FURTHER STUDIES ON THE SURVIVAL AND DEVELOPMENT IN VITRO OF A MALARIAL PARASITE

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The desirability of systematic studies of the effects of various environmental conditions on the survival *in vitro* of malarial parasites has been discussed in the first paper of this series (1). In that paper, experiments were reported which showed that the survival of the avian malaria parasite *Plasmodium lophurae* was favored by a balanced salt solution of high potassium content, by aeration but not by a very high oxygen tension, by an optimal density of parasites per cubic millimeter, by frequent renewal of the suspending medium, by concentrated red cell extract, and by optimal concentrations of plasma or serum, of chick embryo extract, of glucose or glycogen, and of glutathione. In the best preparations parasites survived 5 days as judged by exflagellation of the male gametocytes and 6 days as judged by infectivity under a set of standard conditions.

By adding calcium pantothenate to the suspending medium and by providing the parasites with better aeration and with fresh red blood cells every other day, it has now been possible to obtain survivals of 10 to 16 days at 40–41°C.

Methods

The strains of *P. lophurae* were the same that had been used in the previous studies except that the strain maintained in chicks had been passed once through mosquitoes (*Aedes aegypti*).

In all the experiments reported in the present paper, 25 ml. Erlenmeyer flasks containing 2 ml. of medium were used in the manner previously detailed (1). Every day ⅓ to ½ of the volume of medium in each flask was drawn off and an equal quantity of fresh medium, first warmed to 38–40°C., was added. In some experiments the flasks were held in a simple rocking device actuated by a windshield wiper and giving about 20 up and down movements per minute. Such flasks were removed from the rocker daily and held stationary in an upright position for about 2 hours. This permitted most of the red cells to settle to the bottom so that a portion of medium could be drawn off and replaced by fresh medium. When fresh red cells were to be added, heparinized chicken or duck blood was centrifuged in a graduated centrifuge tube. The plasma was removed. A measured quantity of erythrocytes was drawn up from the bottom of the tube and suspended in 3 times its volume of the balanced salt solu-

tion K[1] (1) used in the media contained in the flasks under treatment. 0.1 ml. of the red cell suspension was then added to each flask.

The basic medium was red cell extract. Chicken red cell extract was used for tests with parasites from chickens, duck red cell extract for tests with parasites from ducks. Duck red cell extract was prepared in the following way. Blood was taken aseptically by heart puncture from ducks not over 2 months old and was defibrinated by shaking with glass beads. The blood was centrifuged 5 minutes at a low speed and the serum drawn off. The cells were frozen by immersion of the centrifuge tube in a dry ice-alcohol mixture, and they were then allowed to thaw slowly in an incubator at 40°C. The resulting dark red viscous liquid was thoroughly mixed with 1⅓ its volume of diluent, consisting of 2 parts of the homologous serum to 1 part of salt solution K (or a modification of it). The mixture was then centrifuged at the high speed of an ordinary centrifuge for 1 hour. Somewhat over ⅓ the total volume of mixture could be drawn off as a clear deep red liquid which constituted the red cell extract. Centrifugation in a high speed angle centrifuge did not increase the yield of clear fluid. Chicken red cell extract was prepared in the same manner, except that the frozen and thawed red cells were suspended in a diluent consisting of only 1 part of chicken serum to 1 part of solution K. Chicken serum in a more than 50 per cent concentration was unfavorable to survival, but this was not true of duck serum. In most of the later experiments, 0.1 per cent glutathione was incorporated in the solution K. Frequently the glucose in solution K was replaced by 0.2 per cent glycogen. Various growth factors tested were added as small amounts of sterile aqueous solutions of the pure compounds. Chick embryo extract in solution K was prepared as previously described (1). The minced tissue of the 10 day old embryos was suspended in solution K, used in the proportion of 1.5 ml. per embryo, and the mixture was centrifuged 5 minutes at low speed. The opalescent supernatant liquid constituted the extract and was always used at a concentration of 10 per cent by volume.

