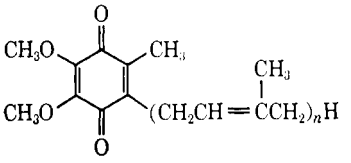
Nishimura, S., Harada, F., Narushima, V., and Seno, T. (1967), *Biochim. Biophys. Acta 142*, 133.
Ofengand, E. J., Dieckmann, M., and Berg, P. (1961), *J. Biol. Chem. 236*, 1741.
Otaka, E., and Osawa, S. (1960), *Nature 185*, 921.
Petrovic, J., and Petrovic, S. (1966), *Anal. Biochem. 15*, 187.
Rajbhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoshinson, R. M. and Khorana, H. G. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 751.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
Singer, B., and Fraenkel-Conrat, H. (1962), *Biochemistry 1*, 852.
Spirin, A. S. (1963), *Progr. Nucleic Acid Res. 1*, 301.
Sueoka, N., and Cheng, T. Y. (1962), *J. Mol. Biol. 4*, 161.
Tissiéres, A. (1959), *J. Mol. Biol. 1*, 365.
Wacker, W. E. C., Gordon, M. P., and Huff, W. (1963), *Biochemistry 2*, 716.
Wacker, W. E. C., and Vallee, B. L. (1959), *J. Biol. Chem. 234*, 3257.
Watson, J. D., and Ralph, R. K. (1966), *J. Mol. Biol. 22*, 67.
Wicks, W. D., Greenman, D. L., and Kenney, F. T. (1965), *J. Biol. Chem. 240*, 4417.
Zachau, H., Dutting, D., and Feldman, H. (1966), *Angew. Chem. 78*, 392.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Biosynthesis of Ubiquinones by Malarial Parasites. I. Isolation of [^{14}C]Ubiquinones from Cultures of Rhesus Monkey Blood Infected with *Plasmodium knowlesi**

Frederick S. Skelton,† Kenneth D. Lunan, Karl Folkers,† Jerome V. Schnell, Wasim A. Siddiqui, and Quentin M. Geiman

ABSTRACT: It has been demonstrated that *Plasmodium knowlesi* biosynthesizes [^{14}C]ubiquinones-8, -9, and perhaps -7 from [^{14}C]*p*-hydroxybenzoic acid by the isolation of these ubiquinones from parasitized (*P. knowlesi*) rhesus monkey blood cells cultured in synthetic medium containing [^{14}C]*p*-hydroxybenzoic acid. Only [^{14}C]ubiquinone-10 was isolated from control cultures of unparasitized rhesus monkey blood cells and was shown to be associated with the leucocytes.

The occurrence of ubiquinones-8 and -9 (I, n = 8 and 9, respectively) in duck blood infected with *Plasmodium lophurae*, in contrast to only ubiquinone-10 (I, n = 10) in normal duck blood, has been reported (Rietz *et al.*, 1967). A similar analysis (F. S. Skelton, P. J. Rietz, and K. Folkers, 1968, unpublished data) performed on rhesus monkey blood infected with either *Plasmodium knowlesi* or *Plasmodium cynomolgi* also showed only ubiquinone-10 in the normal blood and ubiquinones-8 and -9 in the infected blood.



I

p-Hydroxybenzoic acid has been found to be a biosynthetic precursor of ubiquinone in a variety of microorganisms and animals (Rudney and Parson, 1963; Parson and Rudney, 1964; Olson *et al.*, 1963; Aiyar and Olson, 1964). We now report the incorporation of [^{14}C]*p*-hydroxybenzoic acid (uniformally labeled) into ubiquinones-8 and -9 by parasitized (*P. knowlesi*) rhesus monkey erythrocytes cultured *in vitro* using the rocker dilution technique (Geiman *et al.*, 1946). Only [^{14}C]ubiquinone-10 was identified in the normal blood cells, and is believed to be associated with the leucocytes.

