Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
Vorbeck, M. L., Albury, M. N., Mattick, L. R., Lee, F. A., and Pederson, C. S. (1963), *J. Food Sci. 28*, 495.
Vorbeck, M. L., and Marinetti, G. V. (1964), *Federation Proc. 23*, 375.
Vorbeck, M. L., and Marinetti, G. V. (1965), *J. Lipid Res. 6*, 3.
Wade, H. E., and Morgan, D. M. (1953), *Nature 171*, 529.
Weenink, R. O. (1961), *J. Sci. Food Agr. 12*, 34.
Weibull, C. (1957), *Acta Chem. Scand. 11*, 881.
Williams, W. L., Broquist, H. P., and Snell, E. E. (1947), *J. Biol. Chem. 170*, 619.
Yudkin, M. D. (1962), *Biochem. J. 82*, 40P.
Zill, L. P., and Harmon, E. A. (1962), *Biochim. Biophys. Acta 57*, 573.

Components with Redox Potentiality in the "Neutral Lipid" Fraction from Beef Heart Mitochondria*

G. L. Sottocasa† and F. L. Crane‡

ABSTRACT: Four components have been regularly observed in the neutral lipid fraction from mitochondria which show the ability to undergo reversible oxidation and reduction. The first is coenzyme Q. The second is found in the fraction which contains α-tocopherol. The third is a compound which shows higher R_F values on thin-layer chromatograms than any of the known redox components, but the identity of the compound is not clear.

The fourth shows low R_F on thin-layer chromatography and has the ultraviolet spectrum of a benzoquinone. The relation of these components to previous studies of the effects of lipids on the electron transport system is discussed.

The importance of lipid material in the function of the mitochondrion has been many times underlined. Most attention has been focused on the phospholipids as important constituents of biologial membranes. Besides this major class of lipid-soluble material, however, there exists a neutral lipid fraction in mitochondria whose composition has never been well defined.

With the name "neutral lipid fraction" we refer to the petroleum ether–soluble fraction after removal of the phospholipids. The best known component of this fraction is coenzyme Q_{10}, whose involvement in the mitochondrial respiratory chain as an electron carrier has been widely studied (Crane *et al.*, 1959a,b; Lester *et al.*, 1959; Hatefi *et al.*, 1959; De Bernard *et al.*, 1960; Szarkowska and Klingenberg, 1963; Chance 1965). α-Tocopherol is another constant component which has been well recognized and studied, whose intervention as a catalyst in the respiratory chain has been suggested many times (Bouman and Slater, 1957; Bouman *et al.*, 1958; Cowlishaw *et al.*, 1957; Nason and Lehman, 1956; Donaldson and Nason, 1957; Pollard and Bieri, 1960; Nason *et al.*, 1963). Its role is still not clear (Symposium on Vitamin E, 1962). Also vitamins of the K group (Martius, 1961) have been suggested many times as important components of mitochondria and proposals have been made that they are involved in electron transport or in energy coupling to respiration, but all the direct attempts to demonstrate their presence or their function in mitochondria have failed.

Recently it was possible to demonstrate that two undefined factors present in the neutral lipid fraction were necessary to restore completely the succinate oxidase system of mitochondria or submitochondrial particles which had been extracted with isooctane or acetone. The activity of these factors was in addition to the effect of coenzyme Q in restoration of the oxidase (Crane *et al.*, 1959a,b; Hendlin and Cook, 1960; Lester and Fleischer, 1961).

Some other suggestions of a role of lipid material in the mitochondrial function can be found in the literature (Marinetti *et al.*, 1957; Crawford *et al.*, 1959; Redfearn and King, 1964).

Because of the studies mentioned it seemed important to study the neutral lipid fraction of beef heart mitochondria. The most promising components would be

* From the Wenner Gren Institute, University of Stockholm. *Received October 1, 1964.* This investigation was supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council.

† Research carried out during the tenure of a Swedish Institute fellowship.

