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Studies on Reduced and Oxidized Ubiquinones. I. Simultaneous Determination of Reduced and Oxidized Ubiquinones in Tissues and Mitochondria by High Performance Liquid Chromatography

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A high performance liquid chromatographic method with both an ultraviolet spectrometric detector (UVD) and an electrochemical detector (ECD) has been developed for the simultaneous determination of reduced and oxidized ubiquinones in biological materials. This method is based on extraction from animal tissues or mitochondrial fractions with ethanol–*n*-hexane mixture, followed by quantitation on a reversed-phase column with UVD and ECD. The detection limits by ECD or UVD were 100pg, 2ng, 150pg and 2ng for ubiquinol-9, ubiquinone-9, ubiquinol-10 and ubiquinone-10, respectively. The persentages of reduced ubiquinones (ubiquinols) to total ubiquinones were 41.6, 32.4 and 45.2% for guinea pig heart, rat heart and heart mitochondrial fraction of guinea pig, respectively, with added succinate as a substrate.

Keywords——high performance liquid chromatography; ultraviolet spectrometric detector; electrochemical detector; determination; reduced ubiquinones; oxidized ubiquinones; animal tissues; mitochondria

The ubiquinones (UQ)[1] are a family of lipid-soluble benzoquinones that are widely distributed in living organisms. UQ are found in relatively high concentrations in various animals and mitochondria, where they appear to function as electron and proton carriers in the respiratory chain[2] or to be related to energy production *via* the proton-motive force on mitochondria.[3]

Several reports have indicated that local deficiencies of UQ were observed in cardiac patients,[4] in the liver and kidney of rats acutely poisoned by treatment with carbon tetrachloride or mercuric chloride[5] and in the case of warm ischemia of the rat kidney.[6]

It was also suggested that UQ_{Red} might act as an antioxidant of mitochondrial lipid.[8] Therefore, it is important to determine not only UQ_{Ox} but also UQ_{Red} in these biological fluids. Recently, quantitative analyses of individual UQ homologs in biological samples have been performed by high performance liquid chromatography (HPLC) combined with an ultraviolet spectrometric detector (UVD)[8] or mass spectrometry (MS).[9] In addition, an electrochemical detector (ECD) for HPLC was confirmed to be simple and sensitive for the determination of UQ.[10] However, only UQ_{Ox} was determined by these method. For the determination of UQ_{Red} and UQ_{Ox} in mitochondria, submitochondrial particle and cell-free bacterial homogenates, the dual-wavelength spectrometric method which was developed by Hatefi,[11] Redfearn,[12] Crane[13] and Klingenberg[14] has been generally used. The method, however, cannot simultaneously measure the amounts of UQ_{Red} and UQ_{Ox} in whole tissues owing to the presence of vitamin A and other interfering compounds which absorb in the same spectral region as UQ and undergo an absorption change on chemical reduction. Moreover, the dual-wavelength spectrometric method cannot separately determine individual UQ homologs.

The analytical procedure reported here was developed to provide a rapid, sensitive and direct assay method for UQ_{Red} and UQ_{Ox} in biological materials. This method is based on extraction from tissues or mitochondrial fraction with organic solvents, followed by quantitation by means of reversed-phase chromatography with UVD and ECD.

Experimental

Apparatus——The HPLC system consisted of a YANACO L-2000 pump (Yanagimoto Manufactory Co., Ltd. Japan) with a Rheodyne loop injector. The UVD and ECD were a JASCO UVIDEC 100 UV detector (Japan Spectroscopic Co., Japan) and a YANACO VMD-101 electrochemical detector, respectively. The ECD was connected to the outlet of the UVD. Reversed-phase chromatography was carried out a Nucleosil C-18 column (15 cm × 4.0 mm I.D., Machery-Nagel Co., Germany, 5 μm). The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 1000 ml of ethanol–methanol–70% $HClO_4$ (700: 300: 1). The flow rate was 1.2 ml/min. The HPLC measurements were performed at 30 ± 0.1°. In order to prevent oxidation by dissolved oxygen during separation, the mobile phase was deaerated by nitrogen gas bubbling.

