[Chem. Pharm. Bull.
13(2) 136~142 (1965)]

UDC 547.567.2.07

19. Isuke Imada and Hiroshi Morimoto : Photochemical Reaction
of Ubiquinone (35). VI.*¹ Coenzymatic Activity of
Ubiquinone (35) and Related Compounds.*²

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The authors have clarified the chemical properties of ubiquinone (35) and related compounds over the preceding five reports.^{1~5)} The present paper deals with the biochemical properties of these compounds and some ubiquinone homologues investigated by the following two methods.

Chart 1.

*¹ This paper constitutes Part VII of a series entitled "On the Components of Yeast"; Part VI : This Bulletin, 13, 130 (1965).

*² Brief reports of a part of this work were published as a Communication to the Editor in this Bulletin, 11, 815 (1963); *Ibid.*, 12, 739 (1964).

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1) I. Imada, Y. Sanno, H. Morimoto : This Bulletin, 12, 1042 (1964).

2) I. Imada, H. Morimoto : *Ibid.*, 12, 1047 (1964).

3) *Idem* : *Ibid.*, 12, 1051 (1964).

4) I. Imada, Y. Sanno, H. Morimoto : *Ibid.*, 12, 1056 (1964).

5) I. Imada, H. Morimoto : *Ibid.*, 13, 130 (1965).

6) I. Imada, S. Wada, H. Shimazono, N. Miyata, M. Miwa : Nippon Nôgei-Kagaku Kaishi, 37, 580 (1963).

Reactivation of Succinate Oxidase System Inactivated by Extraction with Acetone

Since Nason and Lehman⁷⁾ introduced the technique of solvent extraction of respiratory particles for investigating the activity of electron carriers in cells, some improved methods have been reported. In short, in these methods it is necessary to conduct the extraction under mildest possible conditions, and in this sense the method of Lester, *et al.*⁸⁾ employing beef-heart mitochondria inactivated with acetone containing 4% of water is most suitable. Although Weber, *et al.* and Redfearn, *et al.*⁹⁾ have reported that succinic-cytochrome c reductase activity in solvent-extracted mitochondria could be restored by addition of vitamin K₁ or vitamin E, in the present study these vitamins could not reactivate the enzyme preparation obtained by the method of Lester, *et al.*, and only ubiquinone was effective in restoring the activity specifically. However, the reactivation by ubiquinone was not so complete as reported by Lester, *et al.* The supplementation of the system with phospholipid fractions extracted from beef-heart mitochondria and

TABLE I. Reactivation of Succinate Oxidase System of Extracted Mitochondria with Ubiquinone and Related Compounds

Enzyme	Addition (γ)	Specific activity (μ moles of succinate/min./mg. protein)	
		Expt. No. 1	Expt. No. 2
Lyophilized mitochondria	none	0.45	0.43
Extracted mitochondria	none	0.01	0.07
	I (300)	0.16	0.18
	(200)	0.13	—
	(100)	0.08	—
	mitochondrial phospholipid (2 mg.)	0.02	—
	I (300)+mitochondrial phospholipid (2 mg.)	0.17	—
	III (300)	—	0.18
	vitamin K ₁ (300)	—	0.02
	vitamin E (300)	—	0.04

from *Candida utilis* were without any beneficial effect. Moreover, ubiquinone (50) (III) showed the same effect as ubiquinone (35) (I) (Table I) and a proportional relation was observed between the reactivation and the amount of added (I) (Fig. 1). So the compounds shown in Chart 1 were investigated by this method (Table II) and it was made clear that the reactivation caused by these compounds was inhibited by antimycin A,^{*4} indicating that like ubiquinone these compounds are actually participating in the respiratory mechanism of mitochondria. Especially the following points were made clear.

Fig. 1. Reactivation of Succinate Oxidase System as a Function of I Concentration in Acetone Treated Mitochondria

*4 Antimycin A was kindly given by Dr. A. Miyake of the research laboratories.

7) A. Nason, I. R. Lehman: *Science*, **122**, 19 (1955); *Idem*: *J. Biol. Chem.*, **222**, 511 (1956).

8) R. L. Lester, S. Fleischer: *Biochim. Biophys. Acta*, **47**, 358 (1961).

9) F. Weber, U. Gloor, O. Wiss: *Helv. Chim. Acta*, **41**, 1038 (1958); *Idem*: *Ibid.*, **41**, 1046 (1958); F. Weber, O. Wiss: *Ibid.*, **42**, 1292 (1959); E. R. Redfearn, A. M. Pumphrey, G. H. Fynn: *Biochim. Biophys. Acta*, **44**, 404 (1960).

