Biosynthesis of Amino Acids from ^{14}C-U Glucose, Pyruvate, and Acetate by Erythrocytic Forms of *P. knowlesi, in Vitro** (34073)

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Previous investigations have shown that erythrocytic forms of malaria plasmodia use two sources of amino acids for nutrition (1): amino acids derived from digestion of host cell hemoglobin (2, 3) and extracellular amino acids (4–6). A third possible source, amino acids synthesized from glucose was investigated in the present report, by growing erythrocytic forms of *P. knowlesi in vitro* in the presence of uniformly ^{14}C-labeled glucose, pyruvate, or acetate and measuring the radioactivity of the amino acids of digested plasmodial protein.

Materials and Methods. Growth of plasmodia in ^{14}C-labeled substrates. Cultivation of erythrocytic forms of *P. knowlesi* for one schizogonic cycle, described previously (7), was modified only in that 4-oz Teflon bottles[1] were used instead of 12-ml culture tubes. The former contained 11 times more medium and cells than the latter. Parasitized cells (0.33 ml of packed cells per bottle) were incubated for 12–16 hr in the presence of 200 μCi glucose ^{14}C-U (specific activity 307 mCi/mmoles), 50 μCi sodium pyruvate-^{14}C-U (specific activity 16.6 mCi/moles) or 50 μCi sodium acetate-^{14}C-U (specific activity 32.9 mCi/mmole)[2] per bottle, added at time zero. Teflon bottles containing nonparasitized (1 ml of packed cells per bottle) cells harvested from monkeys with similar white blood cell counts as the infected monkeys[3] (10,000–13,000/mm^3) were incubated simultaneously. The growth medium contained all of the amino acids, including the nonessential ones, of Eagle's medium (8). Growth of plasmodia in the absence of amino acids would have been most appropriate for our study. However axenic cultures of plasmodia are not available at the present. Furthermore, plasmodia grown in media depleted of amino acids derive amino acids for nutrition from digested hemoglobin of the erythrocytic host (2,3).

At the end of incubation the cells were collected by centrifugation, washed once with saline and incubated for 1 hr at 37° in 5 ml of 0.9% NaCl and 0.1% saponine (in 12-ml polypropylene tubes) to lyse the erythrocytes. The free plasmodia, leukocytes, and red cell rests were spun to a pellet for 20 min at 46,000*g*, the supernatant portion was discarded and the pellet was washed with saline. A Giemsa-stained smear of the pellet showed a sheet of free plasmodia with a few leukocytes. Most, but not all of the white cells could be removed by passing the saponized cell suspension through a Millipore filter of 8-μ pore size with gentle to moderate pressure. The pellet was then washed with a mixture of 100 vol acetone and 1 vol 1 *N* HCl to remove the pigment (1). Protein was isolated as described previously (7) and dried. One bottle of parasitized cells contained 2–5 mg protein and one bottle of nonparasitized cells 0.2–0.7 mg. The pellet was then digested and prepared for amino acid analysis as described by Moore and Stein (9).

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[1] Nalge Corp., Rochester, N. Y. Catalog No. 1600.

[2] Amersham/Searle Corp., Des Plaines, Ill.

[3] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Quantitative and radioactive assay of the amino acids. An aliquot of each sample was used for assay by the ion-exchange chromatographic method of Moore and Stein (10) and Spackman *et al.* (11), with modifications, some of which were published by Rosen *et al.* (12). The present study utilized "Technicon" photometers with a "Bristol" three-point recorder and a Technicon Autograd gradient device for measurement of amino acids. A single column (140 × 0.9 cm) of ion-exchange resin was used and required two buffers: (1) 0.2 *M* sodium citrate plus citric acid, adjusted with concentrated HCl to a pH of 3.41; (2) 0.58 *M* sodium citrate–sodium hydroxide with a pH of 6.30. A longer flow cell system was used and required only one-half of the cyanide concentration (11). The ninhydrin reagent required less buffering than that previously described for gradient elution (3) and was prepared as follows: 40 g of sodium propionate and 40 ml of propionic acid brought to a volume of 200 ml with water. To this was added 800 ml of ethylene glycol monomethyl ether and 10 g of ninhydrin. Each assay was performed at a buffer and ninhydrin pumping speed of 55 ml/hr and 30 ml/hr respectively, and required 17 hr.