At least 9 ml. of medium per flask was prepared at one time. This provided 2 ml. for the original preparation and 1 ml. of replacement fluid daily for 7 days. If survival continued beyond the 7th day, enough fresh medium was prepared to suffice for several additional days.

Parasite material was obtained from young chickens or ducks, inoculated 4 to 5 days previously with *P. lophurae*. As in the previous work, the chickens weighed about 300 gm. and were rendered more susceptible to the infection by the intraperitoneal injection of carbon ink. The blood of the infected animal was taken aseptically from the heart into a small amount of heparin-sodium chloride solution (0.05 ml. per ml. of blood). The blood was centrifuged and the plasma discarded. A measured volume of cells was drawn up from the bottom and suspended, together with an equal volume of similarly prepared red cells from an uninfected animal, in twice the volume of solution K. 0.2 ml. of this suspension was inoculated to each flask containing 2 ml. of medium.

An initial stained film and a red cell count were made on the contents of each flask.

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[1] Solution K contained, in gm. per liter, NaCl 3.039, KCl 4.100, $NaH_2PO_4 \cdot H_2O$ 0.690, K_2HPO_4 2.613, $NaHCO_3$ 0.168, $CaCl_2$ 0.166, $MgSO_4 \cdot 7H_2O$ 0.370, $MgCl_2 \cdot 6H_2O$ 0.407, *d*-glucose 2.377.

Every day a sample was removed from each flask, after the addition of fresh medium, and used for making a stained smear, a wet film, and in some experiments suitable dilutions for infectivity tests and for red cell counts. If fresh red cells were to be added a drop was first removed and a stained smear made. The flask was then left stationary in the incubator for 2 hours, after which the fresh medium and red cells were added and the usual sample taken for a smear, wet film, etc. When infectivity tests were performed the technique previously described was followed exactly. It was assumed that the minimum numbers of parasites required to produce detectable infections under the conditions used were still the same as had been determined before (1). The figures in the tables representing approximate percentage survival are based on this assumption and on the highest dilution which produced an infection in the test chicks. Since only 2 or 3 dilutions were tested each day, if these were not correctly chosen, as occasionally happened, a higher percentage survival might appear on the 4th day than on the 3rd day. But usually the results were sufficiently consistent for their purpose.

Since it was observed that infectivity almost always persisted at least a day beyond exflagellation, infectivity was abandoned as a criterion of survival in the more recent experiments, and the very simple and rapid exflagellation test was relied on mainly. This was roughly standardized by always preparing the wet film in as even a manner as possible, using 0.02 ml. of material and a No. 1 coverslip. The preparation, after standing at room temperature for 25 to 30 minutes, was searched for 5 minutes with a 4 mm. objective and 15 × oculars. The motile exflagellating male gametocytes found during this period were counted.

Parasite counts were made from the Giemsa-stained films. Since the red cell nuclei persist for some time after the red cell is hemolyzed, more accurate relative parasite counts could be obtained by determining the parasites per 1000 red cell nuclei, rather than per 1000 intact red cells. In the better preparations, among those not receiving fresh red cells, the red cell count remained at about its original level for the first 3 days, in spite of the likelihood of loss of some cells during the daily withdrawal of a portion of the medium. This indicated little hemolysis. In making the parasite counts the number of very young parasites and segmenters (4 or more nuclei) was noted.

RESULTS

The preservation of human erythrocytes at low temperature in various media, all of which represent but slight modifications of the glucose-citrate-plasma medium first described by Rous and Turner (2), suggested that such a medium might be suitable for the maintenance of malaria parasites within surviving erythrocytes. A number of experiments showed clearly that such a medium did not give as good survival of *P. lophurae* as did the already developed red cell extract medium. The results presented in Table I show that: (1) equal parts of citrated chicken plasma and isotonic glucose were better than 3 parts of citrated plasma to 1 part of glucose; (2) the inclusion of red cell extract in the citrated plasma-glucose medium favored survival; (3) red cell extract in solution K with embryo extract gave the best survival; (4) equal

parts of citrated plasma and isotonic sucrose did not give nearly as good survival as citrated plasma and glucose, in agreement with the fact that sucrose also could not replace glucose in the red cell extract-embryo extract medium.