TABLE II. Reactivation of Succinate Oxidase System of Extracted Mitochondria with Ubiquinone and Related Compounds

Compound	Addition (γ)	Relative activity to I (250 γ) (%)	Compound	Addition (γ)	Relative activity to I (250 γ) (%)
I	250	100	VII	250	7 $\pm$ 2
	125	71 $\pm$ 15		125	4 $\pm$ 1
II	250	95	X	250	100
	125	72		125	75
III	250	96	XI	250	0
	125	70		125	0
IV	250	1	XII	250	46
	125	3		125	18
V	250	117	XIII	250	35 $\pm$ 15
	125	78		125	25 $\pm$ 15
VI	250	3	XIV	250	26 $\pm$ 10
	125	2		125	15 $\pm$ 10
VII	250	34			
	125	22			

i) The compounds such as ubihydroquinone(35) diacetate(VI) and ubichromenol(35) (VII), in which the quinone nucleus was converted into the benzene ring have no activity. Ubihydroquinone(35) diphosphate showed a slight activity, probably due to the formation of ubihydroquinone(35) by the action of phosphatase of contaminating microsomes. From these facts it is evident that a quinone structure is essential for the restoration of activity¹⁰⁾.

ii) Demethylubiquinone (35) (X) was active but it lost the activity by conversion of the hydroxyl to acetoxyl, and therefore there seems to be no influence on the activity whether position 3 of the quinone nucleus has hydroxyl or methoxyl.*⁵ As mentioned above, both X and I are active and in the diluted state the former is rather stronger than the latter in activity. This may be explained by an increased polarity of X owing to its hydroxyl and by its easier accessibility to the active site of the enzyme system

TABLE III. Reactivation of Succinate Oxidase System of Extracted Mitochondria with Ubiquinone and Related Compounds

Enzyme	Addition (γ)	Specific activity (μ moles of succinate/min./mg. protein)	
		Expt. No. 1	Expt. No. 2
Lyophilized mitochondria	none	0.432	0.440
Extracted mitochondria	none	0.046	0.041
	antimycin A (10)	—	0.017
	I (300)	0.239	0.222
	(200)	0.184	—
	(150)	—	0.156
	(100)	0.114	—
	X (300)	0.209	0.208
	(200)	0.205	—
	(150)	—	0.180
	(100)	0.185	—
	X (300) + antimycin A (10)	—	0.024

*⁵ After the authors' communication on this point, Ozawa, *et al.* reported their studies in this field; H. Ozawa, K. Momose, S. Natori, H. Ogawa, K. Yamaguchi: *Biochim. Biophys. Acta*, 86, 395 (1964).

10) D. Hendlin, T. M. Cook: *J. Biol. Chem.*, 235, 1187 (1960).

(Table III, Fig. 2). As described in the preceding report⁴⁾ this compound forms a complex compound with magnesium. To investigate the influence of the complex combination on activity, 1% ethanolic magnesium acetate solution of X was added, but no change was observed in the restoration of activity. As is known,¹¹⁾ hydroxyquinones undergo conversion of (A) $\rightleftharpoons$ (B) according to the pH of the medium and such a conversion (C) $\rightleftharpoons$ (D) is conceivable also in X, and therefore it is possible that pH exerts influence over activity.

iii) It was found that I, ubiquinone (45) (II) and (III) showed no difference in

Fig. 2. Reactivation of Succinate Oxidase System with I and X

————— Expt. No.1 - - - - - Expt. No. 2

activity. This fact shows that in the present test method, difference in the number of isoprene unit in the side chain has no influence on activity within the limit, but 5-methyl-2,3-dimethoxy-*p*-benzoquinone (IV) which has no isoprene side chain, exhibited no activity. In perhydropubiquinone (35) (XII), in which the side chain is saturated, the activity decreased by half. Difference between this result and that of Hendlin, *et al.*¹⁰⁾ must be due to the difference in the enzyme preparation used in the test. As seen in isoubiquinone (35) (XIII) and γ -hydroxyisoubiquinone (35) (XIV), it is noteworthy that when one of the double bonds in the isoprene side chain conjugated with the quinone nucleus, the activity lowers to 1/3.

In XII as there were produced many new asymmetric centers, their effects on the activity of this compound are to be clarified in future. In XIII and XIV the effects of the conjugated double bonds in their side chains on quinone $\rightleftharpoons$ hydroquinone system and their geometric configuration remain to be made clear hereafter.