* From the Stanford Research Institute, Menlo Park, California 94025 (F. S. S., K. D. L., and K. F.) and the Department of Preventive Medicine, Stanford University School of Medicine, Stanford, California 94305 (J. V. S., W. A. S., Q. M. G.). *Received October 3, 1968.* Coenzyme Q. CXII. This investigation was supported by U. S. Army Medical Research and Development Command Contracts DA-49-193-MD-2587 and DA-49-193-MD-2784. This is Contribution No. 461 from the Army Research Program on Malaria.

† Present address: Institute for Biomedical Research, The University of Texas, Austin, Texas 78712.

Experimental Section

In Vitro Culture Methods. The blood samples were drawn from uninfected rhesus monkeys (*Macaca mulatta*) and rhesus monkeys infected with *P. knowlesi*. *P. knowlesi*[1] was maintained by weekly intravenous inoculation of erythrocytic stages into rhesus monkeys. The techniques for maintaining, counting, and evaluating the parasites were based on those described (Geiman *et al.*, 1946). Cultures were maintained under bacterial free conditions in all experiments.

The incorporation of [^{14}C]*p*-hydroxybenzoic acid into ubiquinones was measured under conditions designed for the intracellular *in vitro* cultivation of malarial) parasites (Geiman *et al.*, 1946). The synthetic medium was based upon that described by Anfinsen *et al.* (1946), with modifications according to Geiman *et al.* (1966). Stearic acid was used to replace plasma (Siddiqui *et al.*, 1967).

Biosynthesis of [^{14}C]*Ubiquinones from* [^{14}C]*p-Hydroxybenzoic Acid by Parasitized Blood Cells.* The infected blood samples (1.5 ml of blood cells in 9 ml of medium, and the corresponding control samples (containing no parasites) were incubated (in duplicate) together at 37° for a specified length of time. [^{14}C]*p*-Hydroxybenzoic acid (Parson and Rudney, 1964) (9.0×10^4 cpm sample) was added at the start of incubation. At the end of the specified incubation period, the cells from each sample were isolated by centrifugation, washed with modified Ringer's solution,[2] and frozen in a Dry Ice–acetone bath. These cells were stored frozen until they were processed.

Biosynthesis of [^{14}C]*Ubiquinone, from* [^{14}C]*p-Hydroxybenzoic Acid, by Normal Blood Cells.* After incubation with [^{14}C]*p*-hydroxybenzoic acid, three duplicate normal blood samples were centrifuged at 500 rpm for 10 min. The supernatant was removed and the packed cells were washed twice with modified Ringer's solution. The upper three-quarters and the lower one-quarter of the packed cells were separated and the two fractions were then frozen until they were processed.

Isolation of the Ubiquinones from Blood Cells. The frozen blood cells were lyophilized and then extracted for 9 hr with 25 ml of *n*-hexane. The solid material was filtered, and the hexane extract was evaporated to dryness *in vacuo*. Unlabeled ubiquinone-10 (140 μg) was added to the residue as a carrier, and the mixture was twice chromatographed (multiple pass) on thin-layer, silica gel G plates[3] using ethyl ether–*n*-hexane (10:90, v/v), as the solvent. The ubiquinone area adjacent to a ubiquinone-10 reference was scraped from the plate and eluted with ether. After evaporation of the ether, the radioactivity in the residue was measured using a Nuclear-Chicago liquid scintillation spectrophotometer.

Identification of the Ubiquinone(s). Appropriate reference ubiquinones and the residue containing the isolated ubiquinones with the ubiquinone-10 carrier were applied to a Whatman No. 3MM chromatographic paper impregnated with Dow Corning No. 550 silicone oil. Reversed-phase paper chromatography in *n*-propyl alcohol–water (70:30, v v), which will separate ubiquinones with isoprenyl side chain lengths 10–7 (I, $n = 7$), was performed.

