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Studies on Vitamin E

4. THE SIMULTANEOUS DETERMINATIONS OF TOCOPHEROLS, UBIQUINONES AND UBICHROMENOLS (SUBSTANCE SC) IN ANIMAL TISSUES: A RECONSIDERATION OF THE KEILIN–HARTREE HEART PREPARATION*

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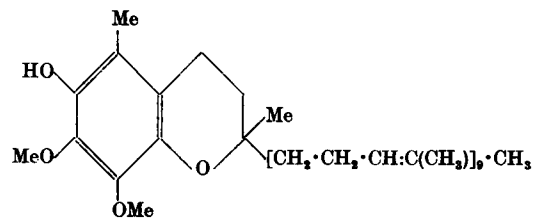
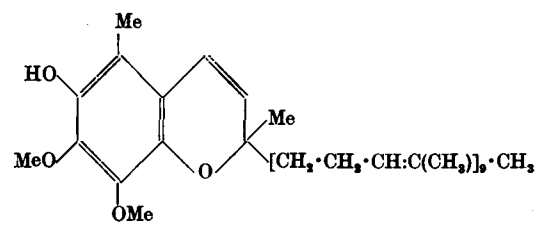
Bouman & Slater (1957), in a study of the Keilin–Hartree heart preparation, observed that it contained appreciable amounts of a substance they believed to be α-tocopherol. They found further that by treating the lipid extract with a mixture of ascorbic acid and ethanolic hydrochloric acid, a naturally occurring quinone, thought to be tocopherylquinone, was reduced and cyclized to a new substance apparently identical with α-tocopherol (criteria being ultraviolet spectrum and chromatography on zinc carbonate-treated paper). More recently, Bouman, Slater, Rudney & Links (1958) have reconsidered the interpretation of their original observations, in view of their findings that ubiquinone 50, a naturally occurring quinone [in particularly high concentration in normal heart, see Lester & Crane (1959)] could be reduced and cyclized to give a product with the same ultraviolet maximum and apparently the same chromatographic behaviour as α-tocopherol. The nature of this product has not been confirmed, but it may have the structure of the chromanol (I). In a personal communication, Professor R. A. Morton has informed us that the nature of the cyclized products may depend on the reagents used, certain reagents apparently causing alterations in the side chain. We have therefore preferred for the present to call the product RC-ubiquinone. Bouman *et al.* (1958) concluded that the formation of this product accounted quantitatively for the earlier results of Bouman & Slater (1957) and were of the opinion that the presence of α-tocopherylquinone in heart remained to be established.

Several factors combined to urge a reinvestigation of these findings. Our own studies indicated that the chromatographic data given by Bouman *et al.* (1958) could hardly be correct. Work by Edwin, Diplock, Bunyan & Green (1960) has shown that animal tissues contain a reducing substance that simulates α-tocopherol (from which it can, however, be separated by two-dimensional paper chromatography) and that this substance runs near ubiquinone on chromatograms. Meanwhile Laidman, Morton, Paterson & Pennock (1959) have shown that a reducing substance isolated from human kidney and from several other animal tissues (Lowe, Morton & Harrison, 1953; Cunningham & Morton, 1959) has the structure (II).



(I)



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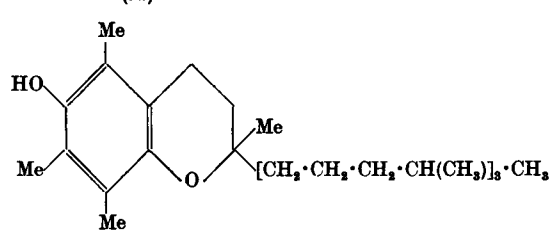

(III)

This substance, previously designated by Morton and co-workers as substance SC, and now called by them ubichromenol, is obviously related to RC-ubiquinone 50 and may indeed be the 3:4-dehydro derivative of the latter. Its chemical similarities to α-tocopherol (III) are equally apparent and the experiments that follow indicate that the ubichromenols are the main non-tocopherol-reducing substances remaining in animal tissues after classical methods of purification of tissue extracts for vitamin E analysis. This paper compares the

* Part 3: Bunyan, Green, Edwin & Diplock (1960).

chromatographic behaviour of ubiquinones, ubichromenols, RC-ubiquinone, tocopherols and certain other substances. It also describes an analytical procedure for the simultaneous determination of the three groups of compounds. The Keilin–Hartree heart preparation has been reinvestigated.