TABLE I

Effects of Glucose-Citrate-Plasma, Red Cell Extract, and Embryo Extract

(Parasites from chick. 51,000 parasites per c.mm. Temperature 41–41.5°C.)

Flask No.	Medium	Infectivity* after days:			Exflagellants‡ after days:				
		3	4	5	1	2	3	4	5
1	Equal parts citrated chicken plasma and isotonic (5.4%) glucose	10%	−		4	0	0		
2	3 parts of citrated plasma to 1 part of isotonic glucose	−	−		1	0	0		
3	Chicken red cell extract prepared in equal parts of citrated plasma and isotonic glucose	2%	++	−	3	4	0	0	0
4	Equal parts of citrated plasma and isotonic (10.3%) sucrose	−	−		0	0			
5	Chicken red cell extract in equal parts plasma and solution K with citrate	2%	+	−	0	11	0	1	0
6	Same as 5, + 10% by volume chick embryo extract	20%	++	+	30	3	0	0	0

* In this and succeeding tables, a number followed by % indicates that the material was infective at such a dilution as to show that the given percentages of the original parasites were still capable of producing infection. ++ indicates infection apparent 7 days after inoculation of 2 day old chicks intracerebrally with 0.05 ml. of undiluted material, while + indicates infection apparent 11 days after such inoculation, but not 7 days after. − indicates no infection after 11 days.

‡ The number of exflagellants seen in a 5 minute examination of a wet mount allowed to stand 25 to 30 minutes at room temperature.

Since lipids are important constituents of the red cell membrane, the effect of adding cholesterol or phospholipid to the red cell extract-embryo extract medium was tried. Cholesterol at concentrations of 0.025 mg. per ml. and higher had either no effect or a deleterious effect. Crude egg phospholipid, prepared after the manner of Lardy and Phillips (3), had a harmful effect at high concentrations but a favorable effect at low concentrations (Table II). This effect has not yet been studied in greater detail.

In the earlier work (1), it was observed that very low concentrations of liver extract or yeast extract favored survival. This effect has been confirmed and

TABLE II

Effects of Egg Phospholipid

(Parasites from duck. 90,000 parasites per c.mm. Temperature 41–41.5°C.)

Flask No.	Medium	Infectivity after days:				Exflagellants after days:					
		3	4	5	6	1	2	3	4	5	6
1	Duck red cell extract in equal parts solution K and serum, + chick embryo extract	20%	1%	−	−	11	18	44	40	3	0
2	Same as 1, + crude egg phospholipid 0.2 mg. per ml.	20%	10%	+	−	9	11	24	32	15	0
3	Same as 1, + crude egg phospholipid 0.8 mg. per ml.	2%	10%	+	−	11	10	19	29	10	0

TABLE III

Effect of Yeast Autolysate

(Parasites from chick. 20,000 parasites per c.mm. Temperature 41–41.5°C.)

Flask No.	Medium	Infectivity after days:				Exflagellants after days:					
		3	4	5	6	1	2	3	4	5	6
1	Chick red cell extract in equal parts solution K and serum, + chick embryo extract	4%	1%	−		3	10	2	2	1	0
2	Same as 1, + yeast autolysate, 4% by volume	40%	<1%	−		10	9	3	1	0	0
3	Same, but 0.7% yeast autolysate	20%	20%	+	−	5	0	28	17	2	0
4	Same, but 0.14% yeast autolysate	40%	2%	+	+	8	1	16	16	2	0

it has been found that low concentrations of yeast autolysate also favor survival (Table III). Another interesting observation was made with several survival tests which had become contaminated and which were examined when the growth of contaminating bacteria was still extremely light. In such preparations more exflagellants were observed than in corresponding uncon-

taminated flasks. All these facts suggested that growth factors of the B-complex type might be active in prolonging the *in vitro* survival of *P. lophurae*. The addition of thiamin alone to the red cell extract-gluthathione-embryo extract medium had no effect, but the addition of calcium pantothenate definitely favored survival (Tables IV to VI).

