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*THE REACTIVITY OF A NATURALLY OCCURRING QUINONE
(Q-255) IN PHOTOCHEMICAL REACTIONS OF ISOLATED
CHLOROPLASTS**

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In a recent paper we showed that vitamin K derivatives restored photochemical activity to chloroplasts which had been extracted with petroleum ether. Since the petroleum ether extracts contain mainly carotenoids, Lynch and French¹ initially attributed the effect to β -carotene, but we observed that purified β -carotene was completely inert.^{2, 3} Vitamin K and other typical fat-soluble vitamins could be expected to be removed by the extraction process, and since vitamin K has often been reported to be localized in the chloroplasts, it was logical to assume that this might be the active substance extracted. Furthermore, vitamin K is an effective cofactor for the photophosphorylation catalyzed by chloroplasts.⁴⁻⁷

Added vitamins K₃ and K₅ gave the anticipated activation effect^{2, 3} and therefore we assumed, following the literature, that vitamin K was present in the chloroplasts and that it was the natural reactivating substance removed along with the carotenoids and other lipid-like substances. Since then, we have made analyses of the petroleum ether extracts and of the total pigment content of the chloroplasts. To our surprise, we found little, if any, vitamin K. In its place we obtained a crystalline, yellow compound which can be reversibly oxidized and reduced.⁸ Its color and spectral behavior turned out to be identical with a substance extracted recently by Crane from alfalfa,^{9, 10} which belongs to a newly-discovered group of "ubiquinones." Crane *et al.*,^{9, 10} as well as Folkers and colleagues,¹¹ have obtained suf-

ficient information to characterize this compound as a substituted *p*-benzoquinone: 2,3-dimethyl-5-(3'-methyl-2' butynyl-oktakis-(3'-methyl-2' butynylene)) benzoquinone.

We have now extracted from the chloroplasts of spinach, sugar beet, and swiss chard sufficient quantities of this material to test its ability to restore the photochemical activity of petroleum ether-extracted chloroplasts. Qualitatively the effect of the new benzoquinone is the same as that of the naphthoquinones (vitamin K, etc.). Quantitatively it is much more effective and, so far, the only compound which produces the same or even better rates of the photochemical reaction than the unextracted controls.

Materials and Methods.—The methods for preparing, lyophilizing, and extracting the chloroplasts, the method for determining the photochemical activity (with potassium ferricyanide as oxidant), and the method for the re-addition of various compounds to extracted chloroplasts were the same as previously described.^{2, 3} In addition, we have used dichlorophenol-indophenol to compare the reactivation of the photochemical activity with two Hill oxidants of different characteristics. The reduction of this dye was followed spectrophotometrically after the method of Lynch and French.

The petroleum ether for extraction and chromatography was first treated with sulfuric acid, washed several times with water, dried with anhydrous sodium sulfate, and finally distilled; the fraction going over between 30°C and 40°C was collected and used for extractions. Other solvents were of sufficiently high purity to exclude contaminants which would interfere with the determination of absorption characteristics in the ultraviolet region.

The first step in the fractionation of the extracts was the same as previously described. The powdered sugar column effectively removes the chlorophylls and xanthophylls contained in the extract, and the fraction that comes through the column contains β -carotene and the other lipid-like compounds. It is this fraction which, as previously shown, contains the component(s) responsible for reactivation of chloroplasts rendered inactive by extraction.

For further fractionation and purification, this yellow eluate from the sugar column was evaporated to dryness under vacuum at 35°C, re-dissolved in iso-octane, and placed on a 2 × 15 cm silicic acid column previously washed with iso-octane. The carotenes contained in the sample are carried through the column with a solvent composed of 25 per cent chloroform and 75 per cent iso-octane, while the Q-255 compound remains near the top of the column. With 75 per cent chloroform and 25 per cent iso-octane, the Q-255 moves down the column. As a general practice, this band was never taken completely through the column but was moved only far enough away from other material at the top to permit a manual separation of the bands. After the portion containing the Q-255 was removed from the column, it was eluted with chloroform, reduced to dryness (under vacuum) and the dried material re-dissolved in absolute alcohol. The light absorption curves of such samples were determined between 210 and 300 $m\mu$ before and after the addition of a few grains of sodium borohydride.

