STUDIES ON MALARIAL PARASITES

VIII. Factors Affecting the Growth of Plasmodium knowlesi in Vitro*

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The development of methods (1, 2) for the growth and multiplication of *P. knowlesi in vitro* permits a study of the importance of various nutritional and environmental factors in the life of this malarial parasite. Although some deductions may be made concerning the needs of parasites growing within the host, greater certainty attends observations made upon organisms grown *in vitro*. Indeed, certain questions, such as whether parasites will survive anaerobically, must perforce be answered by experimentation outside of the animal host. We will present in this paper data concerning the effect of certain environmental factors upon the growth of *P. knowlesi in vitro* and some observations on the nutritional requirements of this parasite.

Studies by previous workers have dealt mainly with the survival of parasites in *in vitro* systems where multiplication of the parasite population did not occur (see reference 2 for literature). The most careful experiments have been those of Trager (3, 4) in which survival of *P. lophurae* was observed in cultures for as long as 10 to 16 days. In these experiments, however, the death rate far exceeded the rate of reinvasion particularly after the first few days. Nevertheless, Trager obtained definite evidence of a nutritional need for pantothenic acid and glucose, and observed beneficial effects upon the addition of glutathione, fresh normal red cells, and embryo extract to the cultures. He also pointed out the toxic effects of a high oxygen tension on surviving *P. lophurae*.

Trager's studies have been repeated and, on the whole, confirmed by Hawking (5), although he did not obtain the prolonged survival reported by Trager. Hawking's cultures of erythrocytic stages of *P. gallinaceum* seldom survived for more than 3 or 4 days. The absence of multiplication of parasites even during the first day of culture paralleled Trager's results.

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The ultimate aim of the present investigation was the culture and continued subculture of the erythrocytic form of *P. knowlesi* in a medium containing only substances of known structure and purity. It has been possible to obtain normal growth, segmentation, and reinvasion in 24 hours with such a medium. However, to obtain subcultures, a small amount of normal blood or serum must be added. Under these conditions, subculture has been obtained for seven generations, the infectivity of the final generation being demonstrated by inoculation into a normal monkey.

Methods

The apparatus employed in these growth studies has been described in detail previously (1, 2). Two types of apparatus were used. In the first, termed the rocker-dilution technique, parasitized cells are suspended in the nutrient medium to be tested. In the second type, called the rocker-perfusion technique, the infected red cells are separated from the nutrient medium by a cellophane membrane. In the latter type, metabolic products are removed as they are formed and nutrients are constantly supplied across the membrane. Deficiencies in the nutritional medium are thus more readily observed with the rocker-perfusion type of apparatus, since constituents of the whole blood not supplied by the medium are rapidly diluted or removed.

Careful morphological control was maintained for all the experiments by the methods previously described (2). This control included parasite counts, differential counts of parasite stages, and evaluations of parasite condition. Morphological observations were made at the beginning and end of each experiment and, in a number of runs, at various intervals during development. In crucial experiments, sufficient parasites were counted to reduce counting errors to 10 per cent. In the majority of the experiments, the differential counts were made by direct observation of one hundred parasites.

The composition of the standard culture medium is given in Table I. In a previous publication (1), this medium was described as containing proteose peptone but not *p*-aminobenzoic acid. As will be seen later in this paper, *p*-aminobenzoic acid appears to replace completely the proteose peptone originally included in the medium. Since our aim was the preparation of an adequate nutritional medium containing only known components, proteose peptone has been replaced by *p*-aminobenzoic acid in all our later growth experiments.

The inorganic composition of the medium was the result of analyses of normal monkey plasma (7) and the pH of the medium was maintained at 7.45 ± 0.10 by equilibrating the bicarbonate-containing solution with 5 per cent CO_2–95 per cent air.

The composition of the culture medium with respect to its organic components is, in part, a result of preliminary respiratory and glycolytic studies on *P. knowlesi* (7), but chiefly the result of a consideration of general nutritional experience with other organisms. Consequently, the solution may well include substances not required for the normal growth and multiplication of the parasite.