TABLE IV

Effect of Calcium Pantothenate

(For flasks 1 to 3: parasites from chick; 43,200 parasites per c.mm. For flasks 4 and 5: parasites from duck; 89,000 parasites per c.mm. Flasks 4 and 5 held in rocker, received fresh duck erythrocytes every 2nd day. Temperature 41.2–41.8°C.)

Flask No.	Medium	Infectivity after days:						Exflagellants after days:									
		3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10
1	Chicken red cell extract in equal parts solution K with 0.1% glutathione and glygen in place of glucose, and serum, + chick embryo extract	4%	20%	+	−			0	1	4	2	0	0				
2	Same as 1, + liver extract, 0.14% by volume	4%	2%	++	+			0	10	16	5	0	0				
3	Chicken red cell extract in equal parts solution K with 0.1% glutathione, and serum, + chick embryo extract + Ca pantothenate 0.02 mg. per ml.	40%	10%	++	+	−	−	6	15	23	20	12	11	1	0		
4	Duck red cell extract in 1 part solution K with 0.1% glutathione and glycogen in place of glucose, and 2 parts serum, + chick embryo extract + Ca pantothenate 0.02 mg. per ml.	4%	2%	10%	++	+	+	28	100	56	98	11	14	18	2	1	0
5	Same as 4, but Ca pantothenate replaced by liver extract, 0.14% by volume	4%	2%	+	+	−	+	14	18	18	49	9	3	0	1	0	0

In Table IV flasks 1 and 2 illustrate again the favorable effect of liver extract, while flask 3 was the first flask, held in a stationary position and not receiving any fresh red cells, ever to show exflagellants after the 5th day. Flasks 4 and 5, which were held in a rocker and received fresh duck erythrocytes every other day, showed the better survival, as judged both by infectivity and by exflagellation, with added calcium pantothenate than with the optimal concentration of liver extract. Table IV also serves to illustrate the higher num-

bers of exflagellants which are always to be seen with duck material than with chick material, a fact which is probably related to the greater suitability of the duck as a host for *P. lophurae*. As was to be expected, the frequent ad-

TABLE V

Effect of Calcium Pantothenate

(Parasites from duck. 79,000 parasites per c.mm. Flasks held in rocker and received fresh duck erythrocytes daily. Temperature 40.0–40.5°C.)

Flask No.	Medium	Exflagellants after days:												Parasites per 1000 red cell nuclei after days:*			
		1	2	3	4	5	6	7	8	9	10	11	12	0	1	2	3
1	Duck red cell extract in 1 part solution K with glucose replaced by glycogen and 0.1% glutathione to 2 parts serum, + embryo extract	27	22	60	47	7	34	C‡						230–2–4	234–4–3 124–6–4	144–10–7 82–6–2	69–4–6
2	Same as 1, + Ca pantothenate 0.04 mg. per ml.	26	72	73	55	36	30	14	1	23	5	0	0	218–1–5	236–5–0 146–8–1	204–24–8 126–22–6	100–0–16
3	Same as 1, + Ca pantothenate 0.2 mg. per ml.	31	72	68	116	14	39	15	3	31	10	0	0	212–1–12	264–4–4 186–3–2	186–19–12 140–16–6	92–2–16
4	Same as 3, + ascorbic acid 0.005 mg. per ml.	31	107	94	105	26	37	13	1	2	3	0	1	218–2–6	250–7–3 144–2–4	191–18–4 144–26–2	100–4–14

* The upper line of numbers gives, reading from left to right, the number of parasites per 1000 red cell nuclei, the per cent of segmenters (parasites with 4 or more nuclei), and the per cent of very young parasites before the addition of fresh red cells, while the lower line gives the same information after the addition of fresh red cells.