Materials——Ubiquinone-9 (UQ_9) and ubiquinone-10 (UQ_{10}) were synthesized by Eisai Co., Ltd. and Nisshin Chemical Co., respectively. Ubiquinol-9 (UQ_9H_2) and ubiquinol-10 ($UQ_{10}H_2$) were obtained by the reduction of UQ_9 and UQ_{10} with sodium borohydride. Other chemicals (reagent grade) were used without further purification.

Extraction of UQ from Tissues——A male guinea pig (three or four months old) was sacrificed by decapitation. The liver, heart, adrenals, kidneys and brain were removed as quickly as possible, weighed to the nearest mg, rinsed with ice-cold 0.15 M NaCl solution, and homogenized at 4° with 4 volumes (v/w) of water in a Polytron homogenizer (Hijiri Seiko, Japan) for 20 seconds. One ml of the homogenate was poured into a test tube containing 7 ml of a mixture of ethanol–*n*-hexane (2: 5), and the tube was rapidly shaken for 10 min to extract UQ. This extraction procedure was repeated three times. The combined *n*-hexane layer was evaporated to dryness under a stream of nitrogen. The resulting residue was dissolved in 0.5 ml of ethanol and subjected to HPLC.

Extraction of UQ in Heart Mitochondria of Guinea Pig——Male guinea pig heart was homogenized with 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, and the mitochondrial fraction prepared by the method of Rehncrona[15] was suspended in the buffer described above. The mitochondrial protein content was determined to be 0.25—0.5 mg/ml by the method of Lowry[16] with bovine serum albumin as a standard. UQ in the mitochondrial suspension were extracted by the afore-mentioned method. The residue was dissolved in 0.2 ml of ethanol and subjected to HPLC.

All extraction steps for tissues and mitochondrial fraction were performed in the absence of direct sunlight and incandescent light to minimize the photochemical degradation of UQ. The concentration of each UQ in tissues and mitochondrial fraction was determined by comparing the peak heights on HPLC with those of the corresponding authentic UQ treated in the same manner as the sample.

Results and Discussion

Isolation and Identification of Reduced and Oxidized Ubiquinones

Tentative identifications of $UQ_{10}H_2$ and UQ_{10} present in the quinea pig heart were based on comparisons of the retention times, UV spectra and hydrodynamic voltammograms of the the chromatographic peaks with those of the corresponding authentic compounds. Typical chromatograms obtained from the guinea pig heart (used to identify the peaks) are shown in Fig. 1-b. $UQ_{10}H_2$ and UQ_{10} were clearly separated from each other, and had retention times consistent with those of the authentic compounds at 5.7 min (peak B) and 7.4 min (peak D), respectively. As shown in Table I, the UV spectra and hydrodynamic voltammograms of the corresponding peaks are broadly in accord with those of the authentic compounds. Peak B, corresponding to $UQ_{10}H_2$, was also characterized by the following experiments. Oxidation of the extract with PbO_2 or acetylation with acetic anhydride induced the disappearance of peak B, and the oxidation gave rise to an increase in the peak height of peak D corresponding to the decrease of peak B. Furthermore, we carried out a more rigorous identification of $UQ_{10}H_2$ and UQ_{10} in the guinea pig heart by MS and TLC. The mass fragmentation patterns of the compounds isolated by TLC were similar to those of authentic UQ_{10} and diacetate of $UQ_{10}H_2$. In addition, the reduced and oxidized levels of UQ in the heart mitochondrial fraction of guinea pig generated with succinate or malate as a substrate are broadly in accord with Klingenberg's results obtained by the dual-wavelength spectrometric method.[14] In the rat heart, UQ_9H_2 and UQ_9 were identified as the main UQ components. Therefore, based on a combination of data obtained by various techniques, peak, A B, C and D in Fig. 1 were identified as UQ_9H_2, $UQ_{10}H_2$, UQ_9 and UQ_{10}, respectively.