For determining the amount of Q-255 in a sample, this abbreviated procedure proved to be of importance, since we observed a gradual decomposition of the compound the longer it was held on the column. The chromatographic technique used

for this separation is similar to that described by Crane and co-workers¹⁰ for the Coenzyme Q from mitochondria. The following extinction coefficients were used to determine the amount of Q-255 in each sample: $E_{1\text{ cm}}^{1\text{ per cent}} = 212$, and $E_{1\text{ cm}}^{1\text{ per cent}}$ (oxidized-reduced) = 200. These values are in good agreement with those obtained for "Q₂₅₄" from alfalfa.¹⁰ All spectral measurements reported in this paper were made either with a Beckman DU spectrophotometer or a Zeiss spectrophotometer, Model PMQ II.

Results.—The petroleum ether extracts from lyophilized chloroplasts, whether

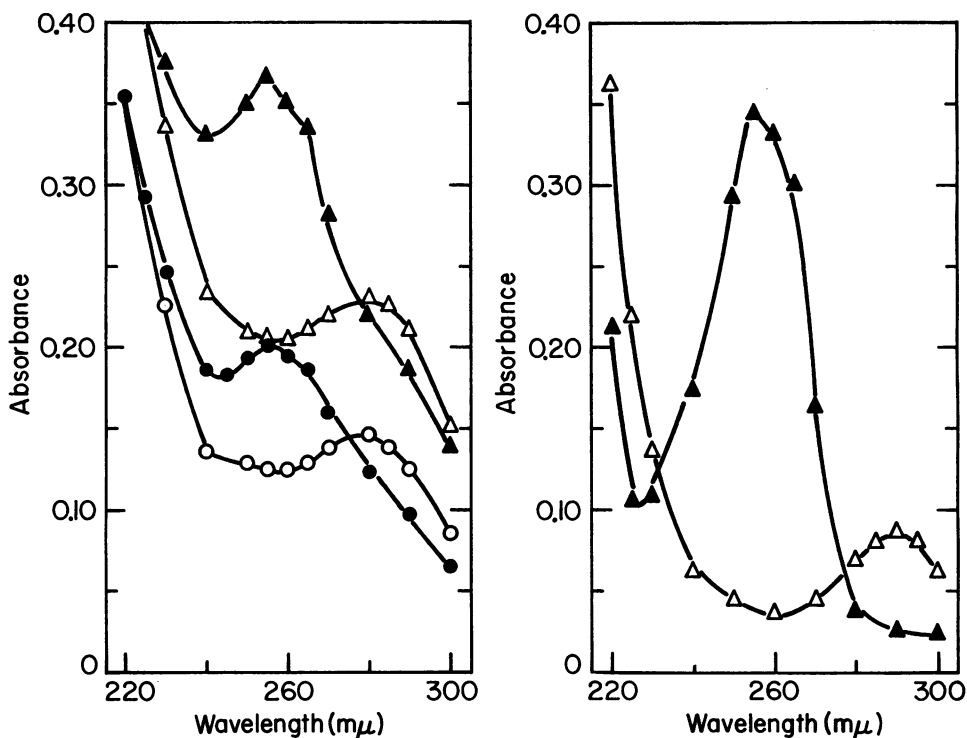


FIG. 1a (left).—Absorption curves of petroleum ether extracts (in absolute ethanol) of lyophilized sugar beet chloroplasts. Extract was divided into two equal parts, one chromatographed on a powdered sugar column (●—● = oxidized, ○—○ = reduced with sodium borohydride), and the other unchromatographed (▲—▲ = oxidized, △—△ = reduced).

FIG. 1b (right).—Absorption curve of purified Q-255 from sugar beet chloroplasts. ▲—▲ = oxidized, △—△ = reduced. 0.04 mg in 3 ml absolute ethanol.

those of sugar beet, spinach, or swiss chard, always contained Q-255. A typical example of such an observation is shown in Figure 1a.

For this curve, 100 mg of freeze-dried chloroplasts from sugar beet leaves were extracted with 100 ml of cold petroleum ether. The petroleum ether was split into two equal portions: One portion was evaporated to dryness and the residue re-suspended in absolute ethanol, while the other sample was chromatographed on a powdered sugar column as indicated in *Methods*. The curves showed that chromatographic treatment to remove the xanthophylls and carotenoids also decreases the amount of Q-255 present. This observation explains why after such chromatographic treatment the extract is not as active in restoring photochemical

activity as it was before such treatment. Previously, Lynch and French had experienced the same difficulty when magnesium oxide was used as the absorbent. In Figure 1b the absorption curves (oxidized and reduced) of purified Q-255 from sugar beet chloroplasts are shown.