EXPERIMENTAL AND RESULTS

Nomenclature

The term ubiquinone was originally proposed by Morton, Wilson, Lowe & Leat (1957) to describe a quinone isolated from liver. It is now known (Lester, Crane & Hatefi, 1958) that a series of such quinones exist in Nature, and five compounds, differing from each other only by isoprenoid units, have already been isolated. The normal ubiquinone found in higher mammalian tissues, such as ox and pig heart, has been considered by Lester & Crane (1959) to have a C_{50} side chain (see also Morton *et al.* 1958), but recently Rüegg *et al.* (1959) have shown that the ubiquinone of rat liver is the C_{45} 'isoprenologue', and Diplock, Edwin, Green, Bunyan & Marcinkiewicz (1960) have demonstrated that the C_{50}, C_{40} and C_{35} compounds also exist in rat liver. In the text below we have indicated, wherever possible, which ubiquinone is being examined by giving the number of carbon atoms in the side chain; thus the compound from pig heart is ubiquinone 50. Where the term ubiquinone is used without a number, either its identity is obvious from the text or else it is used in a generic sense and applies to any of the group of compounds. The term ubichromenol refers to the cyclized chromenol, formally derived from the ubiquinone which is isomeric with it; thus ubichromenol 50 contains a C_{47} side chain etc. Diplock *et al.* (1960) have found that a series of such ubichromenols also exists in animal tissues, the chief one in rat liver being ubichromenol 45. Lester *et al.* (1958) have called the series of ubiquinones, coenzymes Q, but one advantage of the ubiquinone terminology is the convenience with which the quinones may be related to the isomeric ubichromenols.

Materials

Ubiquinone 50 and ubiquinone 30 were kindly given by Professor R. A. Morton.

Ubichromenol 45. This was prepared from the livers of vitamin E-deficient rats, which were a convenient source since an otherwise difficult separation from tocopherol could be avoided. The livers (400 g.) from rats were homogenized at $-70°$ by the sodium sulphate–acetone method of Edwin *et al.* (1960). The lipid was extracted with acetone in a Soxhlet apparatus for 3 hr. and then saponified, sterols were removed and the material was purified by the methods described for vitamin E analysis (Edwin *et al.* 1960). The total extract was chromatographed on 60 cm.-wide $ZnCO_3$-treated paper, after pilot strips had shown that the concentration was low enough to prevent co-chromatography of the ubichromenol with ubiquinone (see below). After a 1 hr. run in *cyclo*hexane–benzene, the bands were viewed under u.v. light and the ubichromenol band was removed. Elution from the paper with ethanol gave ubichromenol ($\lambda_{max.}$ 275 mμ) still somewhat contaminated with ubiquinone. It was further purified by chromatography on a 20 cm. × 1·5 cm. column of alumina [Peter Spence and Sons Ltd., type O; see Cunningham & Morton (1959)]. The ubiquinone was eluted with 10% (v/v) of ethanol in ether. The ubichromenol remained as a pink band on the column and was eluted with 15% (v/v) of ethanol in ether. The material was freed from traces of ubiquinone by two more separations on alumina. The final eluate, on evaporation in N_2, gave ubichromenol as a pale-yellow oil (1·4 mg.). Its u.v. spectrum was very similar to that given for the pure substance by Cunningham & Morton, with $\lambda_{max.}$ at 233, 275 and 332 mμ, an inflexion at 281 mμ and $\lambda_{min.}$ at 259 and 302 mμ. $E^{1\%}_{1\,cm.}$ for the preparation was 71, whereas Professor Morton has informed us that $E^{1\%}_{1\,cm.}$ for pure ubichromenol 50 is 96·1.

Chromatographic studies on zinc carbonate-treated paper

RC-*ubiquinone*. Bouman & Slater (1957) have recorded almost identical R values for the migration of α-tocopherol, ubiquinone 50, RC-ubiquinone 50 and ubiquinol 50 on $ZnCO_3$-treated paper (0·71, 0·77, 0·73 and 0·73 respectively). [Although several workers have preferred to use the term 'R_F value', as defined by Consden, Gordon & Martin (1944), for paper-partition chromatography, we have found it convenient to use the term 'R value' to describe migrations on $ZnCO_3$-treated paper. This is in accordance with the original terminology Le Rosen (1942) applied to adsorption columns where, as on $ZnCO_3$-treated papers, migrations are conventionally measured from the position of maximum concentration, i.e. the head of the band. Moreover, there is some convenience in being able to distinguish in this way between adsorption and partition runs.] It seemed likely, however, that these figures were in part incorrect, if the likely effect of the structural groups that are substituents in the ring system of RC-ubiquinone are considered. In RC-ubiquinone, the accelerating effect of the long unsaturated side chain (compared with α-tocopherol) would be expected to be overcome by a pronounced retarding effect of the nuclear methoxy groups. Thus on $ZnCO_3$-treated paper, with benzene as solvent, the R values of *o*-, *m*- and *p*-cresol are 0·63, 0·44 and 0·44 respectively and those for 2-, 3- and 4-methoxyphenol are 0·26, 0·33 and 0·33 respectively. The *o*-methoxy group, it should be noted, unlike the *o*-methyl group, retards chromatographic migration, probably owing to complexing of the two vicinal oxygen atoms to the zinc atoms through a bridge structure. It would thus be expected that RC-ubiquinone would have an R value in adsorption chromatography nearer to that of γ-tocopherol than to α-tocopherol. When RC-ubiquinone 50 was prepared by the ascorbic acid–HCl method of Bouman & Slater (1957), the product contained only about 20% of the desired substance, the remainder being unchanged ubiquinone. When this product was chromatographed in amounts not exceeding 10 μg., the RC-ubiquinone separ-

