Coenzyme Q. CXXII. Identification of Ubiquinone-8 Biosynthesized by *Plasmodium knowlesi*, *P. cynomolgi*, and *P. berghei*[1]

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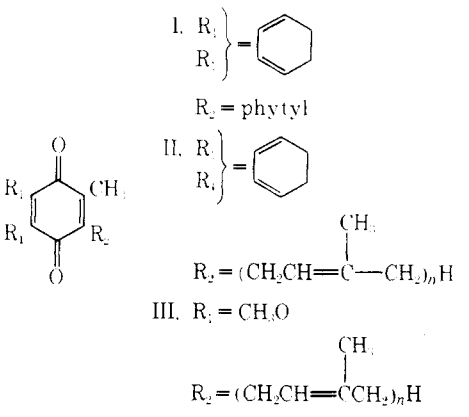
Investigation by reversed-phase paper chromatography and mass spectra of the ubiquinone and vitamin K content of normal Rhesus monkey blood and blood infected with *Plasmodium knowlesi* and *P. cynomolgi* revealed the presence of ubiquinones-8 and -9 in the infected blood. Ubiquinones-8 and -7 were also found in mouse blood infected with *P. berghei*. The presence of vitamin K could not be detected by reversed-phase paper chromatography or mass spectral analysis, nor by a labeling method using *in vitro* cultures of *P. knowlesi* incubated with [^{14}C]shikimic acid.

Vitamins K_1 (I), K_2 (II), and ubiquinone (III) are extensively found in nature and are known to possess



multiple biological function. Their functional role in the respiration of microorganisms and animals has been a subject of much scientific interest for many years and is currently a study of major importance in the laboratories of this country and throughout the world.

In an effort to develop a new and more effective approach to finding antagonists of the oxidative metabolism of the malarial parasite, it was first necessary to establish and confirm the presence of either or both ubiquinone and K vitamins in *Plasmodium*.

The occurrence of ubiquinones-8 and -9 (III, n = 8 and 9) in duck blood infected with *P. lophurae*[3] has been reported in a separate publication, since this avian parasite could be obtained at levels of parasitemia of 90% or higher in the blood. The high levels of isolated ubiquinone from the infected blood facilitated unconditional proof that ubiquinones-8 and -9 were present in addition to only ubiquinone-10 (III, n = 10), found in normal duck blood.

Similar data are presented here for Rhesus monkey blood infected with *P. knowlesi* and *P. cynomolgi* and mouse blood infected with *P. berghei*.

It is desirable to investigate and to compare the presence and type of ubiquinone or K vitamins in as many species of the parasite as is experimentally feasible. The universality of ubiquinone and vitamin K distribution in the various species of *Plasmodium* strengthens the basic thesis for developing inhibitors of either the function of these quinones in electron transport or of their biosynthesis.

Experimental Section

Determination of Ubiquinones in Normal Blood.—Frozen samples of blood (250 ml) of the normal Rhesus monkey and blood (300 ml) of the normal Swiss mouse were allowed to thaw at room temperature and were saponified by boiling under reflux for 15 min with a mixture of EtOH (2 l.), H_2O (300 ml), NaOH (12 g), and pyrogallol (10.6 g). The mixture was rapidly cooled in an ice bath and exhaustively extracted with hexane (10 × 200 ml). The extract was washed with H_2O (3 × 300 ml), and residual H_2O was removed by azeotropic distillation (C_6H_6) under reduced pressure. Each lipid residue (300 mg from Rhesus monkey blood and 328 mg from mouse blood) was fractionated by preparative tlc on silica gel G (4 plates, 20 × 20 cm, 1.0-mm layer) and developed in 35% Et_2O–hexane. The ubiquinone area, located by a reference sample of ubiquinone-10, was removed from the plate and eluted with dry Et_2O. Evaporation of the ether gave crude ubiquinone fractions (1.0 mg from Rhesus monkey blood and 1.2 mg from mouse blood). The amount of ubiquinone in the fractions was determined by a modified Craven's assay[4,5] as follows. An aliquot(0.1–0.25) of the residue was dissolved in 0.2 ml of abs EtOH and then 0.05 ml of ethyl cyanoacetate was added. After 6 min, 0.05 ml of ethanolic KOH (0.2 N) was added, and a microcuvette (0.1 ml) of the Beckman-Spinco Model 151 spectrocolorimeter was filled with the mixture. The ΔE at 625 mμ was determined by reading the E values every minute for about 9 min. The maximum reading was compared with those maxima obtained from measurements of standard solutions. The assay for ubiquinone indicated a value of 0.45 μg/ml for Rhesus monkey blood and 0.96 μg/ml for mouse blood.