‡ C = flask contaminated.

dition of fresh red cells and agitation of the flask in a rocking machine, were favorable to survival. Table V shows an experiment in which exflagellation persisted up to 12 days. The preparation not containing added calcium pantothenate became contaminated on the 6th day, so that its survival time could not be determined. Of great interest, however, is the fact that the

number of parasites increased significantly between days 0 and 1 in flasks 2 to 4 but not in flask 1, and again between days 1 and 2 in flasks 2 and 4 but not in flasks 1 and 3. By the 3rd day the parasite number had maintained itself at a notably higher level in flasks 2 to 4 containing calcium pantothenate than in flask 1 not containing added pantothenate. Also noteworthy are the

TABLE VI

Effect of Calcium Pantothenate

(For flasks 1 to 5: parasites from chick; 100,000 parasites per c.mm. For flasks 6 to 8: parasites from duck; 77,000 parasites per c.mm. Flasks 6 to 8 held in rocker and received fresh duck erythrocytes every other day. Temperature 40–40.5°C.)

Flask No.	Medium	Exflagellants after days:											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Chick red cell extract in equal parts serum and solution K with 0.1% glutathione, + embryo extract	19	42	20	24	2	0	0					
2	Same as 1, + Ca pantothenate 0.02 mg. per ml.	5	33	17	20	13	10	C*					
3	Same as 1, + Ca pantothenate 0.06 mg. per ml.	2	24	22	22	21	2	1	0				
4	Same as 1, + Ca pantothenate 0.2 mg. per ml.	13	18	28	29	16	4	0					
5	Same as 2, + biotin 0.03 γ per ml.	32	6	19	23	20	13	6	7	0	0		
6	Duck red cell extract in 2 parts serum and 1 part solution K with glucose replaced by glycogen, and 0.1% glutathione, + embryo extract, + Ca pantothenate 0.02 mg. per ml.	13	30	109	63	58	10	11	31	25	7	7	3
7	Same as 6, but Ca pantothenate 0.24 mg. per ml.	15	50	74	70	25	38	23	40	31	18	14	12
8	Same as 6, but Ca pantothenate 0.28 mg. per ml.	21	44	65	46	63	80	85	57	35	11	34	C*

* C = flask contaminated.

changes in percentage of segmenters and young forms which occurred in flasks 2 to 4 but not in flask 1. A higher percentage of normal appearing very young parasites on the 3rd day than on previous days can only indicate that segmentation and the infection of new red cells have occurred *in vitro*. The higher numbers of exflagellants seen on some later days than on previous days also indicate that development of male gametocytes has taken place. Table VI again shows the favorable effect of calcium pantothenate on the persistence of exflagellation in stationary flasks containing parasites from a chick (flasks

1 to 4). As usual, in the absence of added pantothenate, no exflagellants were seen after the 5th day, but they were seen through the 7th day in the presence of pantothenate. Concentrations of calcium pantothenate varying from 0.02 to almost 0.3 mg. per ml. seem to be about equally effective. The result with flask 5 suggests a favorable effect of biotin, but this effect has not been obtained with any regularity. Flasks 6 to 8 illustrate long survival as judged by exflagellation in flasks which are rocked and which get fresh erythrocytes every other day. This is still better shown by Table VII, in which exflagellation will be seen to have persisted 14 to 16 days. Flasks 2 and 3 of this experiment contained various B-complex factors other than pantothenate, but no consistently favorable effect of their addition has yet been found.