ated and migrated to a position near that of γ-tocopherol (R 0·45). When large amounts were run, RC-ubiquinone co-chromatographed with ubiquinone and had R 0·70, thus explaining the findings of Bouman & Slater.

Ubiquinones and ubichromenol. Both ubiquinone 50 and ubiquinone 30 have R values on $ZnCO_3$-treated paper of about 0·75 (slightly faster than α-tocopherol). When ubiquinone is chromatographed together with small quantities of tocopherols, RC-ubiquinone or ubichromenol, all these latter substances may co-chromatograph with the ubiquinone, thus exhibiting spurious R values. Ubichromenol is chromatographically indistinguishable from the reduced and cyclized compound prepared from the ubiquinone of the same molecular weight, in the systems used by us.

Separation of ubiquinones, ubichromenols, tocopherols and some other substances by two-dimensional paper chromatography

When preparations of animal tissue (purified by the methods described below) containing ubiquinones, ubichromenols, tocopherols, vitamin K_1 and α-tocopherylquinone are chromatographed in the two-dimensional adsorption–partition system of Green, Marcinkiewicz & Watt (1955), with 95 % ethanol as mobile phase in the second dimension, the substances separate as shown schematically in Fig. 1. Provided that the preliminary steps of purification are rigorously carried out (in particular no traces of fat must remain), the factor that determines the separation pattern is the amount of the main ubiquinone present. As shown by Diplock *et al.* (1960) there may be more than one ubiquinone present in the tissue (particularly so in liver), but there is always one in preponderance. The amount of this ubiquinone is nearly always much greater than that of the other lipid constituents in the extract and because of its high molecular weight, it materially affects the partition not only of other substances but also of itself, this in addition to the co-chromatography sometimes observed in the first dimension. Thus ubichromenols, α-tocopherylquinone and β- and γ-tocopherol, whose true positions are shown by the full lines in Fig. 1, may occasionally occupy the false (but easily recognizable) positions shown by the dotted lines. This in no way interfered with the subsequent removal and determination of the substances, but should better separation be required, it is often sufficient to run the chromatogram again at a lower loading.

The only substantial modification to the chromatographic separation that may be necessary is when an extract from a tissue such as liver is being examined and there is reason to believe that small quantities of lower-molecular-weight ubiquinones or ubichromenols may be present. As indicated above, these mixtures are usually only partially separable on the normal chromatograms. Complete separation can be obtained, however, if the partition run is extended to about 16 hr. and the second dimension of the chromatographic paper increased to about 35 cm. Fig. 2 shows schematically the separation of a rat-liver extract into four ubiquinones and three ubichromenols, after separation on the normal square sheet of paper had shown only one of each type of compound. Under these long-running conditions, provided that the loading is light enough, the molecular weight of each compound can be found by the addition of known markers or by calculation from R_M values.

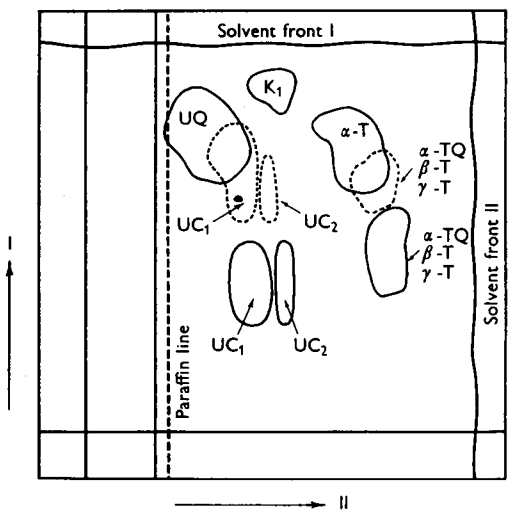

Fig. 1. Two-dimensional paper chromatography of preparations of animal tissues. The positions illustrated by the broken-line spots are those normally occupied by the substances indicated if much ubiquinone is present in the extract (for explanation, see text). K_1, Vitamin K_1; UQ, ubiquinone; UC_1 and UC_2, ubichromenols; α-T, α-tocopherol; α-TQ, α-tocopherylquinone; β-T, β-tocopherol; γ-T, γ-tocopherol.