Since ubiquinones-10 and -8 are the predominant ubiquinones found in parasitized blood samples, the ubiquinone-9 and -7 areas were rechromatographed in order to determine if these ubiquinones were actually present or if the label in these areas were simply radioactive fringes of ubiquinones-10 and -8, respectively. The ubiquinone-9 and -7 areas, cut from the paper chromatogram, were eluted with ether and the residues were applied to separate strips of silicone-impregnated paper. Reversed-phase paper chromatography was run as above with appropriate reference ubiquinones. Strips corresponding to the areas of reference ubiquinones-9 and -7, as well as narrow strips immediately above and below each area, were cut from the paper while still damp with solvent. Each strip was eluted with ether and the radioactivity in each residue was measured by liquid scintillation counting.

Results

The incorporation of [^{14}C]*p*-hydroxybenzoic acid observed in the ubiquinones isolated from samples 1–3 (Table I) was higher than the levels attained in the control samples 4-6 containing uninfected blood cells. The level of incorporation in the infected cells was not increased by incubation periods exceeding 8 hr. Data from similar experiments indicate that maximum incorporation was often attained by time periods as short as 3 hr, but this interval varied from one experiment to another. The variables affecting the levels of incorporation of [^{14}C]*p*-hydroxybenzoic acid in both normal and infected blood samples will be studied further.

The ubiquinones isolated from each sample were separated by reversed-phase paper chromatography for the determination of the length of the isoprenyl side chain and identification of the particular ubiquinone. The results summarized in Figure 1 show that [^{14}C]ubiquinones-8, -9, and -10 were found in the infected samples. The results from scintillation counting also show that the residue from the ubiquinone-9 area appeared to contain more radioactivity than that from each of the bordering areas. It appears, therefore, that ubiquinone-9 is also present as a component and is not a fringe of the ubiquinone-10 area. The residue from the ubiquinone-7 area showed radioactivity which was intermediate between that from each of the border-

[1] The strain used for these studies was originally isolated from a monkey (*Macaca irus*) from Malaya and was given to Q. M. G. in 1965 by E. H. Sadun of the Walter Reed Army Institute of Research, Washington, D. C.

[2] Composition of modified Ringer's solution used for washing blood cells: NaCl, 8.21 g; KCl, 0.30 g; $CaCl_2$, 0.20 g; $MgCl_2$, 0.10 g; and H_2O, 1 l.

[3] Brinkmann precoated silica gel G thin-layer plates (20 × 20 cm) were marked into halves, and each half was marked into four individual thin-layer segments (5 × 10 cm). This allowed four samples to be quickly chromatographed at one time and facilitated isolation of ubiquinone from a minimum quantity of silica gel.

TABLE I: Biosynthesis of [^{14}C]Ubiquinones from [^{14}C]*p*-Hydroxybenzoic Acid by Normal Rhesus Monkey Blood and Blood Infected with *P. knowlesi*.

Sample	Incubn Time With [^{14}C]*p*-Hydroxybenzoic Acid (hr)	Parasitemia (%)	[^{14}C]Ubiquinone Isolated by Thin-Layer Chromatography (total dpm)	Side-Chain Length by Reversed-Phase Paper Chromatography
1	8	3.6	1439	10, 9, 8, and 7[a]
2	18	9.5	1321	10, 9, 8, and 7[a]
3	24	7.4	1439	10, 9, 8, and 7[a]
4	8	0[b]	87	10
5	18	0[b]	147	10
6	24	0[b]	177	10

[a] Presence of ubiquinone-7 is uncertain as shown in Figure 1. See text for discussion. [b] Control samples of normal blood.

ing areas. This area may represent a trace of ubiquinone-7 or just a fringe of the major component, ubiquinone-8. Only [^{14}C]ubiquinone-10 was found in the controls.

The data in Table I and Figure 1 indicate that [^{14}C]*p*-hydroxybenzoic acid is also incorporated into ubiquinone-10 isolated from the control samples of normal blood. In order to determine the site of this incorporation, three duplicate *in vitro* cultures of control blood samples, containing uninfected erythrocytes, were incubated with [^{14}C]*p*-hydroxybenzoic acid. The cells were isolated and fractionated. The data in Table II show that [^{14}C]ubiquinone was found only in the upper fraction of the packed cells containing most of the leucocytes. Labeled ubiquinone was not found in the lower fraction which was shown, microscopically, to be relatively free of leucocytes.