‡ Research carried out during the tenure of a National Science Foundation Senior Postdoctoral Fellowship. Permanent address: Department of Biological Sciences, Purdue University, Lafayette, Ind.

TABLE I: Properties of the Ultraviolet Spectrum of Fractions from a Silicic Acid Column Chromatography of the "Neutral Lipids" from Beef Heart Mitochondria.[a]

Fraction No.	Volume (ml)	Maximal Absorbancy[b] Oxidized (mμ)	Maximal Absorbancy[b] Reduced (mμ)	Isosbestic Points (mμ)	Maximum
1	61	255 S	255 S		
2	25	255 P	255 P		
3	31	255 P	255 P		
4	90	258 P	258 P	234	at 258 mμ + 2.47
5	69	260 S	260 S		
6	61	285 P	285 P	252–319	at 285 mμ − 1
7	34	275 P	290 P	237–293–301	at 275 mμ − 120
8	50	273 P	282 P	237–292–304	at 275 mμ − 12.4
9	80	270 S	270 S	248–290–310	at 270 mμ − 0.6
10	102	270 S	294–264 P	241–312–340	at 263 mμ + 36.25
11	124	270 S	270 S	253–290	at 275 mμ − 0.45
12	120	275 S	275 S	262–283	at 275 mμ − 0.15

[a] Elution sequence: 200 ml chloroform-heptane, 20:80; 200 ml chloroform-heptane, 50:50; 200 ml chloroform-heptane, 80:20; 250 ml chloroform. [b] S = shoulder; P = peak.

those which could undergo oxidoreduction reactions.

Methods

Preparation of Mitochondria and Extraction of Lipids. Mitochondria from beef heart were prepared according to the technique described by Low and Vallin (1963). Only the light fraction was used. The extraction of the lipid fraction was performed, under nitrogen, as follows: One part of the mitochondrial suspension in 0.25 M sucrose, containing about 40 mg of protein/ml, was added to 3 parts of dry acetone in a separatory funnel. Nitrogen was vigorously bubbled into the mixture for 5 minutes through a 10-ml pipet, thus ensuring good agitation. The mixture was extracted five times with 1 part of petroleum ether (30–60°), always under nitrogen. All the operations were carried out at room temperature. The containers were protected against light by means of aluminum foil.

Column Chromatography. CHROMATOGRAPHY OF LIPIDS ON SILICIC ACID. A silicic acid (Mallinckrodt 200 mesh) column 3.3 × 20 cm was used. Silicic acid (60 g) suspended in heptane was carefully packed in the column and washed with heptane. The sample, which contained the extract from 5–10 g of mitochondrial proteins, taken up in heptane, was put on the top of the column. In this solvent the lipids remain at the top as an orange-yellow layer.

The single components were then eluted with increasing concentrations of chloroform in heptane, as described in the tables. Under these conditions all the phospholipids are retained on the column. The fractions from the column were concentrated to dryness *in vacuo* and taken up in absolute alcohol for spectral analysis.

CHROMATOGRAPHY OF LIPIDS ON ALUMINA. The same size column was used for alumina chromatography. Neutral aluminum oxide (Merck) (60 g) containing 6% water was used as absorbent. The same heptane-chloroform solvent system was used to develop the column.

Thin-Layer Chromatography. Thin-layer chromatography plates were prepared by standard technique using Silica Gel G (Merck) as absorbent. The samples were put on the plate in petroleum ether. Different solvents and solvent systems were used to develop the chromatograms. In order to detect the spots of oxidizing components a leucomethylene blue spray was used (Crane and Dilley, 1963). For reducing components the $FeCl_3$-α, α′-dipyridyl or Neotetrazolium sprays were applied to the plate.

Spectral Analysis of the Fractions. The spectrophotometric analysis of the fractions was performed using a Beckman DK-2 recording spectrophotometer. Spectra were recorded, in ethanol, before and after addition of crystals of sodium borohydride.