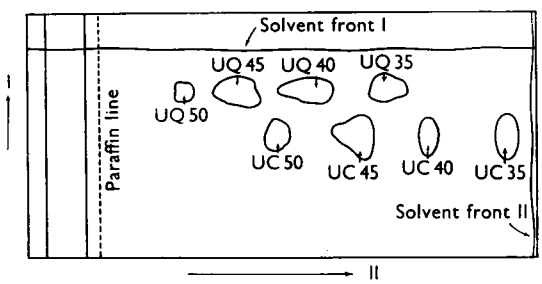

Fig. 2. Separation of a rat-liver extract by extending the partition run and increasing the second dimension of the chromatographic paper. UQ 50, 45, 40, 35, ubiquinones 50, 45, 40, 35 respectively. UC 50, 45, 40, 35, ubichromenols 50, 45, 40, 35 respectively.

All the ubiquinones, ubichromenols, tocopherols and other quinones are readily observed on the dried chromatograms under ultraviolet light. On paper treated with sodium fluorescein (Green, 1959) the limiting amounts observable are: tocopherol and all the quinones, about 1–2 μg; ubichromenols, 5–10 μg.

Colorimetric determination of ubichromenols

Ubichromenols can be determined by the ferric chloride–dipyridyl reaction in the same way as α-tocopherol. Under the conditions of oxidation normally used in these laboratories (Report of the Vitamin E Panel, S.A.C., 1959), the reaction is complete in 2 min., and extinctions at 520 mμ are linear with respect to concentration over the range usually encountered. The spectroscopic factor for the reaction of ubichromenol 45 is 276 at 520 mμ, compared with 98 for α-tocopherol. In view of the molecular-weight ratio between the two substances, it would appear therefore that less Fe^{2+} ion is produced in the oxidation of ubichromenol than in that of α-tocopherol [but see Boyer (1951) for a discussion of the influence of conditions on the mechanism of the ferric chloride–dipyridyl oxidation]. Since Kaunitz & Beaver (1944) found that the ferric chloride oxidation of α-tocopherol is influenced by the presence of certain lipid substances, a study was made of the effect of relatively large amounts of ubiquinone on the determination of ubichromenol (Table 1). Only very slight effects on the colour reaction were observed.

Simultaneous determination of ubiquinones, ubichromenols, tocopherols and vitamin A in animal tissues

The method of analysis is based on that described by Edwin *et al.* (1960) for tocopherols, except that purification is carried out on columns of Decalso F by a modification of the procedure of Crane, Lester, Widmer & Hatefi (1959) and the final paper-chromatographic separation is slightly altered.

(i) The tissue (20 g.) was homogenized with Na_2SO_4–acetone at −70° and extracted in the dark with acetone for 3 hr. After evaporation of the solvent the lipid extract was saponified in the presence of pyrogallol. It is important that saponification is complete. This can be assured by still further increasing the concentration of alkali and extending the time. For each gram of lipid or less, the saponification mixture consisted of 4 ml. of 5% (w/v) pyrogallol in ethanol and 2 ml. of saturated aqueous KOH solution. The mixture was heated in a boiling-water bath for 5 min. and then extracted with ether as usual. One-tenth of the non-saponifiable extract may be removed at this stage for measurement of vitamin A by the $SbCl_3$ method.

(ii) The remainder was dissolved in methanol (10 ml.) and sterols were removed at −15°. Where necessary, and particularly if the sterols after centrifuging were tinged with yellow, the solids were redissolved in a further 1–3 ml. of methanol and recrystallized once or twice.

(iii) The methanol-soluble fraction, after evaporation of solvent, was dissolved in light petroleum (b.p. 40–60°, 5 ml.). This was added to a column of Decalso F (5 g. in a tube 1·3 cm. in diameter) previously washed with the same solvent. After percolation of the extract at a rate of 1–1·5 ml./min. (controlled by a capillary outlet to the tube) the column was washed with a further 25 ml. of light petroleum and then eluted with benzene (35 ml.). The benzene eluate was evaporated at reduced pressure and the residue was dissolved in benzene (1 ml.) for paper chromatography.

(iv) Two-dimensional paper chromatography was carried out on $\frac{1}{4}$–$\frac{1}{3}$ of the total extract, as described by Edwin *et al.* (1960), except that 95% (v/v) ethanol was used as mobile phase for the second dimension. After the running, the papers were dried in a current of air (usually about 1 min.) and the spots are observed under u.v. light. Another chromatogram was sprayed heavily with the ferric chloride–dipyridyl reagent (freshly prepared) to indicate the positions of tocopherol and ubichromenol. As far as possible, exposure of papers and ubiquinone solutions to daylight should be avoided.