To quantify the individual amino acids, the chromatograms of the samples were reproduced with a dry-copy duplicating machine on copy paper of uniform weight per unit area. The individual amino acid peaks were carefully cut out of the reproduced chromatogram and weighed. The weights of the various peaks were then compared with those of similarly reproduced chromatograms of known quantities of amino acids. The amino acids found to be present in concentrations of 3 μmoles or less per 100 ml of sample could not be quantified with sufficient precision by our assay system.

In the ninhydrin reaction the COOH group of the amino acids is split off as CO_2. To measure the radioactivity of the amino acids, the eluent corresponding to each amino acid peak on the chromatogram was collected upon leaving the colorimeter in a stoppered flask. A 1-ml beaker, containing 0.2 ml hyamine hydroxide to trap $^{14}CO_2$, was attached to the stopper with a steel wire. The hyamine hydroxide and 1 ml of the eluent were counted for at least 1000 counts using the method of liquid scintillation counting as described previously (7). Neither hyamine hydroxide nor the eluent caused significant quenching of radioactivity.

The $^{14}CO_2$ formed in the ninhydrin reaction was found to escape through the wall of the Teflon tubing, used to lead the reaction mixture through the hot water bath of the analyzer. Therefore, Teflon was replaced by glass tubing. Formation of gas bubbles in the glass tubing deformed the tracings of the chromatograph recorder, yielding inaccurate quantification of the amino acids. Therefore, each sample was analyzed twice: once with Teflon tubing for measurement of the amino acids and once with glass tubing for measurement of radioactivity in the eluent (using 0.5 mg of digested protein).

Results. Analysis of protein of parasitized cultures showed that protein of *P. knowlesi* contains most of the usual amino acids. No unusual amino acids were found, as has been reported previously by Fulton and Grant (1). We did not examine the eluent for cystine, proline, hydroxyproline, and tryptophane. The plasmodial protein was not pure; it contained small amounts of white blood cell protein, hemoglobin, and malaria pigment.

Assay for radioactivity of the amino acids from digested protein isolated from parasitized blood cell cultures incubated in the presence of ^{14}C uniformly labeled glucose or pyruvate showed ^{14}C labeling of three amino acids; *i.e.*, aspartic acid, glutamic acid, and alanine (Table I). Only aspartic acid and glutamic acid were labeled in plasmodia grown in the presence of ^{14}C-U-acetate. No radioactivity was found in threonine, serine, glycine, valine, methionine, isoleucine, leucine, tyrosine, phynylalanine, histidine, lysine, and arginine. No ^{14}C was detected in the eluent collected between the amino acid peaks of the chromatogram.

The analyzed protein from parasitized cultures contained small amounts of white blood cell protein. Protein isolated from non-parasitized blood cells incubated with the

TABLE I. Radioactive Assay (expressed in counts/minute) of the Eluent Fractions and Their $^{14}CO_2$ Content from the Amino Acid Analyzer, Containing Aspartic Acid, Glutamic Acid, and Alanine.[a]

^{14}C-U precursor	Parasitized cells			Nonparasitized cells		
	Aspartic acid	Glutamic acid	Alanine	Aspartic acid	Glutamic acid	Alanine
Expt. 1						
Glucose						
Eluent	1616	5500	286	24	125	20
CO_2	55	43	10	2	12	—
Pyruvate						
Eluent	800	1320	531	210	1392	105
CO_2	35	20	2	18	21	1
Acetate						
Eluent	418	680	2	3472	16,284	240
CO_2	34	12	—	208	447	20
Expt. 2						
Glucose						
Eluent	550	2079	152	—	—	—
CO_2	23	35	5	—	—	—
Pyruvate						
Eluent	169	756	675	105	184	180
CO_2	10	15	23	44	5	10
Acetate						
Eluent	1092	5073	—	780	2250	140
CO_2	47	70	—	29	66	6