The method of preparation of the culture medium varied according to the type of apparatus used, and the method employed for sterilization. In our earlier experiments, culture medium was sterilized by autoclaving and by Berkefeld (N) or Selas (03) filtration of some of the more unstable components which were subsequently added by syringe through a vaccine port. Subsequently, it was found that filtration of all the components through fritted glass filters (Corning ultra-fine) prevented the formation of toxic substances produced during autoclaving, and resulted in greatly improved growth and appearance of the parasites (Table II). It should be stated that the use of fritted glass filters has been a completely satisfactory method for steriliza-

TABLE I

Composition of Culture Medium

Inorganic	*gm./liter*	*Organic*	
$MgCl_2 \cdot 6H_2O$	0.203	*Group 3*	*μg./liter*
$CaCl_2 \cdot 2H_2O$	0.074	Thiamine	1000
K Cl	0.410	Niacin	1000
NaCl	5.825	Nicotinamide	1000
Na_2HPO_4	0.301	Cocarboxylase	400
$NaHCO_3$*	2.35	*d*-Ca pantothenate	500
Organic		Pyridoxine	500
Group 1		Ribose	500
Glucose	2.50	Riboflavin	500
Amino acids‡	0.056	Choline	500
Glycerol	0.25	Biotin‡	0.4
Sodium acetate $\cdot 3H_2O$	0.25	*Group 4. Purines and Pyrimidines*	
Group 2	*μg./liter*		*ug./liter*
Ascorbic acid	5000	Xanthine	250
p-Aminobenzoic acid	100	Guanine	250
		Thymine	125
		Adenine	250
		Uracil	250
		Cytosine	125

* Added as Na_2CO_3 (1.48 gm. per liter) and converted to bicarbonate by passing CO_2 through the solution.

‡ These values are the corrected figures from those which appeared in a previous paper (1).

This solution has a freezing point of -0.60 ± 0.02°C. and a final pH of 7.45 ± 0.10.

Supplementary Notes for Preparation of Culture Medium

All solutions are prepared using glass-redistilled water.

Inorganic Solution

Made up in a concentration twice that in Table I, equilibrated with CO_2 until clear, and diluted for use.

Organic Solutions

Group 1—Amino acids (other constituents of group 1 measured out as in above table and added directly): 10.6 cc. Stearns' amino acid hydrolysate (15 per cent amino acids) + 100 mg. glycine + 100 mg. histidine made up to 64 cc. with H_2O. Autoclaved 15 minutes. Keep in cold. Use 2 cc. per liter of medium.

Group 2:

Ascorbic acid. Make fresh each experiment. Add to rest of medium 1.0 cc. of a 500 mg. per cent solution per liter of medium. Sterilized with rest of medium through sintered glass filter.

p-Aminobenzoic acid. 10 mg. *p*-aminobenzoic acid made up to 100 cc. with 0.85 per cent NaCl. Autoclave 15 minutes. Use 1 cc. per liter of medium.

Group 3:

Using 0.85 per cent NaCl as diluent, make up 50 cc. containing 1000 times the concentration of each component in the final medium. Add 25 mg. per cent KH_2PO_4 to maintain an acid pH. Warm to dissolve components and store in brown bottle in cold. Make fresh about every month. Can probably be safely autoclaved for permanent storage. Use 1 cc. per liter of medium.

Group 4—Purines and pyrimidines: Using 0.85 per cent NaCl as diluent, make up 50 cc. containing 1000 times the concentration in the final medium. To dissolve, heat after adding 3 to 4 drops 10 N NaOH. Bring to neutrality with dilute HCl. Autoclave 15 minutes. Precipitate which separates in cold should be resuspended before pipetting. Use 1 cc. per liter of medium.

tion. In no case have we been able to trace the contamination of cultures to the filtration technique. The filter flasks are fitted, through the rubber stopper, with a glass tube through which the filtered medium can be equilibrated with sterile 5 per cent CO_2–95 per cent air.

With large quantities of medium, as used in the rocker-perfusion apparatus (type 2), filtration through fritted glass filters becomes too time consuming. With such quantities, the inorganic medium is autoclaved alone for 30 minutes and the other components, made up in concentrated form, are added after the inorganic solution has been thoroughly equilibrated

TABLE II

Growth in Autoclaved and Filtered Media

Perfusion apparatus (type 1)—1 cc. parasitized blood.