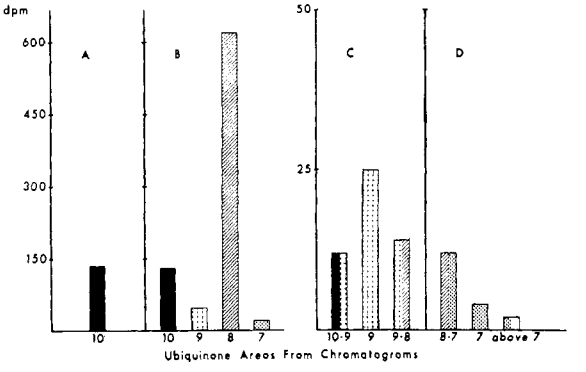


FIGURE 1: [^{14}C]Ubiquinone distribution by reversed-phase chromatography. (A) [^{14}C]Ubiquinone from samples 4, 5, and 6. (B) [^{14}C]Ubiquinone from samples 1, 2, and 3. (C) Rechromatography of ubiquinone-9 area from samples 1, 2, and 3. (D) Rechromatography of ubiquinone-7 area from samples 1, 2, and 3.

Discussion

Several *in vitro* cultivation systems have been developed for *P. lophurae* in duck erythrocytes (Trager, 1941), *Plasmodium gallinaceum* in chick erythrocytes (Anderson, 1953), and *P. knowlesi* in rhesus monkey erythrocytes (Geiman *et al.*, 1946). *P. knowlesi* was chosen for the present study because (a) it has a 24-hr asexual cycle, (b) it is highly pathogenic for monkeys (killing them in approximately 5–8 days with a *falciparum*-like syndrome), (c) it will produce clinical malaria in man (Chin *et al.*, 1965), and (d) it has recently been cultured in plasma-free medium (Siddiqui *et al.*, 1967). The latter is significant for these studies since this culture permits the incorporation of [^{14}C]-*p*-hydroxybenzoic acid into ubiquinone by the *Plasmodium* to proceed in the absence of unlabeled ubiquinone-10 which is normally present in the blood plasma (J. L. G. Nilsson and K. Folkers, 1967, unpublished results).

These experiments establish ubiquinone-8 as the dominant ubiquinone which is biosynthesized by *P. knowlesi*. Since only ubiquinone-10 was found in normal monkey blood, these experiments strongly imply that ubiquinone-8 is the dominant ubiquinone of *P. knowlesi* and small amounts of ubiquinone-9 are also biosynthesized. However, the mechanism of biosynthesis of

TABLE II: Fractionation of Samples of Normal Monkey Blood.

Samples	Incubn Time with [^{14}C]Hydroxybenzoic Acid (hr)	[^{14}C]Ubiquinone-10 in the Upper Three-fourths of Packed Cells (total dpm)	[^{14}C]Ubiquinone-10 in the Lower One-Fourth of Packed Cells (total dpm)
1	10	93	0
2	10	82	0
3	10	98	0

ubiquinone by microorganisms (Friis *et al.*, 1966), which has been reported for the lower homologs, would support the belief that ubiquinone-7 is also biosynthesized by the *Plasmodium*. Similar results have been obtained with the human malarial parasite, *Plasmodium falciparum*, and are reported in detail in part II. Since ubiquinone-8 has previously been found in *P. lophurae* (Rietz *et al.*, 1967) and *P. knowlesi* and *P. cynomolgi* (F. S. Skelton, P. J. Rietz, and K. Folkers, 1968, unpublished data), it may be that ubiquinone-8 is the predominant ubiquinone in all species of *Plasmodium*.