(v) Each clearly separated spot was cut out and eluted. Tocopherols were eluted with 3·5 ml. of 0·07% (w/v) αα′-dipyridyl in ethanol for the usual determination. Ubichromenols, if distinct, were eluted separately and determined with ferric chloride. Combined ubiquinone–ubichromenol spots were normally eluted with 6 ml. of ethanol; half the solution was used for ubichromenol determination and the remainder diluted with ethanol (if necessary) and the ubiquinone determined on a portion by the borohydride-assay method of Crane *et al.* (1959). To 3 ml. of solution was added 0·03 ml. of the KBH_4 solution. These proportions, as indicated by Crane *et al.*, are critical if turbidity is to be avoided. $E^{1\%}_{1\,cm.}$ (oxidized–reduced) at 274·5 mμ was found to be 140 on a pure specimen of ubiquinone 50. Tocopherylquinone and vitamin K_1 are only rarely observed (see below), but when visible can be determined by their ultraviolet spectra.

Table 1. *Effect of ubiquinone 50 on the ferric chloride–dipyridyl determination of ubichromenol 45*

Each substance or mixture of substances was dissolved in ethanol and the determination was in a final volume of 4 ml. of ethanol in a cell of 1 cm. light path.

Ubichromenol (μg.)	Ubiquinone added (μg.)	$E_{520\ m\mu}$
75·4	—	0·208
75·4	230	0·192
75·4	1000	0·227

Quantitative work and recovery experiments

Saponification. Table 2 shows the recovery of two levels of ubiquinone after saponification, assays being by the borohydride method. Even under these drastic conditions, a mean recovery of 85% was obtained.

Column chromatography. Preliminary experiments showed that chromatography on floridin

earth as practised for vitamin E was unsuitable for ubiquinone. Whereas from some batches of adsorbent nearly quantitative recoveries were obtained, other batches destroyed substantial but varying amounts of the adsorbed ubiquinone, depending on the exact method of column preparation. There is also a considerable hazard in the use of floridin earth as an adsorbent when very small amounts of tocopherols are present in the extract. In the examination of vitamin E-deficient tissues, for example, the total extract from 20 g. of tissue may contain as little as 10 μg. of tocopherol, and, at this level, losses often occur on floridin, whereas such quantities may be safely handled on Decalso F. Table 3 gives the results of recovery experiments with Decalso F. Substances investigated were cholesterol, vitamin A, vitamin K_1, β-carotene, α-tocopherol and ubiquinone 50, in amounts chosen as likely to be encountered during the analysis of 20 g. of animal tissue. After adsorption of the individual substances from light petroleum, the columns were eluted successively with 25 ml. lots of benzene and 5% (v/v) ether in light petroleum (b.p. 40–60°). Both tocopherol and ubiquinone were recovered nearly quantitatively and almost entirely in the benzene fraction. (The volume of benzene was increased to 35 ml. in subsequent analyses.) Small amounts of cholesterol and vitamin A could be removed completely by adsorption but could be eluted with more polar solvent mixtures. Vitamin K_1, at the level employed, could not be recovered. β-Carotene spread over all the fractions, but only a small proportion appeared in the benzene fraction, in an amount that in no way interferes with paper-chromatographic analysis.

Paper chromatography. Table 4 gives the results of experiments to determine the recovery of ubiquinone and ubichromenol. Each substance was chromatographed by the two-dimensional method, the ubichromenol being run together with ubiquinone in order to simulate conditions during a normal assay of animal tissue. Amounts of ubiquinone up to 1 mg. could be chromatographed and recovered in about 90% yield; ubichromenol was recovered quantitatively.

Overall assay recoveries. Although the recovery experiments described above gave satisfactory results, it was important to study more exactly the overall recovery of ubiquinones and ubichromenols to be expected from the complete analysis of animal tissue. The results from such experiments are grouped in Tables 5 and 6. In the first experiment, the kidneys from 12 rats were pooled, quartered and divided into two lots that were separately homogenized. Ubiquinone 50 (1 mg.) was added to

Table 2. *Recovery of ubiquinone* 50 *after saponification in the presence of pyrogallol*

Amount taken (μg.)	Amount recovered (μg.)	Recovery (%)
250	222	89
1000	800	80

Table 3. *Recovery of ubiquinone, α-tocopherol and some other substances from Decalso* F *columns*

Each substance was chromatographed in light petroleum (b.p. 40–60°, 25 ml.) and eluted successively with benzene (25 ml.) and 5% (v/v) of ether in light petroleum (25 ml.).