Reservoir contained	Parasite count per 100 R.B.C.		Fold increase	Degenerate and extracellular forms
	Initial count	24 hr. count		
				per cent
40 cc. autoclaved medium	2.2	3.4	1.5	46
40 cc. filtered medium	2.2	10.6	4.8	8

TABLE III

Composition of Concentrates

A	
Glucose	9.56 gm.
Sodium acetate	0.96 gm.
Amino acids	7.7 cc. stock solution*
Purines and pyrimidines	7.7 cc. stock solution*
PABA	3.8 cc. stock solution*
Accessory factors (Table I—group 3)	3.8 cc. stock solution*
Glycerol	0.83 gm.
Water	To make 100 cc.
B	
Na_2CO_3	14.8 gm./100 cc.
Na_2HPO_4	3.01 gm./100 cc.
Add 1 cc./100 cc. of culture medium	

* See Table I for composition.

first with pure CO_2 and then with 5 per cent CO_2–95 per cent air to give a pH of about 7.4. The composition of the concentrate is given in Table III. Thirty cc. is required to make 1 liter of medium. The concentrate is sterilized by filtration through a glass filter. One cc. of a freshly prepared 500 mg. per cent solution of vitamin C, sterilized by filtration, is added to 1 liter of final medium just before use. The compositions of the various stock solutions used in preparing the concentrate are given in Table I.

With smaller amounts (less than 250 cc.) of medium, proportionate amounts of the components given in Table I as well as vitamin C are added directly to the unsterilized salt solution and the complete medium is sterilized by filtration. Since the pH of the carbonate-buffered medium becomes considerably more alkaline during filtration due to the removal of CO_2, the

possibility exists that calcium carbonate or phosphate might precipitate in the pores of the fritted glass disc providing thereby a surface upon which some of the components present in small concentrations might be partially or completely removed (*e.g.*, biotin). For this reason, when filtration was used for sterilization, we have generally employed an inorganic diluent identical with that in Table I, but free of sodium phosphate and of sodium carbonate. These components, in a concentrated solution (Table III) are added with sterile precautions to the empty filter flask before filtration and equilibration with gas is carried out through the equilibrating tube after the rest of the medium has been filtered into the flask. This tube is clamped shut during filtration. This solution is sterilized either by filtration or autoclaving. The precipitate which may form on standing redissolves on gentle warming.

The details of setting up a growth experiment with the different types of apparatus are given in the previous paper of this series (2). When the indispensability of some portion of the medium was being studied, the over-all technique remained the same except for the omission of the component or group of components in question.

RESULTS

Data from typical 24 hour cultures of *P. knowlesi* have been presented previously (1, 2). In general, an average of about fourfold multiplication was obtained in control experiments with the parasite count increasing as much as six- to tenfold in many of the cultures. In these latter experiments, the degree of multiplication was frequently maximal on the basis of merozoite counts made on segmenters in the preceding generation. Indeed, cultures often exhibit a greater multiplication in one asexual cycle than that observed *in vivo* during the same period.

In the sections below are presented the results of experiments dealing with the effect of environmental and nutritional changes on *in vitro* growth.

Ionic Environment.—Our choice of an inorganic salt solution to serve as diluent for the other components of the medium was made on grounds quite different from those mentioned by Trager (3, 4). Although Trager's high potassium medium more nearly resembles the red cell interior, it was felt that a more fundamental necessity for good growth would be the maintenance of the host cell in as good a condition as possible. As was mentioned earlier, the composition of our inorganic solution was based on analyses of normal monkey plasma, rather than on any considerations of the erythrocytic electrolyte pattern.

In Table IV are presented data from an experiment in which the *in vitro* growth of *P. knowlesi* in the sodium-rich, "extracellular," type of medium was compared with that in a medium rich in potassium. Considerably greater multiplication took place in the "extracellular" than in the "intracellular" solution. Preliminary experiments with *P. lophurae* also indicate that with this parasite, fairly normal growth and reinvasion took place using the extracellular type of medium.

Effect of Gas Phase on Growth.—Trager found that high tensions of oxygen greatly inhibited the *in vitro* development of *P. lophurae*. In our studies with

P. knowlesi, high tensions of oxygen markedly affected the respiratory metabolism of parasitized cells (7). The effect of various tensions of oxygen on the *in vitro* growth of the parasite was therefore studied for the purpose of determining optimum conditions for cultivation. In Table V are presented data from several such experiments. These results show that high tensions of oxygen (95 per cent O_2–5 per cent CO_2) are definitely detrimental to growth and that,

TABLE IV

Comparison of in Vitro Growth of P. knowlesi in Complete Synthetic Medium Using "Extracellular" and "Intracellular" Salt Solutions as Diluents
Rocker-Dilution Technique

Medium	Parasite count per 100 R.B.C.		Fold increase
	0 hr.	22 hr.*	
"Extracellular"	1.0	8.5	8.5
"Intracellular"	1.3	4.8	3.7

* Red cells looked normal in both vessels at 22 hours.

