

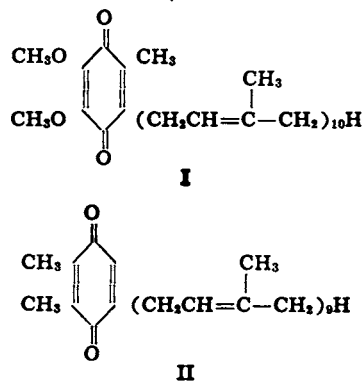
# Coenzyme Q

## XVI. THE ESTIMATION OF THE COENZYME Q<sub>10</sub> CONTENT OF ALFALFA

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Isolated coenzyme Q<sub>10</sub> has been defined (Crane et al., '57; Fahmy et al., '58), and its structure (I) has been elucidated (Wolf et al., '58; Morton et al., '58). Coenzymes Q<sub>6</sub> to Q<sub>9</sub> were also characterized (Lester et al., '58; Wolf et al., '58).

The structure (II) of plastoquinone has also been determined (Trenner et al., '59; Kofler et al., '59); this related quinone had been isolated previously (Kofler, '46; Crane and Lester, '58) from alfalfa and later from commercial alfalfa meal (Trenner et al., '59; Crane, '59a).



The distribution of coenzyme Q<sub>10</sub> and plastoquinone in animal and plant tissue has been studied (Lester and Crane, '59a), and the coenzyme Q<sub>10</sub> content of various dietary constituents has been determined in this laboratory (Page et al., '59) to guide the possible maintenance of animals upon a coenzyme Q-low diet.

The coenzyme Q group of compounds has been shown to be specific for restoration of cytochrome C reductase activity<sup>1</sup> and for restoration of succinoxidase activity<sup>2</sup> in inactivated systems. It appears that the redox functionality of coenzyme Q participates in mitochondrial electron trans-

port, and that of plastoquinone participates in photosynthetic electron transport (Crane, '59b).

The reported occurrence of coenzyme Q<sub>10</sub> in spinach and other white and green plant tissue (Crane, '59b) and in alfalfa meal (Lester and Crane, '59a), in addition to the presence of plastoquinone in alfalfa, seemed rather significant, because of (1) the widespread use of alfalfa and alfalfa meal in animal nutrition; (2) the widespread occurrence of coenzyme Q<sub>10</sub> in mammalian tissue; and (3) the many previous studies (Vavick et al., '53; Scott et al., '53; Hill et al., '53; March et al., '55; Ershoff et al., '59; Ershoff and Hernandez, '59) which had suggested the possible presence of "unidentified factors" in alfalfa to account for the biological responses observed with alfalfa under various test conditions *in vivo*.

We wished to confirm, and have done so, the unexpected presence of both coenzyme Q<sub>10</sub> and plastoquinone in alfalfa, and the apparent absence of other members of the Q group. We also examined freshly collected green alfalfa, because of the possible loss of the rather labile coenzyme Q<sub>10</sub> during the commercial preparation and storage of alfalfa meal which is more commonly used in practical animal nutrition.

### RESULTS

Fresh alfalfa was saponified, and the non-saponifiable lipid material was removed by extraction and purified by chro-

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<sup>1</sup> K. S. Ambe and F. L. Crane 1959 Coenzyme Q specific restoration of cytochrome C reductase activity. *Federation Proc.*, 18: 181 (abstract).

<sup>2</sup> S. Fleischer and R. L. Lester 1959 Restoration of succinoxidase activity by coenzyme Q in acetone-extracted mitochondria. *Federation Proc.*, 18: 227.

matography. The coenzyme  $Q_{10}$  was identified by means of paper chromatographic comparison with an authentic sample and estimated by spectrophotometric (Crane et al., '59; Lester and Crane, '59b; Linn et al., '59) and colorimetric assay (Koniuszy et al., '60). The coenzyme  $Q_{10}$  content of fresh alfalfa was estimated to be 3.4 mg/kg wet weight in the sample studied.

A sample of commercial alfalfa meal was extracted directly with hydrocarbon solvent and the coenzyme  $Q_{10}$  content was estimated. The value of 28 mg/kg is consistent with the value found for fresh alfalfa when adjusted for the probable water content and suggests that in the preparation of commercial alfalfa meal, the coenzyme  $Q_{10}$  content can be essentially retained.

#### EXPERIMENTAL

**Fresh alfalfa.** A sample of fresh alfalfa stems and leaves (1000 gm) was homogenized in a Waring Blendor with 200 ml of 95% ethanol by adding portions of the plant to portions of the solvent and blending for one to two minutes. To the resulting slurry in a 12-l round-bottom flask was added 45 gm of pyrogallol. The mixture was heated to boiling and 220 gm of potassium hydroxide in 400 ml of water was added during 5 minutes, with vigorous stirring. After the addition was complete, the mixture was stirred for 30 minutes under reflux, cooled to room temperature and extracted with  $3 \times 2000$  ml portions of Skellysolve B. Additional water was added as necessary to break persistent emulsions. The combined extracts were washed with water, dried over anhydrous magnesium sulfate and evaporated *in vacuo* to 1.8 gm of orange, semi-solid residue.

A chromatographic column was prepared by adding 40 gm of dry Decalso slowly to a 2.0-cm-diameter glass tube containing Skellysolve B. To the prepared column was added the 1.8 gm of extracted solids dissolved in a minimum volume of Skellysolve B. The column was washed with 250 ml of Skellysolve B and eluted with 1% ether in Skellysolve B, collecting 20 ml fractions at a flow rate of 3 ml/minute. Fractions were assayed by spotting aliquots (10 to 20  $\mu$ l) on filter paper and spraying with LMB reagent (zinc-acetic acid reduced methylene blue) (Page

et al., '59) and characterized by paper chromatography on circles of Whatman no. 1 paper impregnated with vaseline and using dimethylformamide as mobile phase (Linn et al., '59; Page et al., '59).