Material	Amount used (μg.)	Amount eluted (μg.)			Total recovery (%)
		Light petroleum	Benzene	Ether–light petroleum	
Cholesterol	500	None	None	None	—
Vitamin A	50	None	None	None	—
Vitamin K_1	200	None	None	None	—
β-Carotene	70	13·5	11·8	11·3	52
α-Tocopherol	260	None	244	8·1	97
Ubiquinone 50	890	None	816	29·2	95

Table 4. *Recovery of ubiquinone and ubichromenol after two-dimensional chromatography*

Substance	Amount chromatographed (μg.)	Amount recovered (μg.)	Recovery (%)
Ubiquinone 50	48·4	45·0	93·1
	96·8	88·2	91·2
	242	215	89·1
	500	455	91·2
	1000	892	89·2
Ubichromenol 45	49·1*	50·7	103

* Co-chromatographed with 460 μg. of ubiquinone 50.

Table 5. *Overall recovery of ubiquinone in tissue analysis, before and after paper chromatography*

1. Rat kidney (pooled) was divided into two parts, A (27·7 g.) and B (19·9 g.). To B, ubiquinone 50 (1 mg.) was added before extraction. A and B were extracted and A was further divided before chromatography on Decalso F; to one half of A, ubiquinone 50 (1 mg.) was added. Determinations were by the borohydride method, without paper chromatography.

2. Rat kidney (pooled, 22·8 g.) was divided into two equal parts. To one half was added ubiquinone 50 (0·86 mg.). The tissues were then assayed by the full method, including paper-chromatographic analysis.

Expt. no.		Control extract	Control + ubiquinone before extraction	Control + ubiquinone before Decalso	Recovery of added ubiquinone (%)
1A	Ubiquinone (μg. in extract)	685	—	1745	106
1B	Ubiquinone (μg./g. of original tissue)	99	141	—	84
2	Ubiquinone (μg./g. of original tissue)	141	209	—	91

Table 6. *Overall recovery of ubiquinone and ubichromenol in vitamin E-deficient rat liver*

Liver (45 g.) was divided into three equal portions: A was assayed, B was taken through the analytical procedure and added to portion C.

Sample	Ubiquinone* (μg./g.)	Ubichromenol (μg./g.)
A	96·2	46·4
B + C	94·6	44·7
Recovery of added substances (%)	98·0	96·1

* Total of three ubiquinones detected.

Table 7. *Effect of light on the extraction of ubiquinone* 50

Ubiquinone was dissolved in acetone (100 ml.) and the solution refluxed on a steam bath for 3 hr. in diffuse daylight and in the dark. The solutions were then evaporated and assayed by the borohydride method.

Conditions	Ubiquinone used (μg.)	Amount recovered (μg.)	Recovery (%)
Light	50·3	40·4	78·2
	212	154	72·6
Dark	50·3	46·5	90·2
	212	189	90·6

Table 8. *Ubiquinone assays before and after paper chromatography*

Sample	Ubiquinone assay (μg./g.) Before paper chromatography	After paper chromatography
Rat liver 1	129·0	96·2
Rat liver 2	94·0	92·0
Rat kidney	74·6	84·9
Keilin–Hartree preparation	1010	1126

one portion and both were then extracted. The extract from the unsupplemented homogenate was now divided into two parts, one of which was supplemented with ubiquinone 50 (1 mg.). The three extracts were then chromatographed on Decalso F and ubiquinone was measured directly in the eluates. In the second experiment, on a different sample of rat kidney, recovery of added ubiquinone 50 was tested through the complete procedure from extraction to paper chromatography. In a third experiment, in which the recovery of ubichromenol was examined, a different technique was used. Rat liver (45 g.), obtained from vitamin E-deficient animals, was homogenized saponified and divided into three equal portions. Two of the three extracts were carried through the complete analysis, but ubiquinone and ubichromenol were only determined in one extract. The final solution of the other extract was added to the third portion of liver, which was then carried through the analytical procedure again. The recoveries of both ubiquinone and ubichromenol are nearly quantitative (Table 6).

Effect of light on ubiquinone analysis. The recoveries of ubiquinone were materially affected if the preliminary extraction of the tissue was carried out in daylight. In Table 7 are shown the losses caused by heating small amounts of ubiquinone 50 in acetone in diffuse daylight.

Comparison of ubiquinone assays before and after paper chromatography

Crane *et al.* (1959) have commented on the existence of a material in heart lipid that shows increased absorption at 275 mμ after addition of borohydride. In our experience, this material is not always removed by the Decalso columns used in the present procedure (depending mainly on the nature of the tissue being analysed). The material appears to exist in several types of animal tissue

Table 9. *Analyses of lipid constituents before and after storage at −20°*

Tissue	Time of storage before analysis (weeks)	α-Tocopherol (μg./g.)	Ubichromenol (μg./g.)	Ubiquinone (μg./g.)
Keilin–Hartree preparation	0	44·8	23·0*	—
	28	42·6	30·8	—
Rabbit liver†	0	7·0	3·7	56·6
	6	6·9	3·9	59·4

* Assay by modified method, with floridin columns.