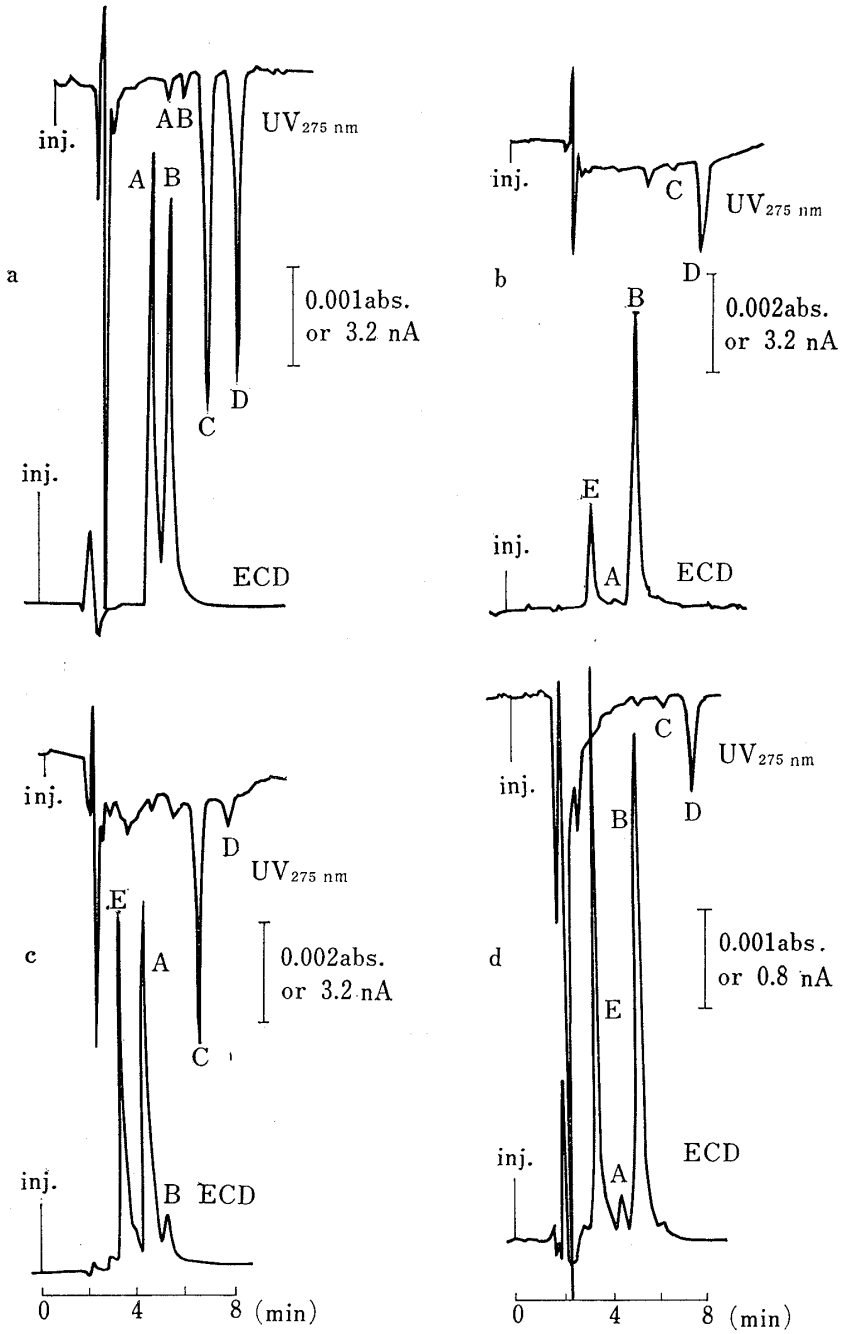

Fig. 1. Charomatograms of Reduced and oxidized Ubiquinones

Column; Nucleosil C-18 (5 μm), 15 cm × 4 mm I.D.
Mobile phase; ethanol–methanol–70% $HClO_4$ (700: 300: 1, containing 0.05 M $NaClO_4$).
Flow rate; 1.2 ml/min.
Detection; UV, 275 nm; ECD, 0.7 V vs. Ag/AgCl.
a, standard (amount injected; 56 ng of each UQ); b, guinea pig heart; c, rat heart; d, mitochondrial fraction of guinea pig heart.
Peak A, UQ_9H_2; peak B, $UQ_{10}H_2$; peak C, UQ_9; peak D, UQ_{10}; peak E, tocopherols and others.