The data in Table 1 give a comparison between the reactivating effects of equal molar concentrations of several different compounds. What is immediately appar-

TABLE 1
COMPARISON OF THE REACTIVATION OF THE FERRICYANIDE REDUCING CAPACITY OF EXTRACTED SUGAR BEET CHLOROPLASTS BY VARIOUS COMPOUNDS

Compound Added	Per cent of Initial Activity		
	a	b	c
Extracted-Control	56	26	35
.36 μ M Q-255	116	97	109
.73 μ M "	96	95	101
.36 μ M Q-275	63	36	53
.73 μ M "	42	31	47
.36 μ M Vitamin K ₃	47	52	53
.73 μ M "	26	49	57
.36 μ M β -Carotene	55	28	32
.73 μ M "	52	26	34

Conditions: Temperature = 15°C; Gas phase = nitrogen; Red light. $[K_3Fe(CN)_6] = 5 \times 10^{-4} M$, $[PO_4] = 0.05 M$ (pH = 6.5), and $[Chlorophyll] = 9.5 \times 10^{-5} M$. Each experiment (a, b, c) was performed on a new preparation of chloroplasts.

ent is the superior reactivating power of Q-255 not only over vitamin K but also over that of the Coenzyme Q compound isolated from mitochondria (Q-275), although structurally the two benzoquinones are quite similar. Menadione has only a slight reactivating effect at the concentration used; and, as reported earlier, purified β -carotene is without effect.

Because Lynch and French used dichlorophenol-indophenol as the oxidant in their experiments, and also since this dye has certain special qualities as a Hill reaction oxidant,¹² the reactivation of the photochemical activity with this dye as the Hill oxidant is compared in Table 2. These are the results of three different experiments,

TABLE 2
COMPARISON OF THE REACTIVATION OF THE DYE* REDUCING CAPACITY OF EXTRACTED SUGAR BEET CHLOROPLASTS BY VARIOUS COMPOUNDS

Compound Added	Per cent of Initial Activity		
	a	b	c
Extracted-Control	46	33	32
.36 μ M Q-255	102	110	101
.72 μ M "	79	93	98
.36 μ M Q-275	61	37	40
.72 μ M "	46	34	39
.36 μ M Vitamin K ₃	51	42	39
.72 μ M "	56	46	43
.36 μ M β -Carotene	43	33	31
.72 μ M "	46	31	30

* Dichlorophenol-indophenol.
Conditions: Temperature = 15°C; Gas phase = nitrogen; Red light. Dichlorophenol-indophenol = $3.33 \times 10^{-5} M$, $[PO_4] = 0.05 M$ (pH = 6.5); and Chlorophyll = $1.9 \times 10^{-5} M$.

and there is no great deviation from the pattern seen in Table 1 where potassium ferricyanide was used as the oxidant.

It is difficult to determine the exact amounts of Q-255 which will restore the original photochemical activity because of the inaccuracy inherent in the method for re-addition of the compound. The procedure, as outlined previously, entails an evaporation of a petroleum ether solution of the substance tested in the presence of the extracted chloroplasts. With such a method there is, a priori, as much chance that the compound will be left on the walls of the container as on the surface of the