† From vitamin E-deficient animals.

Table 10. *Vitamin A recovery after homogenization, extraction and saponification*

Sample of rat liver was analysed by two methods. For details, see text.

Sample	Vitamin A (i.u./g. of tissue) Method A	Method B
Liver	1392	1241
Liver + 4120 i.u. of vitamin A palmitate*	—	5320

* Blue value spectroscopic factor 25·4.

Table 11. *Analysis of the Keilin–Hartree preparation*

Substance	Amount (μg./g. of protein)
α-Tocopherol (before reduction–cyclization)	44·8
α-Tocopherol (after reduction–cyclization)	65·2
Ubiquinone	3560
Ubichromenol	23·0
Ubichromenol + RC-ubiquinone (after reduction–cyclization)*	134·0

* Assuming identical spectrophotometric factors for ubichromenol and RC-ubiquinone.

and to be separable by paper chromatography from ubiquinone. Thus, as shown by some typical results in Table 8, in certain assays the ubiquinone determined after paper chromatography is considerably higher than before. A further source of error (also demonstrated by results in Table 8) may be encountered in some tissues; here the ubiquinone assay is considerably higher before paper chromatography than after. Since it has been adequately demonstrated that the recovery of ubiquinone from chromatograms under assay conditions is virtually quantitative, it would seem that there is another type of material remaining in the extract after Decalso which exhibits a change in *E* on borohydride treatment. Since in certain instances it has been observed that the *E* at 275 mμ actually drops to zero after borohydride addition, it is probable that a substance having fluorescence at this wavelength is the cause of the interference. It must be assumed therefore that assays of ubiquinone after Decalso are incorrect if they do not agree with those after paper chromatography.

Effect of storage of tissues at −20°

In a recent paper, Pollard & Bieri (1959) have commented on the variability of *R* values on $ZnCO_3$-treated paper and we agree with their insistence on the removal of sterols before chromatography. It seems that the 'slow' spot described by them is sometimes due to ubichromenol, although the reducing substance of zero migration must be another substance. Pollard & Bieri found an appreciable decrease of 'apparent' tocopherol after storage of tissues at −20°, and, by implication, a corresponding decrease of ubiquinone. In our experience, such losses only occur in tissues that are continually thawed and refrozen for sampling purposes (as may have been the case in the experiments of Pollard & Beiri). As Table 9 shows, we have found that losses of tocopherol, ubichromenol and ubiquinone are negligible over long periods in cold storage.

Vitamin A. A sample of liver prepared from stock-colony rats was divided into three equal parts. One part was analysed for vitamin A by the method (A) of Ames, Risley & Harris (1954). A second part was analysed by the present method (B) of homogenization, extraction and saponification. To the third part was added vitamin A palmitate and the analysis by method (B) repeated. Determinations were by the antimony trichloride reaction in each case. The results (Table 10) show reasonable agreement between the two methods and a 99 % recovery of added vitamin A. No precautions against the effect of light were taken in these experiments.

Analysis of the Keilin–Hartree heart preparation

The sarcosomal horse-heart preparation (Keilin & Hartree, 1940) was extracted by the two extraction procedures, designated A and B by Bouman & Slater (1957) and the extracts were analysed for the lipid constituents by the paper-chromatographic procedures described above. The chromatogram of the untreated extract (procedure A) clearly showed the presence of α-tocopherylquinone, which was

eluted from the paper and identified by its ultraviolet spectrum (peaks at 261 and 271 mμ in ethanol) and by co-chromatography with the pure substance. Ubiquinone, α-tocopherol and ubichromenol were also detected. The chromatogram of the treated extract (B) contained no tocopherylquinone, but a very large quantity of ubiquinone remained. The quantitative data (Table 11) show that only a small amount of the ubiquinone present can be cyclized by the ascorbic acid–HCl procedure, although even the relatively small amount of RC-ubiquinone formed is more than enough to obscure the true cyclized tocopherylquinone if two-dimensional chromatography is not used.

DISCUSSION

The method described for the simultaneous determination of several important lipid substances has been tested on a variety of animal tissues without any particular difficulty appearing. The only modification that is occasionally necessary is during the analysis of certain samples of liver which may have a high vitamin A content. If this is so, the vitamin A may not be retained by 5 g. of Decalso and will run close to α-tocopherol on the paper, where it will be observed as a strongly fluorescent yellow spot. Since vitamin A interferes with the colorimetric determination of tocopherol, this must be avoided by either increasing the size of the column and volume of eluting solvent appropriately or carrying out a separate tocopherol assay and using floridin earth for purification.