In the high potassium medium, white cells appeared degenerated.

Inorganic diluent for high potassium medium contained the following (gm. per liter): $MgCl_2 \cdot 6H_2O$, 0.203; $CaCl_2 \cdot 2H_2O$, 0.074; $NaHCO_3$, 2.35 (added as Na_2CO_3, 1.45 gm. per liter); K_2HPO_4, 0.369; KCl, 8.95. The final solution was equilibrated with 5 per cent CO_2—95 per cent air.

TABLE V

Effect of Various Oxygen Tensions on the in Vitro Growth of P. knowlesi
Perfusion Apparatus (Type 2)

O_2 content* of gas phase	No. of experiments	Average change in parasite count in 24 hrs.	Degenerate and extracellular forms after 24 hrs.
per cent		*per cent*	*per cent*
0.37	2	+460	5
20.00	4	+310	9
95.0	2	−30	47

* In all cases 5 per cent CO_2 present. Residual gas is nitrogen.

under these conditions, a large proportion of abnormal and extracellular forms are found. At least as good multiplication takes place at very low oxygen tensions as at a level of 20 per cent, even though at 0.37 per cent O_2, respiration went on at a level only 40 per cent of that observed in air. A possibility that must be considered is that, under these more anaerobic conditions, easily oxidizable, essential nutrients are not as rapidly destroyed in the *in vitro* system. We have employed 5 per cent CO_2–95 per cent air (essentially alveolar air) as the standard gas phase in all cultivation experiments, since it approximates closely the physiological conditions encountered *in vivo*.

Subculture.—In Table VI are presented data from an experiment where subculture was carried out for six successive transfers by inoculating a fresh sample of normal monkey blood in the rocker-perfusion apparatus (type 1) with a sample of the terminal blood from the previous asexual cycle, to give an initial count of 1 to 3 per cent. It was found essential in these subcultures to include whole blood or serum in the synthetic medium. In the experiment summarized in Table VI, an equal volume of serum was added to the synthetic medium. In subsequent experiments, good subculture multiplication was obtained when as little as 5 per cent of the total nutrient medium was normal serum or whole blood. Multiplication was very poor or absent after the first generation when blood or serum was not included in the medium and a high percentage of the parasites that did survive were generally abnormal in appearance, exhibiting a

TABLE VI

Subculture of P. knowlesi
Perfusion Apparatus (Type 1)—0.5 Cc. Blood

Generation	I		II		III		IV		V and VI	
Generation time, *hrs.*	0	22	0	19	0	20	0	18	0	43
Cumulative time, *hrs.*	0	22	27	46	52	72	77	95	100	143
R.B.C. in millions	—	4.84	5.13	4.05	4.96	5.00	5.21	5.20	—	4.71
Parasite count, *per cent*	2.1	10.8	2.7	15.0	3.0	13.4	2.1	5.3	1.2	2.2
Fold increase	5.2		5.6		4.5		2.5		1.8	

subnormal cytoplasmic mass and frequently a diminished number of merozoites.

In the experiment above, the fifth and sixth asexual cycles occurred over the weekend in the same vessel without the usual reinoculation into fresh normal blood or replacement of the nutrient medium between generations. This may account in part for the considerable decrease in the degree of multiplication during this 43 hour period. When the usual dosage (6 million per kilo) of parasites from subculture VI was injected into a monkey, the level of parasitemia increased (Fig. 1) at a rate somewhat less than that observed in the usual course obtained upon direct passage of a similar dosage from animal to animal (2). In 3 to 4 days after the inoculation, the animal began to control the infection and, after a short plateau during which the red count fell severely, the animal returned to a normal condition. Subsequent direct passages of our strain of *P. knowlesi* to other animals in this lot of monkeys indicated that spontaneous control even of direct infections occurred in several cases. It seems likely therefore that the abnormal course followed by the monkey receiving sub-

cultured parasites was due to normal defense mechanisms in the host. Additional evidence in support of this conclusion was obtained upon culturing, *in vitro*, a sample of parasitized blood drawn from this monkey during the period when the parasite count in its blood had reached a nearly constant level. At the point indicated by the arrow in Fig. 1, a sample of blood was drawn, diluted