Further purification of the crude ubiquinone fractions by tlc on silica gel G (20 × 20 cm; 0.5-mm layer) was accomplished by developing the plate 5 times with 3% Et_2O–hexane. The ubiquinone region, determined by a reference sample of ubiquinone-10, was removed from the thin-layer plate and eluted with dry Et_2O. A small aliquot of the residue, obtained after evaporation of the ether, was subjected to reversed-phase paper chromatography alongside reference samples of ubiquinone-7, -8, -9, and -10 on Whatman No. 3MM paper impregnated with Dow Corning 550 silicone oil, 5% in $CHCl_3$. The paper was developed with n-PrOH–H_2O (7:3, v/v). After drying in the air, the paper was treated with leucomethylene blue (LMB) reagent[6] which showed the presence of only ubiquinone-10 (R_f 0.15) for Rhesus monkey blood and only ubiquinone-9 (R_f 0.23) for mouse blood. This reagent was made by adding glacial AcOH (1 ml) and Zn dust (1 g) to a solution of methylene blue (100 mg) in EtOH (100

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ml). The lower limit of detection for this spray is about 1 mcg/cm^2.

All of the remaining residue of the purified ubiquinone was subjected to high-resolution mass spectrometry[7] with a Model 20-103C instrument of the Consolidated Electrodynamics Corp. (CEC), which was modified to provide an increased magnetic field, an electron-multiplier Wien-filter detection system, a sampling system permitting direct sample introduction by means of a heated probe, and 1-mm slits to permit unit resolution at *m/e* 1000. After introduction of the sample, it was necessary to hold the sample at a probe temperature of 125° until traces of volatile impurities were eliminated. The mass spectrum, determined at a probe temperature of 380°, showed a weak molecular ion at *m/e* 862 for ubiquinone-10 and the molecular ions of ubiquinone-8 (*m/e* 726) and -9 (*m/e* 794) corresponding to traces of these ubiquinones in Rhesus monkey blood. A strong molecular ion for ubiquinone-9 at *m/e* 794, a weak molecular ion at *m/e* 726 for ubiquinone -8 and a molecular ion at *m/e* 658 showing that mouse blood largely contained ubiquinone-9 with a minor amount of ubiquinone-8 and a trace of ubiquinone-7. All of the molecular ions for the quinones were associated with the molecular ions for their corresponding hydroquinones.

Determination of Ubiquinones in Parasitized Blood.—Frozen samples of Rhesus monkey blood infected with *P. knowlesi* (250 ml, 50% parasitemia), Rhesus monkey blood infected with *P. cynomolgi* (425 ml, 13% parasitemia), and Swiss mouse blood infected with *P. berghei* (275 ml, 70% parasitemia) were allowed to thaw at room temperature and were saponified by the same procedures used for the normal blood samples. The ubiquinone fractions, 5.0 mg, from Rhesus monkey blood infected with *P. knowlesi*, 3.5 mg from Rhesus monkey blood infected with *P. cynomolgi*, and 1.2 mg from Swiss mouse blood infected with *P. berghei*, which were obtained in the same manner as the normal blood, gave a total ubiquinone content of 0.62 μg/ml for blood containing *P. knowlesi*, 0.45 μg/ml for blood containing *P. cynomolgi*, and 1.03 μg/ml for blood containing *P. berghei* by the modified Craven's assay. Reversed-phase paper chromatography of the purified ubiquinone on Whatman No. 3MM paper impregnated with Dow Corning 550 silicone oil (5% in $CHCl_3$) using *n*-PrOH–H_2O (7:3, v:v), showed the presence of ubiquinone-10 and a lesser amount of ubiquinone-8 (R_f 0.32) for blood infected with *P. knowlesi* and *P. cynomolgi*, and ubiquinone-9 and a lesser amount of ubiquinone-8 for blood infected with *P. berghei*. The mass spectrum of the purified ubiquinone obtained from blood infected with *P. knowlesi* and *P. cynomolgi*, determined at a probe temperature of 380°, showed a molecular ion at *m/e* 726 for ubiquinone-8 and ions at *m/e* 794 and *m/e* 862 for ubiquinones-9 and -10. The mass spectrum of the purified ubiquinone obtained from mouse blood infected with *P. berghei* showed ions for ubiquinones-7, -8, and -9. All of the molecular ions for the quinones were associated with the molecular ions for their hydroquinones.

