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Malaria: Extracellular Amino Acid Requirements for *in Vitro* Growth of Erythrocytic Forms of *Plasmodium knowlesi** (32666)

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Previous investigations showed that *dl*-methionine was essential for both *in vivo* and *in vitro* growth and multiplication of erythrocytic forms of *Plasmodium knowlesi* (3,4). The growth medium of those *in vitro* experiments contained monkey serum which could supply other amino acids required for plasmodial growth. In the present paper the amino acid requirements of *P. knowlesi* were investigated further by studying plasmodial growth in media depleted of various amino acids.

Materials and Methods. The preparation of plasmodial cultures, described previously (5), was modified as follows:

Parasites. Blood was obtained from Rhesus monkeys (*Macaca mulatta*), infected with *P. knowlesi* by blood passage, when the parasitemia had reached 10–30% rings or trophozoites. The number of parasitized cells was reduced to 10% by mixing with uninfected monkey erythrocytes, when the parasitemia was higher than 10%. The erythrocytes were washed three times in 10 volumes of ice-cold Hanks' salt solution containing 100 mg/100 ml glucose. Each culture contained 11 ml of medium and 0.03 ml packed parasitized erythrocytes. Cultures were incubated at 37°C for 18–20 hours until the majority of the parasites of the control cultures had developed to immature schizonts and a few segmenters. Each culture was made in duplicate and all media were tested for parasitic growth in one experiment, repeated six times.

Medium. Thirteen lots of Eagle's basal medium (1) were made, differing from each other only in that one of the following 13 amino acids was missing: *l*-cystine, *l*-arginine, *l*-tryptophan, *l*-histidine, *l*-leucine, *l*-isoleucine, *l*-lysine, *l*-methionine, *l*-glutamine, *l*-tyrosine, *l*-threonine, *l*-valine, *l*-phenylalanine. A fourteenth medium containing all of the mentioned amino acids served as a control. All media contained 0.2 m*M* of each of the following amino acids: *l*-alanine, *l*-aspartic acid, *l*-glutamic acid, *l*-proline, *l*-serine, and glycine.[1] The media were supplemented with 10% fresh human AB serum, dialysed against saline until free from amino acids as tested by complete disappearance of *l*-leucine-^{14}C, *l*-isoleucine-^{14}C, or *l*-histidine-^{14}C added to the serum prior to dialysis.

Determination of DNA. In a second set of three experiments, 1 μC orotic-6-^{14}C acid[2] hydrate (specific activity 3.88 mC/mmole) was added to each culture at the onset of incubation and DNA was extracted at the

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[1] Nutritional Biochemicals Corp., Cleveland, Ohio and Sigma Chemical Co., St. Louis, Mo.

[2] New England Nuclear Corp., Boston, Mass.

end of incubation by the method of Smith *et al.* (2) as follows:

Cells were dissolved in a solution of 0.05% lauryl sulfate in water at 3°C, 0.1 mg carrier DNA per culture was added and trichloracetic acid (TCA) was added to a final concentration of 10%. The cell fragments were centrifuged to a pellet at 46,000*g* for 30 min at 3°C. The pellet was washed twice with 10% TCA, twice with ethanol, twice with a 3:1 mixture of ethanol and ether. RNA was extracted with 0.3 *N* KOH at 37°C for 1 hour, followed by precipitation of the cell rests in 5% TCA at 46,000*g* for 30 min at 3°C. The pellet was washed once with 5% TCA. All solutions used had a temperature of 3°C or less. The pellet was dissolved in 0.1 *N* NaOH plated in duplicate, dried and counted to at least 1000 counts in a low background gas flow counter.[3]

Results. After ring forms or trophozoites had been incubated for 18–20 hours, immature schizonts and segmenters appeared in all cultures except in cultures where *l*-isoleucine had been deleted from the growth medium. In the latter cultures all of the parasites failed to grow beyond the ring or trophozoite stage in all experiments performed.

Using the number of immature schizonts and segmenters present at the end of incubation as a parameter of growth in the cultures missing one of the other amino acids tested, it was found that the results varied from one experiment to the other. Therefore, the amount of orotic-6-^{14}C acid incorporated into plasmodial DNA during an 18-hour incubation period was used as a measure of growth. The results, illustrated in Table I, show that orotic-6-^{14}C acid incorporation into DNA during an 18-hour incubation period was significantly inhibited in the absence of *l*-isoleucine, *l*-methionine, *l*-cystine, *l*-tyrosine, *l*-arginine, *l*-glutamine, *l*-histidine or *l*-lysine; this inhibition was particularly marked in the absence of the first three amino acids.