Effects on Mitochondrial Succinate Oxidase System

Jacobs, *et al.* and Smith, *et al.*¹²⁾ have reported that IV and 5-methyl-6-bromo-2,3-dimethoxy-*p*-benzoquinone inhibited irreversibly the succinate oxidase and pyruvate

11) L. F. Fieser, J. L. Hartwell: J. Am. Chem. Soc., 57, 1484 (1935); L. F. Fieser, M. Fieser: *Ibid.*, 61, 596 (1939).

12) E. E. Jacobs, F. L. Crane: Biochem. Biophys. Research Commun., 3, 333 (1960); A. L. Smith, R. L. Lester: Biochim. Biophys. Acta, 48, 547 (1961).

oxidase activities of beef-heart mitochondria as well as phosphorylation coupled to these oxidase activities. The compounds shown in Table IV were added to intact beef-heart mitochondria, and inhibition of the activity of the succinate oxidase system by these compounds was investigated. It was thus found that, except for I and X which showed no inhibition, all the compounds tested were inhibitory. As XII, XIII, and XIV in Table IV group showed reactivation of solvent-extracted mitochondria as mentioned above they seem to inhibit competitively with I or a part of electrons to be carried through succinate oxidase system may be leaked to these compounds. Since the inhibition by VIII was not observed with its acetate (IX), the hydroxyl seems to participate in the inhibition.

TABLE IV. Effect of Various Supplements on Succinate Oxidase System of Beef-Heart Mitochondria

Compound	Addition (γ)	Specific activity (μ moles of succinate/min./mg. protein)	Respiration rate (%)
	none	0.377	100
I	250	0.401	107 $\pm$ 2
VI	250	0.352	92
VII	250	0.342	91
VIII	250	0.311	83 $\pm$ 1
	125	0.332	88
IX	250	0.385	102
XII	250	0.339	91
XIII	250	0.322	86 $\pm$ 3
	125	0.332	88
XIV	250	0.326	87
	125	0.320	85

The results reported in the present paper, in which the action of I and related compounds on the succinate oxidase system are described, have revealed a facet of the biochemical properties of these compounds. However, the results are still preliminary and it seems necessary to clarify further the relation between the physiology of intact mitochondria and acetone-treated mitochondria. Since VIII, X, and XIII have been obtained from I by photochemical reactions, and as it is reported¹³⁾ that substances similar to X and XIII are present in photosynthetic bacteria, their role in photosynthesis is very interesting. Further, as it was reported that the homologues of VIII have vitamin E-like activity,¹⁴⁾ the authors wish to have a chance to investigate further physiological activities of individual compounds.

Experimental^{*6}

Mitochondria—The mitochondria prepared as described by Crane, *et al.*¹⁵⁾ from beef-heart muscle and they were suspended in 0.25M sucrose to a concentration of 20 mg. enzyme protein/ml. and stored at -15° . The suspension was used within 10 days after preparation.

^{*6} Melting point is uncorrected.

- 13) J. Glover, D.R. Threlfall: *Biochem. J.*, **85**, 14 p (1962); I. Chmielewska: *Biochim. Biophys. Acta.*, **39**, 170 (1960).
- 14) B.C. Johnson, Q. Crider, C.H. Shunk, B.O. Linn, E.L. Wong, K. Folkers: *Biochem. Biophys. Research Commun.*, **5**, 309 (1961); A.C. Page, Jr., M.C. Smith, P.H. Gale, D. Polin, K. Folkers: *Ibid.*, **6**, 141 (1961); E. Sndergaard, M.L. Scott, H. Dam: *J. Nutrition*, **78**, 15 (1962); J.S. Dinning, C.D. Fitch, C.H. Shunk, K. Folkers: *J. Am. Chem. Soc.*, **84**, 2007 (1962); J.S. Dinning, A.S. Majaj, S.A. Azzam, W.J. Darby, C.H. Shunk, K. Folkers: *Am. J. Clin. Nutr.*, **13**, 169 (1963).
- 15) F.L. Crane, J.L. Glenn, D.E. Green: *Biochim. Biophys. Acta*, **22**, 475 (1956).

Treatment of Mitochondria with Acetone—According to the method of Lester, *et al.*⁸⁾ the suspension of mitochondria (2 ml.) was added dropwise to acetone (50 ml.) over a period of 5 min. with stirring. Stirring was continued for additional 5 min. and then the suspension was centrifuged at 20000 *g* for 10 min. The sedimented precipitate was resuspended in acetone (50 ml.), the suspension was treated as above and the resulting precipitate was suspended in 0.25*M* sucrose (50 ml.). The latter suspension was stirred for 5 min. and centrifuged at 20,000 *g* for 10 min. and the precipitate thus obtained was preserved as a suspension in 0.25*M* sucrose (1 ml.) under cooling with ice and used within 6 hr. after preparation. The above process was conducted at 3°, and the acetone was cooled to -15° beforehand.