Search for the Presence of Vitamin K in Normal and Parasitized Blood. A. Method of Lester and Ramasarma.[8] Fresh samples of blood (120 ml) of the normal Rhesus monkey and Swiss mouse were lyophilized and exhaustively extracted, under subdued light, for 10 hr with EtOH–Et_2O (1:3, v:v) (1 l.). After filtration and evaporation of the solvents, the lipid residue was fractionated by preparative tlc on silica gel G (20 × 20 cm plate; 1-mm layer) and developed 3 times in 3% Et_2O–hexane and once in 15% Et_2O–hexane. The region for K vitamins, determined by reference samples of vitamin $K_{1(20)}$ and vitamins $K_{2(35)}$ and $K_{2(40)}$, was scraped from the thin-layer plate and eluted with ether. The residue, obtained after evaporation of Et_2O, was subjected to reversed-phase paper chromatography as for residues containing the ubiquinones. The paper was developed with *n*-PrOH–H_2O (85:15, v:v). No K vitamins could be detected in either the Rhesus monkey or mouse blood by the method of Lester and Ramasarma.[8] If any K vitamins were present the level would have been less than about 9.0 μg, the limit of sensitivity for this reagent.

B. The Irreverre–Sullivan Assay.[9]—The lipid fraction from fresh samples of lyophilized Rhesus monkey and mouse blood (120 ml each) was fractionated for K vitamins, as above, and the residue was assayed for K vitamins by the Irreverre–Sullivan method[9] as follows. Each residue was dissolved in 0.2 ml of abs EtOH containing 0.05 ml of a fresh 5% solution of sodium diethyl dithiocarbonate in abs EtOH (w:v); then, 0.05 ml of NaOEt (1 g of Na in 50 ml of abs EtOH) was added. The absorption of light at 575 mμ was followed over a 10-min period by comparing extinction coefficients. Standard samples of menaquinone-9, as low as 4 μg, could be detected by this method. This method showed no change in E_{max} values for the residues when compared with the E_{max} values obtained by the measurement of standard solutions of menaquinone-9. Therefore, no K vitamins could be detected in the residues from Rhesus monkey and mouse blood.

The lipid residues from fresh samples (120 ml) of Rhesus blood infected with *P. knowlesi* (50% parasitemia) and *P. cynomolgi* (13% parasitemia) and mouse blood infected with *P. berghei* (70% parasitemia) were fractionated for K vitamins, as above. The method of Lester[8] and the Irreverre–Sullivan[9] assay indicated no K vitamins could be detected in the parasitized bloods.

C. Mass Spectral Analysis.—Samples of menaquinone-9 of known weight were chromatographed by tlc; developed 3 times with 3% Et_2O–hexane and once with 15% Et_2O–hexane. The best method for introducing the sample into the mass spectrometer in order to obtain maximum sensitivity was to pack the silica gel, holding the menaquinone-9 sample, loosely in a probe designed to accommodate different amounts of silica gel. Determinations were made at a probe temperature of 160°. It was possible to correlate the base peak oxonium ion fragment (*m/e* 225) intensity with the sample size by the use of this method.

Samples of vitamin K_1 (2.0, 4.5, 9.0, 18.0, and 30.0 μg) were added to Rhesus monkey blood (10 ml) and again isolated and chromatographed as above. The vitamin K_1-fractionated residue was not isolated from the silica gel. Mass spectral analysis of these vitamins K_1–silica gel mixtures, using the above method, gave a reproducible standard curve for the oxonium ion fragment (*m/e* 225) intensities.

The K vitamins fractionated lipid residues obtained from fresh samples (120 ml each) of normal Rhesus monkey blood and normal mouse blood were subjected to mass spectral analysis. The determinations were made at probe temperatures from 150 to 180°. No base peak oxonium (pyrylium) ion fragment (*m/e* 225) could be observed in the samples.