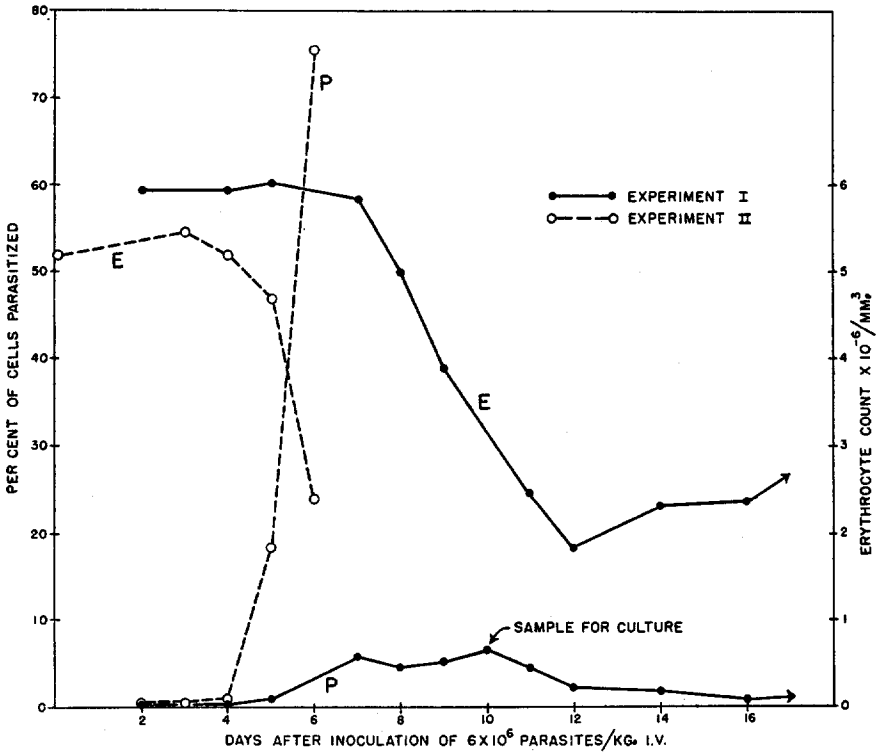

FIG. 1. The course of infection in monkey 9-4 (broken line) and monkey 7-4 (solid line) after receiving inoculations of parasites cultured *in vitro* for seven and six generations, respectively. Dosage: 6×10^6 parasites per kilo intravenously. At the point indicated by the arrow in the solid curve, a sample was withdrawn for *in vitro* cultivation as described in Table VII.

with fresh normal blood, and cultured for 24 hours by the rocker-dilution and rocker-perfusion (type 1) techniques. Complete synthetic medium was used as the source of nutrients. In both types of apparatus excellent growth resulted (Table VII), while in the monkey, a drop in parasite count was observed during the same period.

In a subsequent subculture experiment in which blood from the seventh subculture was inoculated into a monkey at a dosage of 6 million parasites per kilo,

the animal died of the infection in somewhat less than the usual time after inoculation and ran an abnormally severe course (see Fig. 1). The virulence of of the infection was retested by direct passage of a standard dose of parasitized cells from this monkey to another animal which again succumbed in less than the usual period. These results suggest that the parasites from extended *in vitro* subcultures do not decrease in virulence.

TABLE VII

In Vivo and in Vitro Growth of P. knowlesi from Monkey 7-4 during the Control of Infection in the Host

Parasite count per 100 R.B.C.	*In vitro*		*In vivo*
	Rocker-dilution	Rocker-perfusion* (type 1)	Monkey 7-4
0 hr.	1.5	2.2	5.0
24 hrs.	11.8	10.6	4.6
Fold increase	7.9	4.9	None

* The nutrient medium consisted of 20 cc. synthetic medium and 10 cc. of whole, normal monkey blood.

