of added folinic acid at a concentration of about 5 μg. per ml. The development of the human malaria parasite *P. falciparum* intracellularly in suspensions of human erythrocytes was better in a medium with a high than in one with a low concentration of folic acid.

In the early extracellular development of *P. lophurae in vitro* erythrocyte extract could be partially replaced by certain yeast protein preparations and by a non-dialyzable fraction, free from hemoglobin, prepared from duck erythrocyte extract by means of starch electrophoresis.

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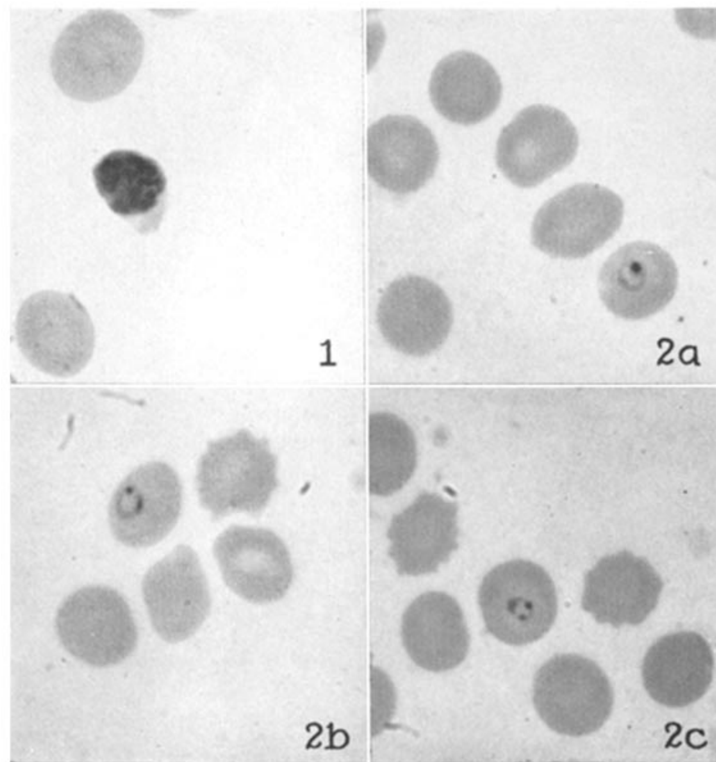
EXPLANATION OF PLATE 48

FIG. 1. *Plasmodium falciparum*. Schizont after 1 day in culture in suspension of human erythrocytes. × 776.

FIGS. 2 *a* to 2 *c*. Rings of *P. falciparum* after 2 days in culture in the presence of a high concentration of folic acid. × 776.



(Trager: Nutrition of malaria parasites)