Chromatographic Detection

UQ_{Ox} homologs have usualy been detected by HPLC with UV[8] or ECD in the cathodic mode.[10] UQ_{Rcd} homologs, however, have not been investigated.

Preliminary trials with measurement of the absorption at 290 nm, which is the maximum of UQ_{Rcd}, were totally unsuccessful for tissue samples because of interference by numerous ultraviolet-absorbing compounds having similar retention times, such as retinyl palmitate.

Table I. Chemical and Physical Data for Ubiquinones from Guinea Pig Heart

		Peak B[a]	Peak D[a]
HPLC[b]	(t_R, min)	5.4	7.6
TLC[c]	(Rf)	0.29[e]	0.40
E_p[d]	(V *vs.* Ag/AgCl)	0.70	−0.40
UV	(λ_{max}, nm)	290	275
MS	(m/e)	M^+ 948, M^+−acetyl 905, M^+ 862 M^+−diacetyl 862	
Identification		$UQ_{10}H_2$	UQ_{10}

a) Peaks B and D are the same as in Fig. 1-b.
b) HPLC conditions were the same as in Fig. 1.
c) HPTLC Silica Gel 60 F_{254} (E. Merck, Germany), *n*-hexane–isopropyl ether (1:1), UV_{254nm}.
d) Peak potential obtained from the hydrodymanic voltammogram.
e) Diacetate of peak B.

However, the applied potential of 0.7 V *vs.* Ag/AgCl was low enough to eliminate the retinyl palmitate contamination in the determination of $UQ_{10}H_2$. Ubichromenol, which was eluted at 5.9 min, was not present (Fig. 1-b). ECD in the anodic mode was utilized for the determination of UQ_9H_2 and $UQ_{10}H_2$.

For UQ_{ox} such as UQ_9 and UQ_{10}, since the millimolar extinction coefficient (ε=14.6 mM^{-1} cm^{-1}) at λ_{max}=275 nm for UQ_{10} was higher than that (ε=4.1 mM^{-1} cm^{-1}) at λ_{max}=290 nm for $UQ_{10}H_2$ and ultraviolet-absorbing compounds having the same retention time were absent, HPLC detection was performed with UVD at 275 nm.

In Fig. 1-a, typical chromatograms with both UVD and ECD detections are shown for a standard mixture containing UQ_9H_2, UQ_9, $UQ_{10}H_2$ and UQ_{10}. A similar mobile phase was used for the determination of UQ in human blood.[10] The peak heights of UQ_{Red} and UQ_{ox} were linear in the ranges of 0.5 ng to 100 ng of $UQ_{10}H_2$ injected and 5 ng to 200 ng of UQ_{10} injected. The detection limits of UQ_9H_2, UQ_9, $UQ_{10}H_2$ and UQ_{10} were 100 pg, 2 ng, 150 pg and 2 ng, respectively. Conversion from UQ_{Red} to UQ_{ox} during the HPLC analysis amounted to less than 2% of the UQ_{Red} injected.

Extraction of Reduced and Oxidized Ubiquinones from Biological Materials

There are two basic methods which have been employed for the extraction of UQ in tissues and mitochondrial fractions. The first involves saponification of the tissue lipids with alcoholic alkali in the presence of pyrogallol, followed by extraction of the nonsaponifiable lipid fraction.[8b] The second is based on direct extraction, for example, with ethanol-ether or acetone.[13,17] The saponification method has been used most extensively as a general method applicable to a great variety of materials. As shown in Table II, however, the bulk of UQ extracted by the saponification method was in UQ_{ox} form.

Direct extraction with organic solvents has the advantage that UQ is not exposed to strong alkali. Therefore, this method is useful as an approach to the determination of both UQ_{Red} and UQ_{ox} in tissues or mitochondrial fractions. Care must be taken to ensure complete extraction of UQ. Among several solvents and extraction systems examined, the ethanol–*n*-hexane mixture proved to be most efficient for this purpose. Table II shows the effect of various ethanol concentrations on the *n*-hexane extraction of both UQ_{Red} and UQ_{ox}. Ethanol was added to remove protein by partition and denature enzymes in the mitochondria. In view of these results, the ethanol–*n*-hexane (2:5, v/v) mixture was selected as a direct extraction solvent. To ensure complete extraction of UQ, extraction was performed three times with the ethanol–*n*-hexane mixture and complete extraction was confirmed by comparison with the results of the saponification method.