TABLE VIII

Stimulatory Effect of Serum and Whole Blood on in Vitro Growth in the Rocker-Perfusion Apparatus (Type 1)

	Parasite count per 100 R.B.C.		Degenerate and extracellular forms (23 hrs.). Per cent of parasites	Fold increase
	0 hr.	23 hrs.		
1. Synthetic medium 30 cc.	2.8	11.3	11.5	4.0
2. Synthetic medium 20 cc. + serum 10 cc.	2.8	17.0	3.0	6.1
3. Synthetic medium 20 cc. + whole blood 10 cc.	2.8	19.6	1.5	7.0

Stimulatory Effect of Added Blood and Serum on Growth.—As was mentioned above, successful prolonged subcultures were possible only when whole blood or serum was added to the synthetic medium. This phenomenon can be demonstrated during the first 24 hours although this stimulatory effect is generally not so striking as in later generations. Table VIII is a summary of data from an experiment using the rocker-perfusion technique (type 1) in which growth was compared in vessels containing the same synthetic medium but with the addition of serum to the second and whole blood to the third. It will be observed

that not only better multiplication takes place in vessels 2 and 3, but that a decrease in the percentage of extracellular and degenerate forms occurs, being lowest in the culture containing whole blood. This experiment makes it clear that even with the present, fairly complex medium, certain factors necessary for a maximum development of the parasites are lacking.

That these unknown factors may be present in a greater amount in normal than in parasitized blood is suggested by experiments such as those presented in Table IX. Here the bloods used for cultivation were prepared by replacing the plasma of the mixture of normal and parasitized blood (see reference 2), in one case with plasma from the parasitized monkey and in the other from a normal monkey. The poorer development in the former case may have been due to

TABLE IX

Effects of Normal and Parasitized Plasma on Growth of P. knowlesi

Rocker-dilution technique. Cells washed with two volumes of cold, modified Ringer's solution (see Table X).

Vessel	Parasite count per 100 R.B.C.		Normal parasites		Fold increase
	0 hr.	21 hrs.	0 hr.	21 hrs.	
			per cent	*per cent*	
1. Control	1.7	6.9	96	92	4.1
2. Replaced with normal plasma	1.8	6.9	96	82	3.8
3. Replaced with "parasitized" plasma	1.9	5.1	88	54	2.7
4. Replaced with heated "parasitized" plasma	1.6	6.1	92	90	3.8

the immunological effects or to nutritional deficiencies. Previous experiments (6) indicate that parasitized monkeys have very low vitamin C blood levels and that vitamin C-deficient normal monkeys are highly resistant to severe infections. This makes the nutritional interpretation a distinct possibility. That immunological effects are also involved, however, is clear upon examination of the growth in vessel 4. In this case when the parasitized plasma used for replacement was first heated at 56°C. for 30 minutes the growth was almost as good as in the control vessel.

It should be mentioned that the degree of dilution of parasitized blood with normal blood in the preparation of the blood sample for cultivation is reflected in the multiplication obtained in *in vitro* cultures. A greater proportion of normal blood added gives, in general, greater multiplication. This fact again suggests the nutrient rôle of normal blood components.

Replacement of Plasma with Albumin.—As will be described in the next section, attempts to fractionate "blocks" of nutrients (*e.g.*, vitamins, amino acids) were not successful. It was felt that this might be due, in part, to the masking effect of components in the plasma of the whole blood under study. We, therefore, performed growth experiments with cells which had been washed with a modified Ringer's solution and resuspended in complete synthetic medium containing purified serum albumin at a level of 1 to 5 per cent (kindly furnished by Dr. E. J. Cohn). Although growth approaching normal was obtained (Table X), adequate experiments to test the sensitivity of the system to omissions of

TABLE X

Growth of P. knowlesi in a Plasma-Free System
Rocker-Dilution Technique

Tube	Suspending media for cells	Parasite count per 100 R.B.C.		Fold increase	Degenerate and extracellular forms
		0 hrs.	24 hrs.		
					per cent
1	Synthetic medium + plasma	1.3	5.4	4.2	2
2	Synthetic medium alone	1.0	2.6	2.6	2
3	Synthetic medium + 1 per cent serum albumin	1.0	6.3	6.3	9
4	Synthetic medium + 5 per cent serum albumin	1.0	2.0	2.0	23

Composition of modified Ringer's solution used for washing cells: NaCl, 0.910 per cent; KCl, 0.030 per cent; $CaCl_2$ (anhydrous), 0.020 per cent; $MgCl_2 \cdot 6H_2O$, 0.010 per cent; glucose, 0.100 per cent.

single components of the various blocks of nutrients have not been performed. To achieve this end, it may be necessary to attempt cultivation experiments on "naked" parasites where the red cell host cell is ruled out as a source of nutrient materials. Preliminary trials indicate, however, that the plasma replacement technique may furnish the required sensitivity.