Fractions 1 to 18 were devoid of any LMB oxidizing substances. Fractions 19 to 21 contained the bulk of the Q-254 while fractions 22 to 24 contained Q-254 and a second faster moving LMB oxidizing substance. Fractions 25 to 65 contained the coenzyme  $Q_{10}$  as well as  $\alpha$ -tocopherol and a trace of Q-254. Fractions 65 to 93 as well as a 25% ether in Skellysolve B eluate were devoid of any LMB oxidizing substances.

Fractions 25 to 65 (0.21 gm) were dissolved in 5 ml of Skellysolve B, cooled to 0° and centrifuged. The supernate was removed and the process repeated twice. The combined supernates were evaporated *in vacuo* to 137 mg of red oil showing  $E_{1\text{cm}}^{1\%} = 29$  at 290  $m\mu$  in ethanol but no maximum at 275  $m\mu$ , characteristic of coenzyme Q (Wolf et al., '58).

A chromatographic column was prepared by adding 10 gm of dry Decalso to a 1.0-cm-diameter buret containing i-octane. A solution of the 137 mg of red oil in a minimum volume of i-octane was added to the column and elution with 1% ether in i-octane was begun immediately, collecting 10 ml fractions at a flow rate of 2 ml/minute. The elution was followed by measuring the absorbance of each fraction at 255, 275 and 290  $m\mu$ . The elution of Q-254 was recognized in fractions 3 to 7 by the peak in 255  $m\mu$  absorbance while the elution of  $\alpha$ -tocopherol was observed in fractions 11 to 16 by the peak in 290  $m\mu$  absorbance. The peak in 275  $m\mu$  absorbance occurred at fractions 24 to 27 which were evaporated to 11.8 mg of red oil.

The red oil was found to have an ultraviolet spectrum in ethanol nearly identical with that of coenzyme  $Q_{10}$  (Wolf et al., '58). It showed a strong LMB oxidizing spot on papergrams which was not separable from that produced by coenzyme  $Q_{10}$  and gave a positive Craven's test under the conditions used for coenzyme  $Q_{10}$ . The  $\Delta E_{1\text{cm}}^{1\%}$  at 275  $m\mu$  obtained on reduction of an ethanol solution with sodium borohydride supported a coenzyme  $Q_{10}$  content

of 12% or a total of 1.4 mg. The amount present in neighboring fractions of the chromatogram was estimated at 1 mg from their absorbance at 275 m $\mu$ . The content of fresh alfalfa is approximately 3.4 mg/kg wet weight.<sup>3</sup>

**Alfalfa meal.** A sample (2000 gm) of commercial alfalfa meal was continuously extracted with Skellysolve B in a Soxhlet apparatus for 24 hours. The extract was evaporated *in vacuo* to a residue (77.8 gm) which was dissolved in 500 mg of Skellysolve B and filtered. The filtrate was applied to a chromatographic column prepared by adding 1500 gm of Decalso to Skellysolve B in a 7.0-cm-diameter column. The column was washed with 4 l of Skellysolve B followed by 8 l of 5% ether in Skellysolve B and eluted with 8 l of 50% ether in Skellysolve B. Removal of the solvent *in vacuo* from the eluate gave 13.5 gm of dark red residue.

A portion (1.35 gm) of the eluate residue was dissolved in 10 ml of i-octane, cooled to 0°, centrifuged and the supernate removed. The procedure was repeated and the combined supernates were applied to a 2.2-cm-diameter column of 50 gm of Decalso in i-octane. The column was washed with 25 ml of i-octane and eluted with 2% ether in i-octane, collected in 10 ml fractions at a flow rate of 1.5 ml/minute. Elution was followed by measuring absorbance at 255, 275 and 290 m $\mu$  as before. The Q-254 peak appeared at fraction 34, the  $\alpha$ -tocopherol peak at fraction 48, and the coenzyme Q<sub>10</sub> peak at fraction 96, after changing the eluting mixture to 5% ether in Skellysolve B at fraction 68. Fractions 83 to 101 were combined and evaporated *in vacuo* to 66.6 mg of red oil. This oil was dissolved in 3 ml of Skellysolve B, cooled in ice, centrifuged and the supernate separated. The procedure was repeated and the supernates combined and evaporated *in vacuo* to 37.1 mg of red oil which showed the same properties as that prepared from fresh alfalfa and assayed 15% of coenzyme Q<sub>10</sub> by spectrophotometric assay. The coenzyme Q<sub>10</sub> content of alfalfa meal is therefore estimated at 28 mg/kg dry weight.

#### SUMMARY

The coexistence of coenzyme Q<sub>10</sub> with plastoquinone in commercial alfalfa meal

has been confirmed, and the content of coenzyme Q<sub>10</sub> was found to be *ca.* 28 mg/kg. The preparation and storage of commercial alfalfa meal can lead to retention of essentially all of the coenzyme Q<sub>10</sub> of growing alfalfa since the content of coenzyme Q<sub>10</sub> of the freshly collected plant was *ca.* 3.4 mg/kg; this value is reasonably consistent with the water content of the green plant.

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<sup>3</sup> A recovery of 70% is assumed. See Page et al. ('59).

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