Table II. Effect of Ethanol Concentration on the Extractability of Ubiquinones from Guinea Pig Heart Homogenates[a]

Solvent ratio (ethanol–*n*-hexane-homogenate, ml)	Extractability		
	$UQ_{10}H_2$ (μg/g)	UQ_{10} (μg/g)	$UQ_{10}H_2$/Total UQ_{10}[b]
Saponification[c]	3.3	219.8	1.4
1 : 5 : 1	47.3	72.8	39.4
2 : 5 : 1	90.2	130.2	40.9
2 : 5 : 1	91.0	129.5	41.2
4 : 5 : 1	88.2	126.9	41.0

a) Homogenates from five guinea pig hearts.
b) Total UQ_{10} is the sum of $UQ_{10}H_2$ and UQ_{10}.
c) Saponification and extraction were carried out as described in ref. 8*c*).

Table III. Recovery of Ubiquinones from Tissue and Mitochondria of Guinea Pig Heart

Sample	Recovery	
	$UQ_{10}H_2$[a] (%)	UQ_{10}[a] (%)
Tissue	95.8±2.0[b]	94.2±1.8
Mitochodria	96.0±2.2	93.8±2.1

a) Each ubiquinone was added (20 μg and 0.9 μg for ubiquinone-depleted tissue and mitochondrial fraction, respectively).
b) The values are means±standard errors of the means for three animals.

The extraction recoveries of UQ in guinea pig heart and mitochondrial fraction were almost quantitative, as shown in Table III. Similar results were obtained for other tissues of guinea pig and rat heart.

It is known that UQ_{Red} itself is moderately oxidized in air. Therefore, it is important to know whether the reduced and oxidized levels of UQ are changerd by extaction and storage. Table IV shows the stability of UQ from guinea pig under various conditions of storage. The percentage of $UQ_{10}H_2$ to the total UQ_{10}, when they were preserved as frozen tissues or as an isopropanol solution of the extracts with the ethanol-*n*-hexane mixture, decreased with time. When UQ components were extracted immediately and stored under a nitrogen gas atmosphere, the percentage remained almost constant. Similar results were obtained in other tissues and mitochondrial fractions.

Determination of Reduced and Oxidized Ubiquinones in Biological Materials

The chromatograms of UQ_{Red} and UQ_{Ox} homologs in guinea pig heart and rat heart are shown in Fig. 1-b and c. Peak E at the retention time of 3.4 min was due to a mixture of tocopherols and other electrochemically active compounds. UQ components found in guinea pig and rat were mainly UQ_{10} and UQ_9 homologs. Table V shows the concentrations of UQ_{Red} and UQ_{Ox} homologs in some tissues. There is no significant difference between the sum of UQ_{Red} and UQ_{Ox} obtained by our method and those reported previously.[9] The percentage of UQ_{Red} to total UQ differs by species and tissues of animals. The physiological significance of these values needs to be clarified in comparison with the values for animals with experimental model diseases such as ischemia and anoxia.

Fig. 1-d shows the chromatograms of UQ_{Red} and UQ_{Ox} in the heart mitochondral fraction of guinea pig after the addition of succinate. Table VI shows the percentage of UQ_{Red} to total UQ after the addition of succinate or malate. $UQ_{10}H_2$ in the mitochondrial fraction amounted to 3.2—3.9% before addition of the substrates. It appears that UQ_{Red} might change to UQ_{Ox}. UQ_{Red} was increased by the additions of succinate and malate as substrates for

Table IV. Stabilities of Ubiquinones in Guinea Pig Heart and Liver on Storage under Various Conditions