These experiments also show that the leucocytes of normal monkey blood have the capacity to biosynthesize [^{14}C]ubiquinone-10 since only the upper and not the lower fraction of packed blood cells yielded the labeled quinone. Furthermore, approximately the same amount of [^{14}C]ubiquinone-10 was found in both the normal and the infected monkey blood. Leucocyte counts of these fractions showed that the upper fraction contained nearly all of the leucocytes. The erythrocytes did not contain detectable amounts of ubiquinone-10. The presence of ubiquinone-10 in leucocytes is expected since these cells contain well-defined mitochondria (Bessis and Thiéry, 1955). The isolation of [^{14}C]-ubiquinone-10 from purified leucocytes of normal human and monkey blood will be reported elsewhere.

The isolation of [^{14}C]ubiquinones from normal monkey blood and from monkey blood infected with *P. knowlesi* after incubation with [^{14}C]*p*-hydroxybenzoic acid is in accord with the known role of *p*-hydroxybenzoic acid as a specific precursor of ubiquinone in other organisms (Rudney and Parson, 1963; Parson and Rudney, 1964; Olson *et al.*, 1963; Aiyar and Olson, 1964). The rapid incorporation of [^{14}C]*p*-hydroxybenzoic acid into ubiquinone-8 in developing malarial parasites implies a continuing biosynthesis of mitochondrial components. The presence of mitochondria in species of *Plasmodium* (Hepler *et al.*, 1966; Rudzinska and Trager, 1957) and the dependence of the parasite upon a gas phase containing a given concentration of oxygen is well known (Anfinsen *et al.*, 1946).

These experiments show the utility of the *in vitro* culture system to study various metabolic processes in the *Plasmodium* such as the role of ubiquinone in its oxidative metabolism. Substances which inhibit the biosynthesis of ubiquinone-8 or its role in the oxidative metabolism of the *Plasmodium* might be useful antimalarial agents in medicine.

Acknowledgments

We wish to thank W. F. Argall, Mrs. Esther Montero, Mrs. Kathleen Edelman, Miss Elizabeth Riddleberger, and Miss Paula Harris for excellent technical assistance.

References

Aiyar, A. S., and Olson, R. E. (1964), *Federation Proc. 23*, 425.

Anderson, C. R. (1953), *Am. J. Trop. Med. Hyg. 2*, 234.

Anfinsen, C. B., Geiman, Q. M., McKee, R. W., Ormsbee, R. A., and Ball, E. G. (1946), *J. Exptl. Med. 84*, 607.

Bessis, M., and Thiéry, J. P. (1955), *Rev. Hematol. 10*, 583.

Chin, W., Contacos, P. G., Coatney, C. R., and Kimball H. R. (1965), *Science 149*, 865.

Friis, P., Daves, Jr., G. D., and Folkers, K. (1966), *J. Am. Chem. Soc. 88*, 4754.

Geiman, Q. M., Anfinsen, C. B., McKee, R. W., Ormsbee, R. A., and Ball, E. G. (1946), *J. Exptl. Med. 84*, 583.

Geiman, Q. M., Siddiqui, W. A., and Schnell, J. V. (1966), *Military Med. 131*, 1015.

Hepler, P. K., Huff, C. G., and Sprinz, H. (1966), *J. Cell Biol. 30*, 333.

Olson, R. E., Bentley, R., Aiyar, A. S., Dialameh, G. H., Gold, P. H., Ramsey, V. G., and Springer, C. M. (1963), *J. Biol. Chem. 283*, PC3146.

Parson, W. W., and Rudney, H. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 444.

Rietz, P. J., Skelton, F. S., and Folkers, K. (1967), *Intern. J. Vitamin Res. 37*, 405.

Rudney, H., and Parson, W. W. (1963), *J. Biol. Chem. 283*, PC3137.

Rudzinska, M. A., and Trager, W. (1957), *J. Protozool. 4*, 190.

Siddiqui, W. A., Schnell, J. V., and Geiman, Q. M. (1967), *Science 156*, 1623.

Trager, W. (1941), *J. Exptl. Med. 74*, 441.