Results

Data collected in Table I were obtained from a typical chromatographic separation of lipids from beef heart mitochondria on a silicic acid column. In this table, the wave length of the maximal absorbancy before and after addition of sodium borohydride, the position of the isosbestic points, and the maximal change in optical density upon addition of borohydride are reported. The positive sign before the figures of the last column means an increase of absorbancy as a result of borohydride addition, while the negative sign means a decrease. Figure 1 illustrates the thin-layer chromatography of all the fractions whose spectral properties are described in Table I.

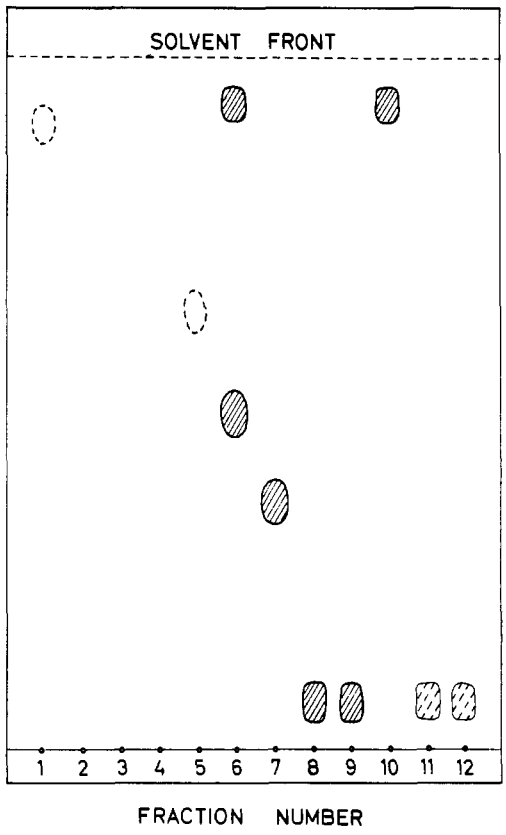

FIGURE 1: Thin-layer chromatography of all the fractions obtained from a silicic acid column, whose spectral properties are described in Table I, as sprayed with reduced methylene blue. Striped spots, blue; open, enclosed by dashed lines, white; striped with dashed lines, slightly blue color. Experimental conditions: absorbent, silica gel G; solvent, benzene; detection of the spots with leucomethylene blue spray.

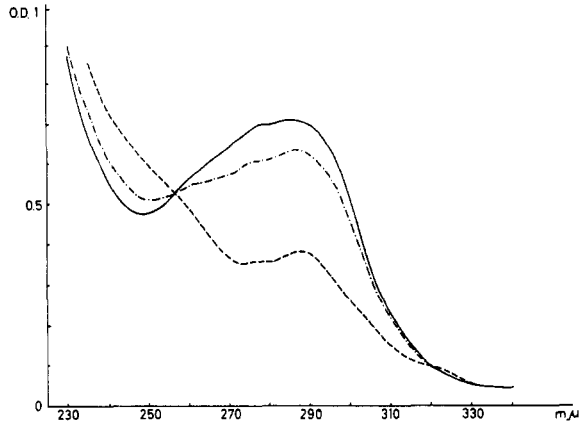

FIGURE 2: Absorption spectrum in ethanol of fraction 6 from the silicic acid column. ———, before addition of borohydride; ---------, 10 minutes after addition of borohydride; –·–·–·, 2 minutes after addition of borohydride.

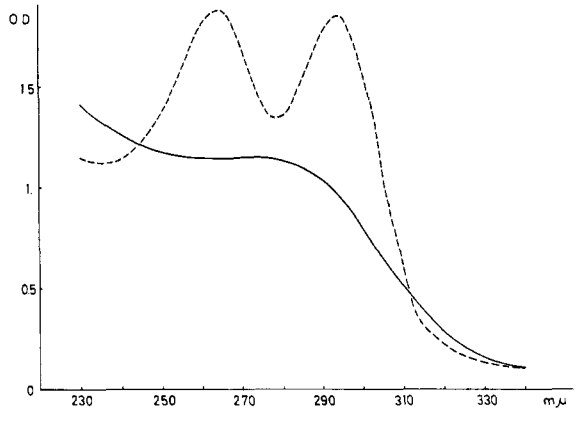

FIGURE 3: Absorption spectrum in ethanol of fraction 10 from the silicic acid column. ———, before addition of borohydride; ---------, after addition of borohydride.