Storage conditions	Temp. (°C)	Days	Heart[b] $UQ_{10}H_2$ (μg/g)	Heart[b] UQ_{10} (μg/g)	Heart[b] $\frac{UQ_{10}H_2}{\text{Total } UQ_{10}}$[c] (%)	Liver[b] $UQ_{10}H_2$ (μg/g)	Liver[b] UQ_{10} (μg/g)	Liver[b] $\frac{UQ_{10}H_2}{\text{Total } UQ_{10}}$ (%)
Frozen tissues[a] (N_2 gas atmosphere)	−20	0	82.8	132.8	38.4	34.7	22.6	60.5
		2	72.3	151.1	32.4	33.0	24.9	57.0
		7	33.7	167.1	16.8	29.2	26.8	52.2
		14	—	—	—	31.2	27.9	52.8
Isopropanol Soln. of Extracts[a]	20	0	82.4	132.8	28.4	34.7	22.6	60.5
		1	55.3	146.7	27.4	16.6	37.7	30.5
		2	49.8	156.7	24.1	16.0	41.2	28.0
		6	28.6	195.0	12.8	3.5	50.4	6.5
Extracts with ethanol–*n*-hexane (N_2 gas atmosphere)	−20	0	84.1	114.6	42.3	26.7	23.4	53.3
		1	—	—	—	25.7	22.3	53.6
		2	82.0	107.3	43.3	26.4	23.1	53.3

a) The same tissue homogenates were used in each case.
b) Extractions were carried out as described in "Experimental".
c) Total UQ_{10} is the sum of $UQ_{10}H_2$ and UQ_{10}.

Table V. Contents of Reduced and Oxidized Ubiquinones in Animal Tissues

Animal	Tissue	Content (μg/g) UQ_9H_2	UQ_9	$UQ_{10}H_2$	UQ_{10}	Total UQ[a]	$\frac{\text{Total } UQ_{Red}}{\text{Total } UQ}$[b] (%)
Guinea pig	Heart (11)	5.2±1.6[c]	7.7±1.5	82.3±11.7	114.7±10.72	210.0±21.2	41.6±3.1
	Liver (7)	1.9±0.4	1.5±0.5	32.8±6.3	24.2±2.4	60.5±7.8	57.2±3.8
	Kidney (5)	6.6±0.7	3.7±0.2	109.6±20.4	52.1±6.8	172.1±27.0	67.4±2.0
	Adrenal (4)	3.6±0.3	2.2±0.3	48.8±6.9	35.6±4.4	89.9±11.0	58.1±1.9
	Brain (4)	Trace	Trace	10.4±2.7	16.0±2.7	23.9±0.9	43.8±9.0
Rat	Heart (4)	76.5±6.6	156.9±7.7	5.0±0.5	13.3±1.1	251.6±18.9	32.4±2.5
	Kidney (4)	71.5±2.0	108.9±8.9	10.1±0.4	15.6±1.4	206.1±19.5	39.8±2.1

a) Total UQ is the sum of UQ_9H_2, UQ_9, $UQ_{10}H_2$ and UQ_{10}.
b) Total UQ_{Red} is the sum of UQ_9H_2 and $UQ_{10}H_2$.
c) The values are means±standard errors of the means for the numbers of animals in parentheses.

Table VI. Reduced and Oxidized Ubiquinone-10 Levels in the Heart Mitochondrial Fraction of Guinea Pigs

	Expl-1[a]	Expl-2[b]
Total UQ_{10}[c] (μg/mg of protein)	3.75	3.75
$\frac{UQ_{10}H_2}{\text{Total } UQ_{10}}$ (%)		
Only buffer	3.9	3.2
Added succinate	45.2	43.1
Added malate	44.6	19.1

a) Fresh mitochondrial fraction of a guinea pig.
b) Aged mitochondrial fraction(−20° for 1 day).
c) Total $UQ_{10}$is the sum of $UQ_{10}H_2$ and UQ_{10}.

succinate-ubiquinone reductase and NADH-ubiquinone reductase, respectvely. The percentage of $UQ_{10}H_2$ to total UQ_{10} after the addition of malate was lower in the aged mitochondrial fraction than in the fresh one. This is probably due to damage to the mitochondrial fraction during storage.

The concentrations of the reduced and oxidized ubiquinones can be simultaneously determined by the method described here. The method is so sensitive that the ubiquinone concentrations can be determined in extracts from 2—5 mg of heart and 15 μg protein of mitochondrial fraction. The enzymological and physiological applications of this method will be described elsewhere.