In the experiment summarized in Table X, whole parasitized blood was added directly to the synthetic medium in tube 1. For the other tubes, whole blood was first centrifuged, the plasma withdrawn, the cells washed once with ice cold modified Ringer's solution, centrifuged, and resuspended in the appropriate medium listed in the table. The results indicate that growth and multiplication are supported but poorly in a protein-free medium (tube 2). The addition of purified serum albumin to give a concentration of 1 per cent greatly enhances

growth (tube 3). Higher concentrations of serum albumin (5 per cent) appear to have a deleterious effect. This effect has been shown to be due to the presence in some samples of albumin of a toxic material which is dialyzable. This toxic material may be the stabilizer, acetyl tryptophane, used in some of the albumin samples.

The improved growth in the plasma-free culture (Table X) over that in the control tube is not frequently observed. The albumin does not appear to act as a nutritional factor but rather in a physical manner. Thus, in the usual experiment of this type, growth in plasma-free cultures is about the same as, or slightly poorer than, in control cultures. In some experiments, when somewhat poorer growth was obtained in plasma-free cultures, growth could be returned to normal by the addition of a small amount of whole blood to the medium. To

TABLE XI

The Effect of Substituting Glycerol for Glucose on in Vitro Growth Rocker-Dilution Technique

Medium	Parasite count per 100 R.B.C.		Fold increase	Degenerate and extracellular forms
	0 hr.	22 hrs.		
				per cent
1. Complete, synthetic medium; glucose 250 mg. per cent, glycerol 25 mg. per cent	1.2	4.5	3.8	11
2. Same as (1) without glucose. Glycerol 155 mg. per cent	1.0	0.3	—	80

this extent, at least the sensitivity of the system is increased by the plasma replacement procedure. The technique may be useful in determining factors in plasma or whole blood, over and above those present in the synthetic medium, which are essential for the parasite nutrition. Efforts in this direction are being continued.

Nutritional Requirements of P. knowlesi Grown in Vitro.—Preliminary studies on the utilization of substrates by the parasite (7) indicated that glucose and glycerol were both oxidized, the latter more rapidly. Consequently, these substances were included in the nutrient medium. We have found, however, that when, of these two, only glycerol was included in the medium, no growth resulted (Table XI), whereas with both glucose and glycerol, normal growth took place. In this experiment, the parasitized blood used as the initial sample was gently centrifuged, the plasma removed, and the cells resuspended in complete synthetic medium modified as described in the table, containing 1 per cent crystalline human serum albumin. A preliminary washing with modified

Ringer's solution (see Table X) at 4–8°C. was carried out before resuspension of the cells in the albumin solution.

It appears likely that the parasite can develop normally using glucose as its only carbohydrate source. Similar experiments have shown that the absence of sodium acetate does not materially affect multiplication when glucose is supplied to satisfy the calorific requirements of the parasite.

Although some growth and multiplication are supported on a medium consisting simply of glucose, *p*-aminobenzoic acid, and an isotonic salt solution, maximum development occurs only when the complete synthetic medium is employed. Attempts have been made to fractionate the complete medium into essential and non-essential components without success. It is even difficult to show the effect of various blocks of nutrients on growth. In Table XII are shown the results of a typical experiment of this sort. Here the addition of

TABLE XII

Effects of Nutrients on Growth
Rocker-Perfusion Apparatus (Type 1)

Tube	Glucose and *p*-aminobenzoic acid	Vitamins	Purines	Pyrimidines	Amino acids	Fold increase 24 hrs.
1	+					3.8
2	+	+				3.5
3	+	+	+			4.2
4	+	+		+		4.2
5	+	+	+	+	+	6.9

vitamins or vitamins plus purines or pyrimidines to the basic medium causes but little improvement in the quantitative aspects of growth. When all the components are present, the multiplication of parasites is markedly increased. The same results are obtained if the order of addition of the various blocks is changed. For example, the addition of amino acids alone or in combination with one other group shows but little stimulation of growth. Purines and pyrimidines when added together seem to produce the best response of any combination of two blocks of nutrients. However, as was mentioned above, we have not been able to fractionate these mixtures further into essential and non-essential components. Table XIII gives data taken from a 22 hour growth experiment using the rocker-perfusion apparatus (type 2) demonstrating the need for purines and pyrimidines.