Measurement of the Activity of Succinate Oxidase System—A conventional Warburg manometer was used, and the specific activity was calculated from the oxygen volume of the gas phase (air) consumed at 37.5° in 30 min. and expressed as μ mole of the succinate consumed in 1 min. per mg. of the enzyme protein.

Composition of the reaction mixture: Main compartment; phosphate buffer (pH 7.5) (100 μ moles), cytochrome *c* (2 mg.), enzyme protein (2 mg.). Side arm; sodium succinate (150 μ moles). Center well; 6*N* KOH (0.2 ml.). Total liquid volume; 3.2 ml. Measurements of the reactivation of the enzyme system inactivated by treatment with acetone and effect on mitochondrial enzyme system were conducted after adding the ethanolic solution (0.05 ml.) of a test sample to the main compartment, but when VII was test sample, its aqueous solution was employed. Inhibition test with antimycin A was carried out after adding its 0.1% ethanolic solution (0.01 ml.) to the main compartment.

Determination of Enzyme Protein—The determination was carried out according to Lowry's method¹⁶⁾ and the result was calculated in terms of egg albumin.

Mitochondrial Phospholipid—To lyophilized mitochondria (4 g.) was added ether- $\text{C}_2\text{H}_5\text{OH}$ (1:3) (100 ml.) at 3° and the mixture was stirred for 5 min. and centrifuged at 5000 *g* for 10 min. The sedimented precipitate was extracted three times in the same manner as above and the combined extracts were evaporated to dryness in N_2 *in vacuo*. The residue was dissolved in $\text{C}_2\text{H}_5\text{OH}$ (10 ml.), five times its volume of acetone was added, and the portion insoluble in acetone was collected and washed twice with acetone to give the phospholipid (150 mg.), which was used as a suspension in H_2O (7.5 ml.).

Yeast Phospholipid—According to the previous paper,⁶⁾ dried cells of *Candida utilis* (1 kg.) was added to warm CH_3OH (4 L.), and the mixture was stirred at 45° for 1 hr. and filtered. The filtrate was concentrated to $\frac{1}{2}$ volume *in vacuo* and left standing at 0°. The resulting white precipitate of ergosterin was filtered off, the lipid in the filtrate was extracted three times with $\frac{1}{2}$ its volume of hexane, and the extract was evaporated to dryness *in vacuo* to afford a residue (10 g.), which, after washing three times with $\text{C}_2\text{H}_5\text{OH}$ (20 ml.) gave yeast phospholipid chiefly consisting of lecithin and containing no ubiquinone (35) (I). As in the case of mitochondrial phospholipid, the product was used as a suspension in water.

Ubiquinone (50) (III)—A fresh beef-heart free of fat (400 g.) was minced finely and refluxed under heating for 30 min. with pyrogallol (30 g.) and 10% methanolic NaOH solution (600 ml.). The hydrolysate was cooled and extracted three times with heptane (300 ml.) and the combined extract (875 ml.), after washing thrice with H_2O (500 ml.) and drying over Na_2SO_4 , was evaporated to dryness *in vacuo*. The residue was dissolved in $\text{C}_2\text{H}_5\text{OH}$ (10 ml.), the solution was kept at 5° overnight, the resulting white precipitate of cholesterol was removed, and the clear supernatant was left standing at 0°, separating III m.p. 48.8°, as yellow plates (30 mg., 0.0075%).

Ubiquinol (35) (V)—To a solution of I (490 mg.) in $\text{C}_2\text{H}_5\text{OH}$ -ether (1:1) (50 ml.) was added dropwise 10% $\text{Na}_2\text{S}_2\text{O}_4$ solution (20 ml.) and the mixture was shaken well in a separatory funnel in an atmosphere of N_2 , giving a colorless solution. H_2O (50 ml.) was added to the solution, the ether layer was separated, and the aqueous layer was again extracted three times with ether (30 ml.). The ether solution was washed three times with water saturated with MgSO_4 , dried and evaporated to dryness in N_2 to yield V as a nearly colorless oil (458 mg., 93.2%). UV $\lambda_{\text{max}}^{\text{EtOH}}$ m μ : 290 (hydroquinone). IR $\nu_{\text{max}}^{\text{liquid}}$ cm^{-1} : 3600, 3500 (OH); no quinone absorption.