As indicated in Table I, the first three fractions obtained do not show any spectral modification upon addition of sodium borohydride. Fraction 4 shows an increase of optical density with a maximum at 258 mμ. Fraction 6 showed a very slow modification of the ultraviolet spectrum upon addition of borohydride. This modification is indicated in Table I as a decrease at 285 mμ, but, as shown in Figure 2, it covers a fairly large range between 270 and 290 mμ.

From the chromatographic pattern in Figure 1, it appears that fraction 6 contains two components which can oxidize leucomethylene blue. The spectral properties of fraction 7 correspond to coenzyme Q_{10} while the properties of the subsequent fractions are slightly different than those of CoQ_{10} (the peak is shifted toward shorter wavelengths). Also, from the chromatographic point of view, fractions 8 and 9 are different from CoQ_{10} in that they show a blue spot with leucomethylene blue at a lower R_F value.

Fraction 10 is completely different from all others: it runs on the column as a brown band and it shows the characteristic spectrum reported in Figure 3. Upon addition of borohydride to this fraction a double-peak curve appears with maxima at 264 and 294 mμ.

Fractions 11 and 12 contain a very small amount of material. After purification by thin-layer chromatography, fraction 11 shows the spectrum reported in Figure 4, which is characterized by a peak at 274 mμ in the oxidized state and by a peak at 287 mμ in the reduced state.

The same type of experiment was repeated using aluminum oxide as adsorbent instead of silicic acid. This study was performed in order to determine whether silicic acid could produce some artifact during the long elution time (4–6 hours) required to remove many of the compounds from the column.

Table II and Figure 5 illustrate the results of this study. It is evident that the general pattern is the same. Although silicic acid allows a better resolution of the

TABLE II: Properties of the Ultraviolet Spectrum of Fractions from an Alumina Column Chromatography of the "Neutral Lipids" from Mitochondria.[a]

Fraction No.	Volume (ml)	Maximal Absorbancy[b] Oxidized (mμ)	Maximal Absorbancy[b] Reduced (mμ)	Isosbestic Points (mμ)	Maximum
1	22	255 P	255 P		
2	30	255 S	255 S		
3	36	268 P	268 P		at 268 mμ + 0.225
4	18	258 P	258 P		at 260 mμ + 0.200
5	55	258 S	258 S		at 258 mμ + 0.300
6	50	255 S	255 S	248–268	at 255 mμ − 0.110
7	67	273 P	288 P	238–293–302	at 275 mμ − 146
8	57	275 P	290 P	238–293–303	at 275 mμ − 57
9	35	270 P	270 P	245–302	at 270 mμ − 2.12
10	10	270 S	270 S	245–298	at 270 mμ − 1.4
11	35	270 S	270 S	292	at 270 mμ − 1.3
12	88	270 S	270 S	295	at 270 mμ − 0.45

[a] Elution sequence: 70 ml of heptane; 100 ml of chloroform-heptane, 20:80; 100 ml of chloroform-heptane, 50:50; 100 ml chloroform-heptane, 80:20; 100 ml chloroform. [b] S = shoulder; P = peak.