The K vitamins fractionated lipid residues from Rhesus monkey blood (100 ml each) infected with *P. knowlesi* (35% parasitemia) and *P. cynomolgi* (16% parasitemia) and mouse blood (150 ml) infected with *P. berghei* (70% parasitemia) were subjected to mass spectral analysis as above. No oxonium ion fragment could be observed in any of the samples.

D. [^{14}C] Shikimic Acid Incorporation Studies.—[^{14}C]Shikimic acid (uniformly labeled, 2.5 × 10^4 cpm/culture) was added to synthetic medium,[10] and 6 duplicate *in vitro* suspensions (1.5 ml of blood cells in 9 ml of medium) of normal Rhesus monkey blood cells[11] and those infected with *P. knowlesi* were incubated for 8 hr at 37°.[12] The incorporation of [^{14}C]shikimic acid was measured under conditions designed for the intracellular *in vitro* cultivation of malarial parasites.[13] The synthetic medium was based upon that described by Anfinsen, *et al.*,[14] with modifications according to Geiman, *et al.*[10] Stearic acid was used to replace plasma.[15]

After incubation, the blood cultures were centrifuged, and the normal cells were combined as were the infected cells. The cells were washed twice with modified Ringer's solution,[16] lyophilized, and exhaustively extracted with hexane as described by Skelton, *et al.*[12] The residue, obtained upon evaporation of the hexane extracts, was subjected to tlc on silica gel G, and developed 3

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

(11) The blood samples were drawn from uninfected Rhesus monkeys (*Macaca mulatta*) and Rhesus monkeys infected with *P. knowlesi*, which was originally isolated from a monkey (*M. irus*) from Malaya, and maintained by weekly intravenous inoculation of erythrocytic stages into Rhesus monkeys. Cultures were maintained under bacterial-free conditions.

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

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

(16) 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.