TABLE VII

Prolonged Survival of Plasmodium lophurae in Vitro

(Each flask contained 2.3 ml. of medium + 0.25 ml. of parasite suspension from duck. 112,000 parasites per c.mm. Flasks held on rocker. Temperature 40.3–40.7°C. Medium consisted of duck red cell extract in 2 parts serum and 1 part solution K with 0.1% glutathione and embryo extract + Ca pantothenate 0.03 mg. per ml. Flasks 2 and 3 each contained in addition, per ml., 0.01 mg. thiamin, 0.01 mg. pyridoxin, 0.05 mg. nicotinic acid, and 0.0005 mg. riboflavin, while flask 3 also contained 0.1 γ biotin per ml. Each flask received fresh duck erythrocytes every 2nd day.)

Flask No.	Exflagellants after days:																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	17	49	119	15	75	—	32	8	8	0	7	0	0	2	C*		
2	33	123	148	31	72	—	67	2	16	0	14	6	20	6	1	0	0
3	42	80	125	64	180	—	85	8	6	0	15	0	5	4	0	1	0

* C = flask contaminated.

DISCUSSION

It seems reasonable to conclude, from the results presented in this paper, that the presence of calcium pantothenate is an additional factor which favors the survival *in vitro* of *Plasmodium lophurae*. The lowest concentration of calcium pantothenate tested was 0.02 mg. per ml., which is far in excess of the concentration of pantothenate in the blood of chickens (average of 360 mγ per ml. from 3 chickens determined by Snell (4), average of 308 mγ per ml. from 20 chickens determined by Trager) or ducks (average of 293 mγ per ml. from 3 ducks determined by Trager). Since 0.02 mg. per ml. of added pantothenate was about as effective as higher concentrations, it is possible that lower concentrations would also be effective. It is also possible that only a small proportion of the added pantothenate can get into the red cell where it would first affect the malaria parasite. Of considerable interest here is the fact that the related intracellular parasite *Eimeria nieschulzi* grows better in rats fed a diet adequate in pantothenate than in rats fed a diet deficient

in this growth factor (5, 6). This effect may well occur *via* some complex mechanism in the host, whereas the effect on *P. lophurae in vitro* must be either on the erythrocytes or directly on the malaria parasites themselves.

The results also show that sometimes, under the most favorable conditions for prolonged survival, *P. lophurae* continues to develop *in vitro* for a week or more and may even multiply during the first few days. In some of these preparations, such as flasks 2 to 4 of Table V, films made as late as the 10th day show entirely normal appearing parasites of all stages, including very young forms. Certainly development must be continuing in such preparations, but the death rate, after the first few days, far exceeds the birth rate and the parasites finally die out after about 2 weeks. Coulston (7) has reported briefly somewhat similar results with *P. circumflexum.* In his best preparations, gametocytes survived up to 7 days, while infectivity persisted up to 13 days. Coulston's medium consisted of heparinized blood with Baker's solution, which contains blood digest, hemoglobin, gluthathione, and a variety of other substances, and is not unlike the medium used for *P. lophurae.* He agitated the tubes and supplied them with oxygen and fresh red cells daily. It is unfortunate that his methods and results have not yet been published in detail sufficient to permit a direct comparison between the survival of *P. lophurae* under his conditions and under those described in this paper.

SUMMARY

The survival of *Plasmodium lophurae in vitro* is favored by the presence of calcium pantothenate (0.02 mg. per ml). Survival of about 2 weeks *in vitro* at 40–41°C. has been obtained under the following conditions: a medium consisting of duck red cell extract in balanced salt solution with glutathione and glucose or glycogen, serum, embryo extract, and calcium pantothenate; daily replacement of about half of the medium with fresh medium; addition of fresh uninfected erythrocytes every 2nd day; gentle agitation of the preparation on a rocking machine. In some of these preparations significant increases in male gametocytes and, more rarely, in total numbers of parasites occurred during the first few days.

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