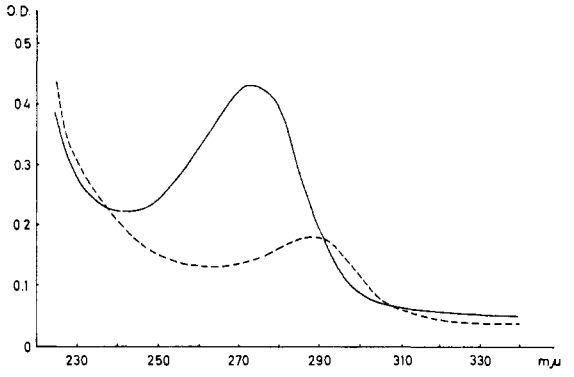

FIGURE 4: Absorption spectrum in ethanol of purified fraction 11 from silicic acid column. ———, before addition of borohydride; ---------, after addition of borohydride.

fractions, the only net difference was the total absence of the fraction which corresponds to number 10 of the previous study, whose characteristic spectrum is reported in Figure 3. As with the separation of lipids on silicic acid columns, one can see also with alumina four components giving a blue spot with leucomethylene blue. After reduction with sodium borohydride not only the fractions number 6 and 7, but also the fractions number 10 and 11 gave a positive reaction with $FeCl_3$-dipyridyl reagent.

A sample of the total lipid extract (T.L. in Figure 5) was also put on the thin-layer plate in order to compare the components present with those obtained in fractions from the column. As shown in Figure 5, the phospholipids present in the crude extract do not allow a good

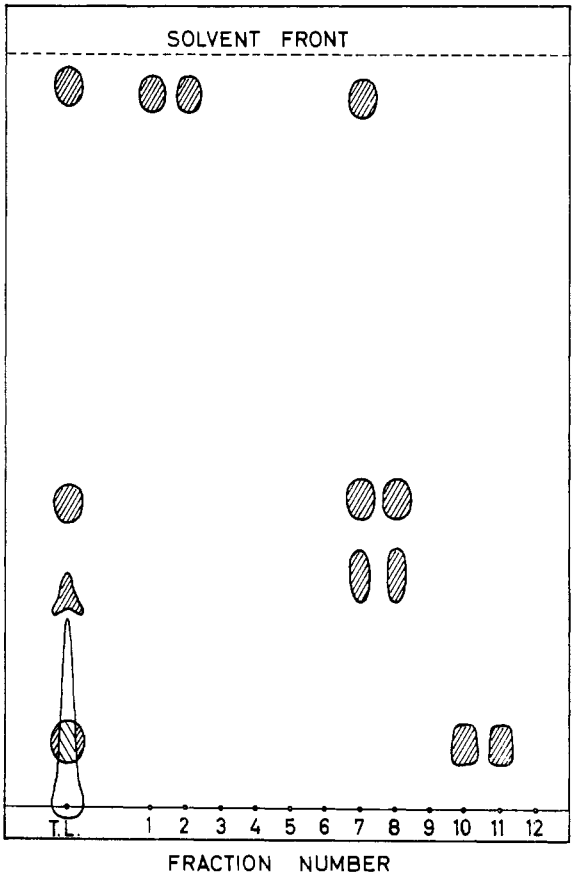

FIGURE 5: Thin-layer chromatography of the total lipid extract (T.L.) and of all the fraction from wet alumina column. Experimental conditions: absorbent, silica gel G; solvent, benzene; detection of the spots with leucomethylene blue spray.

TABLE III: R_F Values of the Four Oxidizing Components of "Neutral Lipid Fraction" of Beef Heart Mitochondria with Different Solvent Systems.

Spot No.	Chloroform	Benzene	Isooctane–Ethyl Ether 70:30	Isooctane–Ethyl Ether 50:50	Toluene	Response to Ultraviolet Irradiation
1	0.19	0.08	0.11	0.23	0.05	Fluorescent
2	0.58	0.35	0.48	0.57	0.26	Quenching
3	[a]	0.46	0.63	0.64[a]	0.38	Fluorescent
4	[a]	0.81	0.74	0.77[a]	0.77	

[a] The spots were not well resolved.

resolution of the material which can oxidize leucomethylene blue.