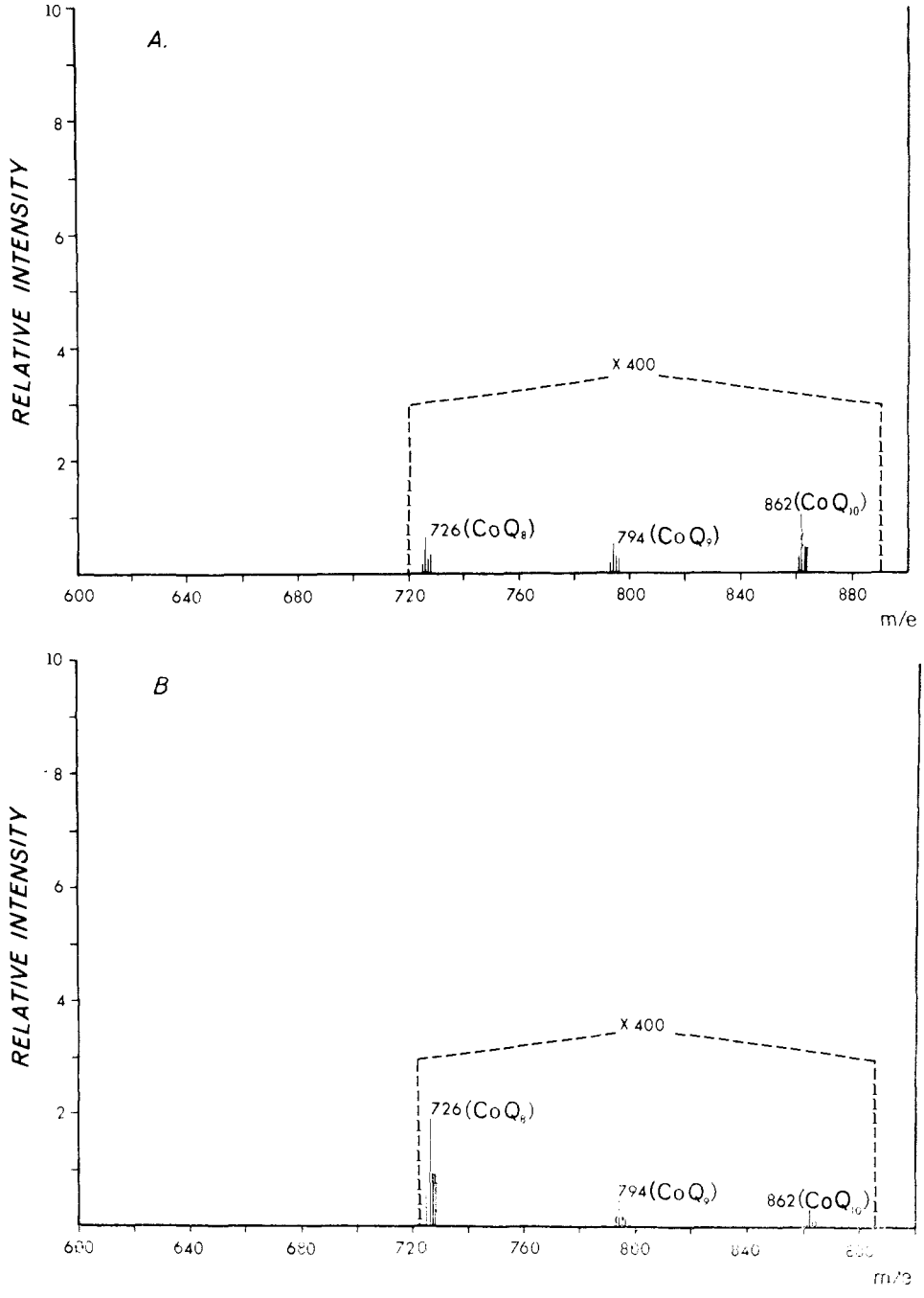

Figure 1.—Mass spectra (high-mass region) of ubiquinones (CoQ) isolated from (A) normal and (B) infected (*P. knowlesi*) Rhesus monkey blood.

times with 3% Et_2O–hexane and once with 15% Et_2O–hexane as described above. The K vitamins region, determined by vitamin K_1, $K_{2(35)}$, and $K_{2(40)}$ references, was eluted with Et_2O and the residue subjected to scintillation counting.[17] No radioactive material and, therefore, no K vitamins could be detected in either the normal or infected blood samples.

Discussion

Ubiquinone-10 (III, $n = 10$) has been identified by reversed-phase paper chromatography (R_f 0.15) and ubiquinones-10, -9, and -8 (III, $n = 9$ and 8) have been identified by high-resolution mass spectrometry in normal Rhesus monkey blood. Ubiquinone-9 (reversed-phase paper chromatography, R_f 0.23) and ubiquinones-9, -8, and possibly -7 (III, $n = 7$) (high-resolution mass spectrometry) were identified in normal mouse blood.

The corresponding ubiquinones were identified in Rhesus monkey blood infected with *P. knowlesi* and *P. cynomolgi* and in mouse blood infected with *P. berghei* but more ubiquinone-8 was found as evidenced by the appearance of ubiquinone-8 in the reversed-phase paper chromatograms of the ubiquinone-fractionated lipid residues from the infected Rhesus and mouse blood. Mass spectral results also indicate that the molecular ion for the more volatile (*i.e.*, ubiquinone-$7 > 8 > 9 > 10$) ubiquinone-8, obtained from the infected bloods, was of higher intensity than that observed for the corresponding ubiquinone-8, obtained from normal bloods (Table I and Figures 1 and 2). Ubiquinone-7 was observed as a weak but well-defined molecular ion

(17) Using a Nuclear-Chicago liquid scintillation spectrophotometer.