Orotic-6-^{14}C acid incorporation into DNA in the absence of *l*-threonine, *l*-phenylalanine, *l*-leucine, *l*-tryptophan, and *l*-valine was not significantly different from the control. Initial experiments had shown that *l*-alanine, *l*-aspartic acid, *l*-glutamic acid, *l*-proline, *l*-serine, and glycine had no effect on parasitic growth.

[3] Model 4342, Nuclear Chicago Corp., Des Plaines, Ill.

TABLE I. Comparison of the Growth Rate of *P. knowlesi* in Culture Media Depleted of Various Amino Acids and the Number of Moles of These Amino Acids in Adult Hemoglobin.

Amino acid depleted from culture media	Orotic-6-^{14}C acid incorporated into DNA of *P. knowlesi*[a]	No. of moles of amino acid per mole of adult human hemoglobin (7)
Control	100 ± 13	
Lysine[b]	88 ± 11	44
Histidine[b]	87 ± 5	38
Glutamine[b]	87 ± 7	30
Arginine[b]	84 ± 8	12
Tyrosine[b]	83 ± 8	12
Cystine[c]	72 ± 13	6
Methionine[c]	54 ± 10	6
Isoleucine[c]	9 ± 4	0

Note.—Comparison among the means was performed by a modification of the method of J. W. Tukey (6). Net counts of radioactivity were used for statistical comparison. The amino acid composition of adult human hemoglobin is given because of its availability. The composition of monkey hemoglobin is believed to be similar (8).

[a] Data are expressed as percentage of the control ± SD. Values are the average of three experiments with duplicate analyses.

[b] Denotes a value which is significantly different from the control value at the 5% level.

[c] Denotes values which are significantly different from the control values at the 1% level.

Discussion. The failure of ring forms or trophozoites to develop in the absence of *l*-isoleucine indicates that this amino acid is essential for growth of erythrocytic forms of *P. knowlesi.*

In the absence of *l*-methionine growth of *P. knowlesi* is reduced to 54%. This is in accord with the findings of McKee and Geiman who showed that *P. knowlesi* requires an extracellular source of methionine for *in vitro* (3) as well as *in vivo* (4) growth.

Significant inhibition of growth of *P. knowlesi* in the absence of *l*-cystine, *l*-tyrosine, *l*-arginine, *l*-glutamine, *l*-histidine, or *l*-lysine suggests that *P. knowlesi* requires an extracellular source of these amino acids for opti-

mal *in vitro* development during one schizogonic cycle.

Finally, when the degree of inhibition of DNA synthesis in the absence of the amino acids, which had a significant effect on parasitic growth, is correlated with the molar amounts of the same amino acids in adult human hemoglobin (7), then it can be seen that inhibition of DNA synthesis is most marked in the absence of these amino acids of which hemoglobin contains the least (Table I). The amino acid composition of human rather than monkey hemoglobin is compared because of its availability. The composition of monkey hemoglobin is believed to be similar (8).

Summary. *l*-Isoleucine and *l*-methionine are essential for growth of erythrocytic forms of *P. knowlesi.* An exoerythrocytic source of *l*-cystine, *l*-tyrosine, *l*-arginine, *l*-glutamine, *l*-histidine, and *l*-lysine is required for optimal *in vitro* development of *P. knowlesi* during one schizogonic cycle.

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Relationship of Chemical Structure to Hemodynamic Properties of Endotoxins* (32667)

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Circulatory changes terminating in irreversible shock represent one of the most typical effects of intravenously administered bacterial endotoxins in mammals (1,2). Hemodynamic alterations are characterized primarily by a marked drop in systemic arterial blood pressure and a concomitant increase in portal vein pressure. In the dog, these changes seem to result from pooling of blood in the liver and diminished venous return to the heart (3).

The unusual variety of endotoxic activities and reactions (4) has stimulated attempts to correlate the chemical structure and biological properties of endotoxins isolated from various gram-negative microorganisms (5,6,7). It appears from such studies that the toxic properties of intact endotoxin preparations depend upon certain minimal chemical and physical–chemical requirements (8,9,10).

Recent observations by Johnson and Nowotny (11) that not all biological activities of endotoxins are related to each other are of considerable interest and suggest the possibility of several chemically active centers in the macromolecule. Studies correlating specifically the hemodynamic effects and chemical composition of endotoxins have not been reported. On the assumption that, for the elicitation of endotoxic reactions, active preparations of a certain critical size contain a large polysaccharide moiety and smaller lipid and peptide components, procedures have been developed in this laboratory (12) for the sepa-

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