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References and Notes

1) The abbreviations used are: UQ, the general term for reduced and oxidized ubiquinone homologs; UQ_{Red}, the general term for reduced ubiquinones; UQ_{Ox}, the general term for oxidized ubiquinones; UQ_9, ubiquinone-9; UQ-10, ubiquinone-10; UQ_9H_2, ubiquinol-9, $UQ_{10}H_2$, ubiquinol-10; total UQ_9, the sum of UQ_9 and UQ_9H_2; total UQ_{10}, the sum of UQ_{10} and $UQ_{10}H_2$; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; UVD, ultraviolet spectrometric detector; ECD, electrochemical detector; MS, mass spectrometry.
2) *a*) R.L. Lester and S. Fleischer, *Arch. Biochem. Biophys.*, **80**, 470 (1959); *b*) L. Szarkowska, *ibid.*, **113**, 519 (1966); *c*) A. Kröger, M. Klingenberg, *Eur. J. Biochem.*, **34**, 358 (1973).
3) P. Mitchell, *Fer. Proc. Fed. Am. Soc. Exp. Biol.*, **26**, 1335 (1967); *b*) E. Quagliariello, and S. Papa, Eds, "Electron Transfer Chains and Oxidative Phosphorylation" North-Holland, Amsterdam, 1975; *c*) F.L. Crane, *Ann. Rev. Biochem.*, **46**, 439 (1977); *d*) J.W. DePierre and L. Ernstein, *ibid.*, **46**, 201 (1977).
4) R. Nakane, G.P. Littarru, K. Folkers, and E.G. Wilkinson, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1456 (1974).
5) G. Kover, L. Telegdyo Kovats, and E.B. Kraszner, *Acta. Biol. Acas. Sci. Hung.*, **26**, 67 (1975).
6) K. Yamada, Y. Tatsukawa, M. Takenaka, T. Iguchi, M. Yamamoto, and T. Kawasaki, "International Symposium on Coenzyme Q," Elsevier Publishing Co., Amsterdam, 1980.
7) *a*) A. Meller and A.L. Tappel, *J. Biol. Chem.*, **241**, 4353 (1966); *b*) S. Sugiyama, M. Kitagawa, T. Ozawa, K. Suzuki, and Y. Izawa, *Experimantia*, **36**, 1002 (1980); *c*) K. Takashige, R. Takayanagi, and S. Minakami, "International Symposium on Coenzyme Q," Elsevier Publishing Co., Amsterdam, 1980.
8) *a*) P.L. Donnahay and F.W. Hemming, *Biochem. Soc. Trans.*, **3**, 775 (1975); *b*) K. Abe, K. Ishibashi, M. Ohmae, K. Kawabe, and G. Katsui, *Vitamins* (Japan), **51**, 111 (1977); *c*) *Idem*, *J. Nutr. Sci. Vitaminol.*, **24**, 555 (1978).
9) S. Imabayashi, T. Nakamura, Y. Sawa, J. Hasegawa, K. Sakaguchi, T. Fujita, Y. Mori, and K. Kawabe, *Anal. Chem.*, **51**, 534 (1979).
10) S. Ikenoya, K. Abe, T. Tsuda, Y. Yamano, O. Hiroshima, M. Ohmae, and K. Kawabe, *Chem. Pharm. Bull.*, **27**, 1237 (1979).
11) Y. Hatefi, *Biochem. Biophys. Acta.*, **31**, 501 (1959).
12) E.R. Redfern and A.M. Pumphery, *Biochem. J.*, **76**, 61 (1960).
13) F.L. Crane and R. Barr, "Methods in Enzymology," D.B. MacCormick and L.D. Wright, Ed., Academic Press, New York, 1971, Vol. 18.
14) *a*) L. Szarkowska and M. Klingenberg, *Biochem. Z.*, **338**, 674 (1963); *b*) K. Kröger and M. Klingenberg, *ibid.*, **344**, 317 (1966).
15) S. Rehncrona, L. Mela, and B. Chance, *Fed. Proc.*, **38**, 2489 (1979).
16) O.H. Lowry, N.J. Rosengrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17) F.L. Crane and R.A. Dilley, "Methods of Biochemical Analysis," D. Glick, Vol. XI, Interscience Publishers, New York, 1967.