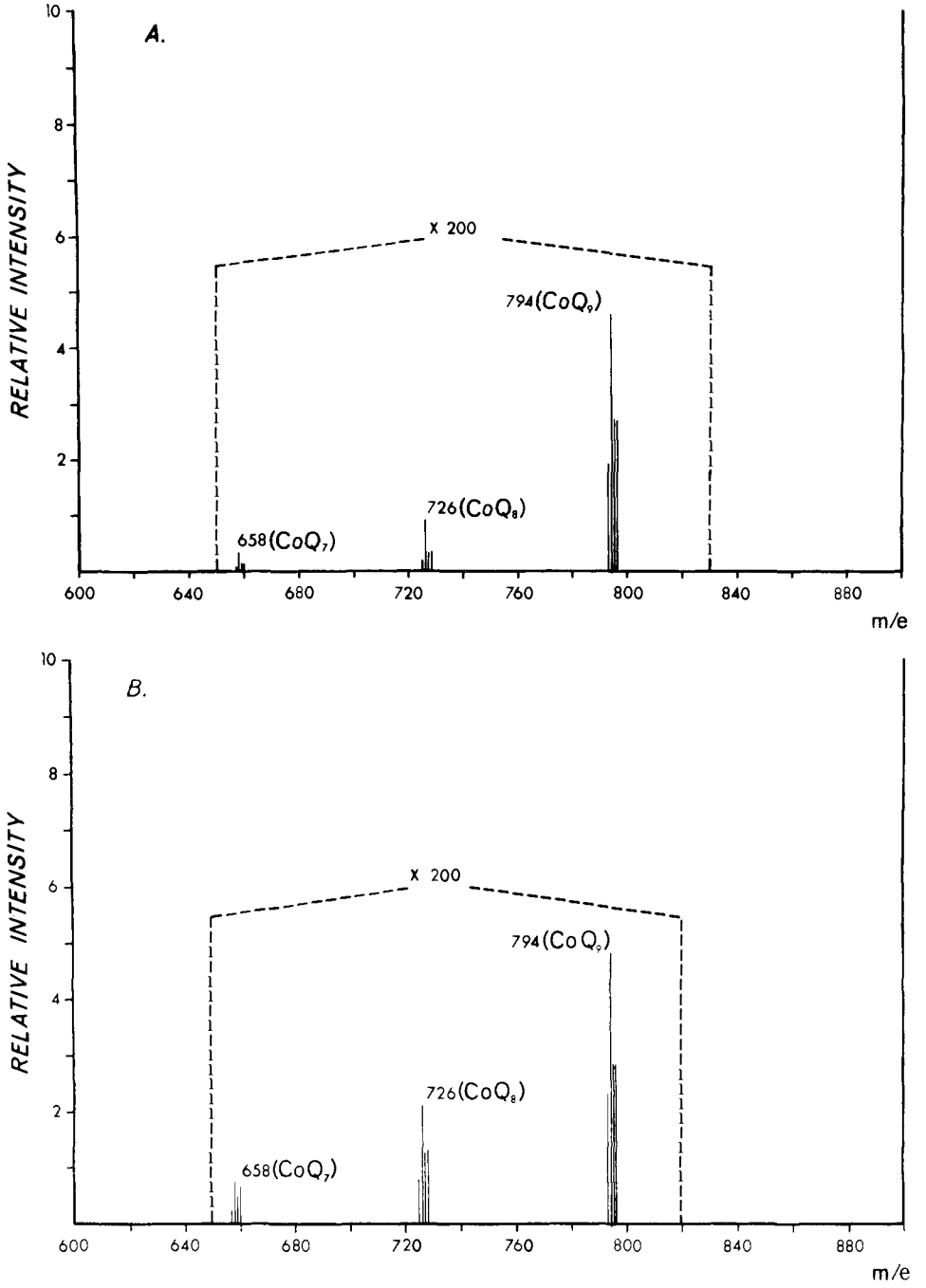

Figure 2.—Mass spectra (high-mass region) of ubiquinones (CoQ) isolated from (A) normal and (B) infected (*P. berghei*) mouse blood.

in the mass spectrum of ubiquinone-fractionated lipid residues obtained from mouse blood infected with *P. berghei*. Only a slight trace of ubiquinone-7 was observed in the mass spectrum of the residue from normal mouse blood.

These results are in accord with the finding of ubiquinones-8 and -9 in duck blood infected with *P. lophurae*.[3] The same ^{14}C-labeled ubiquinones were isolated from Rhesus monkey blood cells infected with *P. knowlesi* and *P. falciparum* incubated with [^{14}C]-*p*-$HOC_6H_4CO_2H$ in synthetic medium.[12,18] The universality of distribution of ubiquinones-8 and -9 among various species of *Plasmodium* is supported by these results.

It is interesting to note that ubiquinone-7 was detected (mass spectral analysis) in mouse blood infected with *P. berghei*. This ubiquinone has also been demonstrated in rat liver by Linn, *et al.*,[6] and Lawson, *et al.*[19] The recently reported study of the *in vitro* incorporation of [^{14}C]-*p*-hydroxybenzoic acid into ubiquinones in cultures of Rhesus monkey blood infected with *P. knowlesi*[12] did not show, unequivocally, that ubiquinone-7 is present in *P. knowlesi*. The finding of ubiquinone-7 in mouse blood infected with *P. berghei* may be exogenous to the parasite and endemic to liver tissue.