In order to study better the properties of the four substances giving a positive reaction with leucomethylene blue, we decided first to pass the crude lipids, dissolved in chloroform, through a very short column of wet alumina to remove phospholipids, and then further to resolve the fractions by thin-layer chromatography on silica gel G with different solvent systems. Table III shows the R_F values obtained and reports also the response of the fractions under ultraviolet irradiation. The same results could be obtained using rapid chromatography on silicic acid to remove phospholipids. Number 4 spot was very fast in all the solvent systems; it is yellow (β-carotene), but the blue spot does not coincide exactly with the yellow although not one of the solvents used succeeded in completely separating the yellow from the blue spot.

Number 3 spot becomes blue immediately after spraying with leucomethylene blue and then it turns yellow-green. When it is sprayed with $FeCl_3$-dipyridyl or with neotetrazolium reagents it becomes red. Its R_F value is identical with α-tocopherol standard which also gives the same reactions with all the reagents used.

Number 2 spot was identified as CoQ_{10} by its chromatographic and spectral properties. Number 1 spot is partially overlapped by a white area which can be seen also without any treatment. The spectrum of the substance corresponds to that reported in Figure 4 which resembles that of quinones of the coenzyme Q group except that the maximum is at 273 mμ in ethanol.

Discussion

The existence of CoQ_{10} in the neutral lipid fraction does not merit comment since its presence and its function have been widely described and studied. Also, the presence of α-tocopherol in mitochondrial lipids has been repeatedly observed although its function is not yet clear. One could wonder why this compound can so readily cause the oxidation of leucomethylene blue. Possibly the reaction is due to a cooxidation of the leuco-dye and of the substance in the particular experimental conditions (acid *p*H, silicic acid as catalyst). A sample of authentic α-tocopherol gave identical reactions.

The nature and the significance of the other two components which show a positive reaction with leucomethylene blue is still to be considered. The first compound (number 4 in Table III) behaves chromatographically in a fashion similar to known members of the K_2 group such as vitamin K_2 (25) or (30). Evidence for a role of vitamin K_2 in phosphorylation in mitochondria has been presented by Martius (1961), Beyer (1959), and Anderson and Dallam (1959), but it was difficult to demonstrate the presence of vitamin K_2 in mitochondria by chemical methods. Green *et al.* (1956) have reported a small amount of vitamin K in beef liver mitochondria by using a biological assay. Recently Hall and Crane (1964) found a vitamin K–like material in beef heart mitochondria which was required to restore the NADH oxidase system of particles extracted with ether. This material showed an R_F on silica gel G similar to that of the compound reported here.

The compound found by us is more labile on chromatography or storage than expected for a vitamin K and we never succeeded in demonstrating a spectrum typical for vitamin K in this fraction. However, in the spectrum of fraction number 6, obtained from a silicic acid column (Figure 2) which contains α-tocopherol, as well as the fast component giving the positive reaction with leucomethylene blue (Figure 1), a slow decrease in absorbency in the 260- to 320-mμ region upon addition of borohydride can be seen. The spectral changes shown are consistent with the presence in this fraction of a naphthoquinone derivative except that the increase in the 320- to 330-mμ region is less than expected for this type of compound.

As for the compound with a low R_F value on silica gel (number 1 in Table III), its spectral properties suggest a benzoquinone type of structure. It is to be considered different from CoQ_{10} because it reacts very slowly with leucomethylene blue and it shows a lower R_F value with both column and thin-layer chromatography than any of the natural forms of coenzyme Q ranging from Q_6 to Q_{10}. The compound could be considered as an artifact due to a modification of CoQ_{10}

catalyzed by the adsorbent in special solvent conditions. We feel that this hypothesis is unlikely since the compound could be found using different adsorbents (silicic acid and alumina) and also with different solvent systems. As for the biological significance of this substance, it can be correlated with the properties described for lipid extracts which are required in addition to CoQ_{10} to restore the mitochondrial enzymatic activities after extraction with solvents which have been referred to as NL II (Crane *et al.* 1959a).