It would appear that some sort of paper-chromatographic separation is essential for the precise determination of ubiquinone. The present method seems efficient in that examination of the ultraviolet spectra of ubiquinone spots eluted from the two-dimensional chromatograms of extracts of animal tissues has revealed that in most cases they contain almost pure ubiquinone (the absorption of the small amounts of ubichromenol contributing only slightly to the total absorption). This has been done by comparing the results of the borohydride assay with determinations made from an accurate measurement of the ultraviolet spectra at three points, 265, 274·5 and 282·5 mμ, and comparison with the data from pure ubiquinone.

Recoveries of all the lipid substances have been adequately tested and the use of Decalso columns for purification gives particularly good results with tocopherol and ubichromenol. Although Festenstein, Heaton, Lowe & Morton (1955) and Ward & Moore (1955) found ubiquinone to be alkali-labile in their procedures, the recovery experiments described in the present work indicate that the substance is readily handled in alkali provided that pyrogallol is present. Ubiquinone is, however, light-sensitive and this may account for the results of these earlier workers. There is no need to saponify the whole tissue, which, provided that light is absent, can be subjected to normal lipid extraction.

The present work explains adequately the earlier results of Bouman & Slater (1957) and Bouman *et al.* (1958). It is clear that single-dimensional chromatography on zinc carbonate-treated paper is inadequate for the analysis of animal tissues and, owing to the co-chromatography phenomena we have described, R values can be grossly misleading. Bouman & Slater, by their cyclization procedure, produced the compound we have called RC-ubiquinone, which at first they confused with α-tocopherol. However, the present investigation has shown that their conclusions, i.e. that α-tocopherol and α-tocopherylquinone both exist in the heart preparation, are correct. We have found 44·8 and 20·4 μg./g. of protein respectively of these two substances in the horse-heart preparation; these amounts may well have been concealed by the procedure later described by Bouman *et al.* (1958). There appears to have been some doubt whether α-tocopherylquinone actually exists in tissues. Donaldson & Nason (1957) presumed it was present in their enzyme preparation from rat skeletal muscle but this conclusion was based on the same evidence (acid-cyclization and then chromatography on zinc carbonate-treated paper) as that of Bouman & Slater and is thus unreliable. Neither Draper & Alaupovic (1959) nor Pollard & Bieri (1959) could find the quinone in chick tissues, but Mervyn & Morton (1959) have recently offered positive spectroscopic evidence for its existence in human kidney. Our findings unequivocally support the existence of tocopherylquinone in animal tissue, although so far we have observed it only in the horse-heart preparation. It would not seem possible that quantities of tocopherylquinone of the order found in this preparation could be artifacts. We have never found, during normal laboratory handling, that tocopherols oxidize to give measurable amounts of quinones: degradation, if it occurs, seems usually to proceed beyond the quinone stage. Moreover, analysis of the 'supernatant' from the Keilin–Hartree preparation showed it to contain tocopherol, ubiquinone and ubichromenol in large amounts, but no tocopherylquinone. It would seem therefore that tocopherylquinone is mainly located in those structural components that appear in the final particulate preparation. The quantity observed is very small and this is, no doubt, why examination of whole tissues (where the concentration is relatively much smaller) usually fails to reveal its existence.

There has been a considerable amount of discussion about the presence of vitamin K_1 in animal tissues. Thompson (1957) considers that no direct

chemical evidence exists to indicate the occurrence of vitamin K compounds in animal tissues, and Lester & Crane (1959), who comment on this, looked exhaustively and unsuccessfully for the typical ultraviolet spectrum of vitamin K in their ubiquinone extracts. Nevertheless we have repeatedly observed vitamin K_1 in rat heart (total tissue), the Keilin–Hartree horse-heart preparation and in rat erythrocytes, its identity being confirmed by chromatography and the typical ultraviolet spectrum of the substance. Both quantitatively and qualitatively, however, its observation is uncertain (as indicated by the recovery experiments described above) and obviously depends on modifications of the analytical procedure not subject to control.

SUMMARY

1. A method is given for the simultaneous determinations of tocopherols, ubiquinones and ubichromenols in animal tissues. The substances are separated, after preliminary purification, on a two-dimensional paper-chromatographic system, from which they are eluted and separately measured. Vitamin A and tocopherylquinone can be determined in the same extract.

2. The colorimetric determination of ubichromenol, with ferric chloride and $\alpha\alpha'$-dipyridyl, is described.

3. The chromatographic behaviour of ubiquinones and ubichromenols has been investigated and conditions are given for their separation.

4. The existence of vitamin K_1 in animal tissue has been confirmed.

5. The Keilin–Hartree horse-heart preparation has been reinvestigated and earlier reports on the presence of α-tocopherol and α-tocopherylquinone have been confirmed.

6. No loss of tocopherol, ubiquinone or ubichromenol was found after prolonged storage of tissues at $-20°$.

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