The finding, by mass spectral analysis, of ubiquinones-10, -9, and -8 in normal Rhesus blood, and ubiquinones-9, -8, and possibly -7 in normal mouse blood,

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TABLE I

Plasmodium	Host	Blood sample[a]	Parasitemia (%)	Total ubiquinone (μg/ml of blood) by Craven's assay	Type of ubiquinone (CoQ)	
					Reversed-phase paper chromatography	Mass spectrometry[b]
P. knowlesi	Rhesus monkey	NB		0.45	CoQ_{10} (R_f 0.15)	CoQ_{10}^2 (*m/e* 862), CoQ_9^3 (*m/e* 794), CoQ_8^3 (*m/e* 726)
		PB	50	0.62	CoQ_{10}, CoQ_9 (R_f 0.32)	CoQ_{10}^2, CoQ_9^3, CoQ_8^1
P. cynomolgi	Rhesus monkey	NB		0.45	CoQ_{10}	CoQ_{10}^2, CoQ_9^3, CoQ_8^3
		PB	13	0.45	CoQ_{10}, $1CoQ_8$)	CoQ_{10}^3, CoQ_9^3, CoQ_8^1
P. berghei	Mouse	NB		0.96	CoQ_9 (R_f 0.23)	CoQ_9^1, CoQ_8^2, $(CoQ_7)^3$
		PB	70	1.03	CoQ_9, CoQ_8	CoQ_9^1, CoQ_8^1, CoQ_7^2 (*m/e* 658)

[a] NB, normal blood; PB, parasitized blood. [b] Numerical superscripts refer to the relative degree of molecular ion intensity: (1 = strong molecular ion; 2 = weak molecular ion; and 3 = trace molecular ion).

is in contrast to the finding of only ubiquinone-10 in normal duck blood.[3] Since these differential analyses are performed on the total ubiquinone content of whole blood, any ubiquinone from body tissues which is deposited in the blood reservoir will contribute to the analysis. It is safe to assume that some of these multiple ubiquinones may be blood contaminants from the ubiquinones endemic to various tissues of the body.

Since the Rhesus and mouse bloods, infected with malarial parasites, contain the same multiple ubiquinones as for the normal bloods, it was necessary to study the relative degree of molecular ion intensities of these ubiquinones in the mass spectra. We attempt to show, in Table I and Figures 1 and 2, the molecular ion intensities observed in the mass spectra of the ubiquinone-fractionated residues obtained from the malaria infected blood. The results clearly indicate the dominance of ubiquinone-8 in the mass spectra of the residues from the infected blood and are in agreement with the predominance of ^{14}C-labeled ubiquinone-8 obtained from *in vitro* cultures of *P. knowlesi* and *P. falciparum* incubated in synthetic medium containing [^{14}C]-*p*-hydroxybenzoic acid.[12,18]

Much time was taken and effort given in search of K vitamins in normal and infected blood samples. The chemical tests, as developed by Lester,[8] and Irreverre and Sullivan,[9] were negative for K vitamins as was found for K vitamins fractionated residues from normal and infected duck blood.[3] A high resolution mass spectral method and a ^{14}C-labeling method were then perfected for detecting mere traces of K vitamins. Before K vitamins fractionated residues from blood samples were submitted to high resolution mass spectral analysis, samples of blood were "spiked" with known amounts of vitamin K_1 and the best method was sought for isolating and preparing the residues for analysis. Best results were obtained by directly introducing silica gel containing the vitamin K_1 sample into the probe for analysis. (See Experimental Section.) When this method was applied to K vitamins fractionated residues from normal and infected bloods, no K vitamins could be detected.

The ^{14}C-labeling method involved incubating normal and *P. knowlesi* infected Rhesus mondey blood in synthetic medium[10] containing [^{14}C]shikimic acid, which is a known precursor of vitamin K_2 in *Escherichia coli*.[20]

After incubation the K vitamins residue, obtained from the washed cells, showed no radioactivity by scintillation counting and no K vitamins were detected.

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We are also indebted to Dr. R. F. Muraca and Mrs. J. S. Whittick for their skill in modifying the model 21-103C (CEC) mass spectrometer, and to Mr. Franklin M. Church for obtaining the spectra.

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