Ubiquinol (35) diacetate (VI)—To V (500 mg.) were added acetic anhydride (10 ml.) and pyridine (0.5 ml.) and the mixture was left standing overnight at room temperature. The reaction mixture was poured into ice water (100 ml.) and extracted three times with ether (30 ml.), and the ether solution was washed with water, dried and evaporated to dryness *in vacuo*, leaving VI as a pale yellow oil (490 mg., 86.9%). IR $\nu_{\text{max}}^{\text{liquid}}$ cm^{-1} : 1785, 1190 (CH_3CO).

Sodium Salt (VII) of Ubiquinol (35) diphosphate—To a solution of V (800 mg.) in pyridine (50 ml.) was added POCl_3 (8 ml.) and the mixture was left standing at room temperature for 17 hr. The reaction mixture was evaporated to dryness *in vacuo*, the residue was dissolved in water, and the solution was extracted three times with ether (1.5 L.). The ether solution was evaporated to dryness under reduced pressure, a solution of the residue in H_2O (10 ml.) was adjusted to pH 8.0 with *N* NaOH and evaporated to dryness *in vacuo*, and the residue was reprecipitated from $\text{C}_2\text{H}_5\text{OH}$ - H_2O to give VII as a white

16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall: J. Biol. Chem., 193, 265 (1951).

powder (860 mg., 75.2%). *Anal.* Calcd. for $C_{44}H_{66}O_{10}Na_4P_2 \cdot 2H_2O$: C, 55.93; H, 7.47; P, 6.56. Found: C, 56.37; H, 8.02; P, 6.53.

The authors wish to express their deep gratitude to Dr. K. Tanaka, head of the second division of the research laboratories, for his kind encouragement and to Dr. Y. Sanno of this laboratory for his kind help. They are also indebted to Professor R. Sato, Institute for Protein Research, Osaka University, for his valuable advice.

Summary

1) Using the mitochondria treated with acetone, measurement was made about the activity-restoring effect of ubiquinone (35) (I) and related compounds on the succinate oxidase system, and as the partial structure required for the effect the following points were made clear: i) the quinone nucleus is necessary, ii) the methoxyl at position of 3 may be changed to a hydroxyl but if it is converted to acetoxyl the effect is lost, iii) the length of the isoprene side chain has no influence so long as the number of the isoprene is 7~10, but when the double bonds of the side chain are saturated, the activity decreases to 1/2, and when one of the double bonds is conjugated with the quinone nucleus, the activity lowers to 1/3.

2) When added to beef-heart mitochondria, some substances in Table IV other than ubiquinone (35) (I) and ubiquinone (35) acetate (K) show slight inhibition of the succinate oxidase system.

(Received August 31, 1964)

[Chem. Pharm. Bull.
13(2) 142~155 (1965)]

UDC 547.773.07

20. Akira Takamizawa and Yoshio Hamashima: Syntheses of Pyrazole Derivatives. IX.*¹ Acetylation Products of 7-Aminopyrazolo[1,5-*a*]pyrimidines.

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In general, on an alkylation and acylation of aminopyrimidine derivatives, it has been known¹⁾ that the alkylation proceeds to the ring nitrogen and the acylation to a side amino group. In a previous paper,²⁾ we also confirmed that the methylation of 7-amino-3,6-dimethylpyrazolo[1,5-*a*]pyrimidine (XXXII) gave 7-imino-3,4,6-trimethyl-4,7-dihydropyrazolo[1,5-*a*]pyrimidine (XXXV). Furthermore, the structure of diacetate (XXXIV) which was prepared by an acetylation of XXXII was presumed to be 4-acetyl-7-acetyl-imino-3,6-dimethyl-4,7-dihydropyrazolo[1,5-*a*]pyrimidine. However, later the monoacetate (XXXIII) was obtained by alumina chromatography of XXIV. The ultraviolet absorption spectrum of XXXIII was similar to that of XXXIV and obviously different from XXXV. From these experimental results, we have reinvestigated the structure of diacetate and established that the diacetate was actually 7-diacetylamino-3,6-dimethylpyrazolo[1,5-*a*]pyrimidine (XXXIV).

*¹ Part VIII: A. Takamizawa, H. Sato: *Yakugaku Zasshi*, 85, 158 (1965).

*² Sagisu, Fukushima-ku, Osaka (高見沢 映, 浜島好男).

1) D. J. Brown: "The Pyrimidines," (1962), John Wiley and Sons Inc., New York, N. Y.

2) A. Takamizawa, S. Hayashi: *Yakugaku Zasshi*, 83, 313 (1963).