The reason that this compound has never been described before is probably because it shows a spectrum similar to CoQ_{10}, and is not very easily separated from the latter on a silicic acid column, and also that the thin-layer chromatography technique was introduced only recently as a tool in this field. Another difficulty is that, with use of the latter technique, it is impossible to observe it in a crude extract since the phospholipids must be removed first because they do not allow a good resolution of the spots.

On a quantitative basis, we can say that this new component is responsible, in our best experiments, for about 10% of the total absorbency change at 275 mμ upon addition of borohydride to mitochondrial lipids. If we assume the molar extinction coefficient of a benzoquinone, the concentration of this compound in the mitochondrion is of the same order of magnitude as that of cytochromes.

Acknowledgments

The authors wish to thank Dr. Lars Ernster for his advice and helpful discussions.

References

Anderson, W. W., and Dallam, R. D. (1959), *J. Biol. Chem. 234*, 409.

Beyer, R. E. (1959), *J. Biol. Chem. 234*, 688.

Bouman, J., and Slater, E. C. (1957), *Biochim. Biophys. Acta 26*, 624.

Bouman, J., Slater, E. C., Rudney, H., and Links, J. (1958), *Biochim. Biophys. Acta 29*, 456.

Chance, B. (1965) *in* Biochemistry of the Quinones, Morton, R. A., ed., New York, Academic.

Cowlishaw, B., Søndegaard, E., Prance, I., and Dam, H. (1957), *Biochim. Biophys. Acta 25*, 644.

Crane, F. L., and Dilley, R. A. (1963), *Methods Biochem. Analy. 11*, 279.

Crane, F. L., Fechner, W., and Ambe, K. S. (1959b), *Archiv. Biochem. Biophys. 81*, 277.

Crane, F. L., Hatefi, Y., Lester, R. L., and Widmer, C. (1957), *Biochim. Biophys. Acta 25*, 220.

Crane, F. L., Widmer, C., Lester, R. L., and Hatefi, Y. (1959a), *Biochim. Biophys. Acta 31*, 477.

Crawford, R. B., Morrison, M., and Stotz, E. (1959), *Biochim. Biophys. Acta 33*, 543.

De Bernard, B., Sottocasa, G. L., Ordanini, G. C., and Alliezi, L. (1960), *Rend. Inst. Lombardo Sci. Lettere, B: 94*, 121.

Donaldson, K. O., and Nason, A. (1957), *Proc. Soc. Natl. Sci. U.S. 43*, 364.

Green, J. P., Søndegaard, E., and Dam, H. (1956), *Biochim. Biophys. Acta 19*, 182.

Hall, C. L., and Crane, F. L. (1964), NIH Information Exchange Group 1, Memo 117.

Hatefi, Y., Lester, R. L., Crane, F. L., and Widmer, C. (1959), *Biochim. Biophys. Acta 31*, 490.

Hendlin, D., and Cook, T. M. (1960), *J. Biol. Chem. 235*, 1178.

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

Lester, R. L., Hatefi, Y., Widmer, C., and Crane, F. L. (1959), *Biochim. Biophys. Acta 33*, 169.

Low, H., and Vallin, I. (1963), *Biochim. Biophys. Acta 69*, 361.

Marinetti, G. V., Scaramuzzino, D. J., and Stotz, E. (1957), *J. Biol. Chem. 224*, 819.

Martius, L. (1961), *Ciba Found. Symp. Quinones Electron Transport*, 312.

Nason, A., Garrett, R. H., Nair, P. P., Vasington, F. D., and Detwiler, T. C. (1963), NIH Information Exchange Group 1, Memo 90.

Nason, A., and Lehman, I. R. (1956), *J. Biol. Chem. 222*, 511.

Pollard, C. J., and Bieri, J. G. (1960), *J. Biol. Chem. 235*, 1187.

Redfearn, E. R., and King, T. E. (1964), NIH Information Exchange Group 1, Memo 148.

"Symposium on Vitamin E" (1962), *Vitamins Hormones 20*.

Szarkowska, L., and Klingenberg, M. (1963), *Biochem. Z. 338*, 674.