Similar experiments have demonstrated that the mixtures of vitamins and amino acids used in our synthetic medium are also important for normal growth and multiplication. The need for vitamin C for the growth of parasites *in vivo* has been presented elsewhere (6).

p-Aminobenzoic Acid.—In a previous paper (1), it was stated that proteose peptone was an essential component of the medium. It was subsequently established that *p*-aminobenzoic acid (PABA) was, if not the only, at least the chief component furnished by the peptone (Table XIV). The media used in this experiment were identical with the regular synthetic medium except for the changes noted in the table.

When higher levels of PABA are used (greater than 1000 γ per cent), inhibition of growth begins to appear.

TABLE XIII

The Effect of Purines and Pyrimidines on Growth
Rocker-Perfusion Apparatus (Type 2)

Vessel	Parasite count per 100 R.B.C.		Fold increase	Degenerate and extracellular forms
	0 hr.	22 hrs.		
				per cent
1. Complete synthetic medium	6.0	20.4	3.4	14
2. Same as (1) without purines and pyrimidines	6.0	8.4	1.4	29

TABLE XIV

The Effect of p-Aminobenzoic Acid on Growth
Rocker-Dilution Technique

Tube	Proteose peptone	PABA	Parasite count per 100 R.B.C.		Increase
			0 hr.	24 hrs.	
					per cent
1	150 mg. per cent	0	1.7	11.7	690
2	0	1 γ per cent	1.5	11.8	790
3	0	10 γ per cent	1.6	12.8	800
4	0	0	2.4	7.0	290

Other Factors Tested.—In addition to the nutrients specifically mentioned in the preceding pages, we have tested a number of other substances for possible beneficial effects in the *in vitro* system. These included folic acid, inositol, glucosamine, trace inorganic ions including nickel, cobalt, iron, copper, and zinc, high-lipid and low-lipid containing fractions of plasma proteins, and supplementary additions of amino acids in addition to those already present in the medium. All these additions were without effect on the *in vitro* growth of *P. knowlesi*. Here, again, the failure to observe effects on growth may be due to the fact that the parasites were already multiplying at nearly the maximum rate (as judged by merozoite counts) even in a plasma-free system.

To detect significant effects on growth from minor omissions in the nutrient medium, it may be necessary to combine albumin replacement of plasma with subculture methods. Thus, cumulative deficiencies may become evident in the second and third *in vitro* generations which are impossible to detect in the first.

SUMMARY

1. Methods have been described for the preparation and sterilization of a synthetic nutrient medium which supports normal growth during one 24 hour asexual cycle of the erythrocytic form of *P. knowlesi*.

2. Successive subcultures with good multiplication can be carried out when whole blood or plasma is added to the medium. Data are presented from two such experiments where the sixth and seventh generations, respectively, were injected into normal monkeys. In both cases, the injections produced clinical malaria and in one, an abnormally severe course was observed.

3. A high percentage (95 per cent) of O_2 inhibits multiplication in *in vitro* cultures. A gas phase approximating alveolar air permits normal development. A lower percentage (0.37 per cent) of O_2 in the gas phase supports growth at least as well.

4. Certain experiments indicate that *in vitro* growth is better supported in normal monkey plasma than in plasma from parasitized monkeys. Heating of parasitized plasma to 56°C. for 30 minutes removed the deleterious effect.

5. A nutritional need by the parasite for glucose and *p*-aminobenzoic acid has been shown. Attempts to demonstrate the need for other individual components of the nutrient medium have not been successful though the effect of the absence of blocks of nutrient such as purines and pyrimidines, amino acids, or water-soluble vitamins can be observed.

6. Further assay of the nutritional requirements of the parasite appears to be hampered by the supply of nutrient furnished by the red cells and plasma. Cultivation of parasitized cells washed free of plasma and resuspended in synthetic medium containing 1 per cent serum albumin has been accomplished as one step directed toward the removal of such interfering factors.

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