[a] Blood cells infected with *P. knowlesi* and nonparasitized blood cells were incubated in the presence of glucose-^{14}C-U, pyruvate-^{14}C-U, or acetate-^{14}C-U. Protein of parasitized and nonparasitized cultures was isolated and digested. The amino acids of 0.5 mg of protein of both cultures were quantified on an automatic amino acid analyzer. The eluent corresponding to each amino acid peak of the chromatogram was collected separately and assayed for ^{14}C after $^{14}CO_2$ had been collected and determined as described in Methods. Radioactivity was found only in aspartic acid, glutamic acid, and alanine from 16 amino acids examined.

same ^{14}C-labeled substrates also contained ^{14}C-labeled aspartic acid, glutamic acid, and alanine. In one experiment (Table I, Expt. 2), the leukocyte protein contaminating the plasmodial protein was greatly reduced. Subsequent analysis showed that removal of most of the leukocytes did not significantly reduce the amount of ^{14}C-labeled amino acids. It was further found that protein of parasitized cultures incorporated as much as three times more ^{14}C-U glucose, pyruvate, or acetate than protein isolated from a similar volume of nonparasitized cells. Parasitized and nonparasitized blood contained similar numbers of white blood cells (7). The data in Table I cannot be used to determine the relative amounts of the three ^{14}C-labeled amino acids in plasmodial and white blood cell protein of parasitized cultures, since the data of Table I were obtained from similar weights of protein from parasitized and nonparasitized cultures. We were unable to determine the weight of leukocyte protein present in parasitized cultures.

Discussion. The result, that the same amino acids (aspartic acid, glutamic acid, and alanine) became labeled in the protein of parasitized and nonparasitized blood cells indicated that either both the white blood cells and the plasmodia or the white cells alone synthesized the three amino acids from ^{14}C-labeled glucose, pyruvate, and acetate. The

observation that protein from parasitized blood cells incorporated three times more ^{14}C-labeled glucose, pyruvate, and acetate than protein from an equal volume of non-parasitized blood cells suggested, but did not prove conclusively, that both plasmodia and white blood cells form the three amino acids from the three precursors used. The latter conclusion was supported by the observation that separation of most of the leukocytes from the parasites did not significantly reduce the amount of ^{14}C-labeled aspartic acid, glutamic acid, and alanine in parasite protein (Table I, Expt. 2).

Our experiments suggested that in the presence of extracellular amino acids *P. knowlesi* formed only three amino acids from glucose, pyruvate, and acetate during growth for one intraerythrocytic cycle; *i.e.,* aspartic acid, glutamic acid, and alanine. This indicates that during malaria infection the rapidly growing plasmodium feeds almost exclusively on the amino acids of the host; *i.e.,* the host cell hemoglobin (2, 3) and the serum amino acids (4). That *P. knowlesi* did not synthesize most amino acids in the presence of extracellular amino acids does not prove that the parasite entirely lack the capability to do so, because the extracellular amino acids may inhibit their synthesis from glucose by feedback inhibition or enzyme repression. Further elucidation of the biosynthetic capability of malaria plasmodia would require plasmodial growth in a chemically defined medium free from the erythrocyte, a tool not available at the present.

Summary. Erythrocytic forms of *P. knowlesi* were grown for one or part of one schizogonic cycle in a medium containing ^{14}C-U glucose, pyruvate, or acetate. Radioactive measurement of the amino acids of digested parasitic protein revealed ^{14}C in aspartic acid, glutamic acid, and alanine. Both plasmodia and leukocytes were found to synthesize the three amino acids from the used precursors. No radioactivity was found in threonine, serine, glycine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine.

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