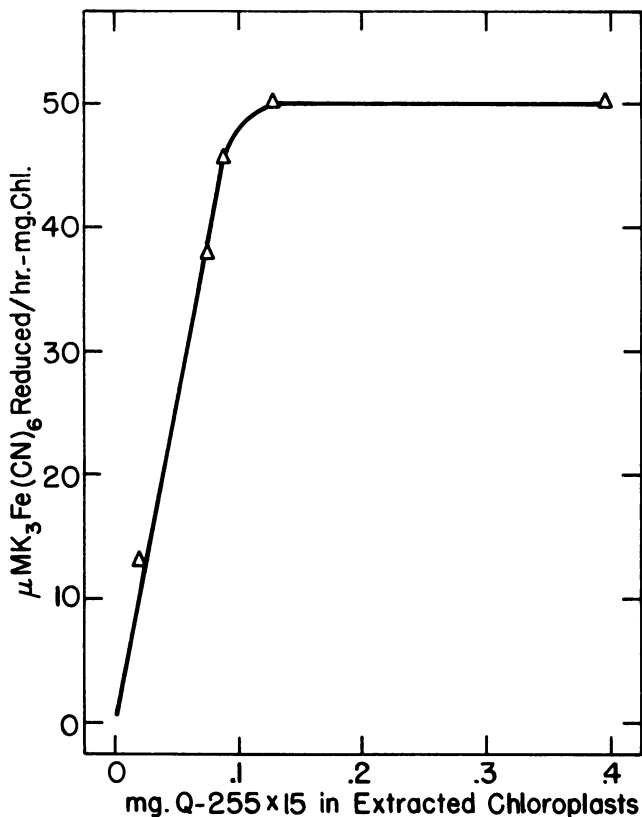


FIG. 2.—Curve showing the relation between Hill reaction activity and content of Q-255 retained in sugar beet chloroplasts after petroleum ether extraction. 100 mg lyophilized chloroplasts extracted with 10 ml, 20 ml, 30 ml, and 40 ml petroleum ether and the photochemical activity of 6.6 mg chloroplasts determined. 100 mg chloroplasts contained 4.11 mg chlorophyll and 0.40 mg Q-255. Temperature = 15°C. Light = saturating red. Gas phase = N₂. (See text.)

chloroplasts. Unfortunately, this direct evaporation is the only way found, so far, which will reactivate extracted chloroplasts.

It is possible, however, to determine the relationship between Q-255 concentration in the chloroplasts and photochemical activity by successively extracting the chloroplasts with petroleum ether and determining, after each washing, the photochemical activity and the amount of Q-255 removed from, or still retained in, the chloroplast. In Figure 2 the results of such an experiment are shown. For this particular experiment the first extraction does not alter the activity of the chloro-

plasts, although about 70 per cent of the Q-255 contained in the sample was removed. It is of interest, however, that the reactivity of the Hill reaction extrapolates to zero for complete extraction of the quinone. For this experiment the saturation rate was attained with only 30 per cent of the total content of the quinone. Presumably, this is due to the low activity of this preparation when compared to the intact chloroplast. Consequently, with chloroplast preparations with greater activity, the saturation rate would be attained at higher concentrations of the quinone.

From the data contained in Figure 2, it is possible to calculate the mole-ratio of Q-255 to chlorophyll. Such a calculation (using an average molecular weight of 906 for chlorophyll and 764 for Q-255) yielded a ratio of Q-255/chlorophyll = 0.12. Similar values were also obtained for spinach and swiss chard chloroplasts. This, we feel, is probably a minimal ratio since a portion of the Q-255 is lost during the extraction and purification procedures. We have extracted the same substituted *p*-benzoquinone from a blue-green alga, *Anacystis nidulans*, and from several green algae, including *Chlorella pyrenoidosa*, *Ankistrodesmus braunii*, *Chlamydomonas moewusii*, and *Scenedesmus obliquus*. In extracts of the photosynthetic bacterium *Rhodospseudomonas spheroides*, this compound was not observed. However, here we observed a reducible substance with an absorption maximum at 272 $m\mu$. It is probably related to the quinone obtained from two other photosynthetic bacteria, *Rhodospirillum rubrum* and *Chromatium*.¹³

Discussion.—In 1937, Robert Hill succeeded in what other experimenters previously had attempted in vain; namely, to divide the process of photosynthesis into at least two characteristically different parts. The existence of dissimilar partial reactions had been deduced earlier from studies involving the entire process. Hill found that properly isolated chloroplasts in the presence of suitable substrates other than carbon dioxide could still evolve oxygen photochemically, while the artificial substrates themselves became reduced.¹⁴ During the twenty-odd years since that discovery, much additional information has been gathered about this process, often called the "Hill reaction."^{15, 16} Beyond the fact, however, that native chlorophyll is involved, only one other substance, manganese ion, has been shown to be essential for this process.^{17, 18} Recently, as said above, Lynch and French discovered the reversible inactivation of chloroplast activity by petroleum ether extraction. This exciting observation amounts to a further subdivision of the system, since it demonstrates that a component essential for the release of oxygen can be reversibly removed.

When we re-examined this problem, we found that β -carotene, the first substance under suspicion,¹ was not the responsible entity. Griffiths *et al.*,¹⁹ working with photosynthetic bacteria, found that in their organisms the carotenoids participate in a process unrelated in essence to that assumed by Lynch and French. They reported that carotenoids prevented the photooxidation of the chlorophyll, an effect previously observed with chlorophyll in organic solution.¹⁵

Our results obtained with vitamin K derivatives appeared to be the definitive answer, except that, as stated in this paper, no significant amount of vitamin K could be found in the chloroplast extracts. In its place we discovered comparatively large amounts of Q-255, a benzoquinone. What is the exact mode of action of this substance in the photosynthetic process? It would be of little practical value to

formulate an involved reaction mechanism involving this benzoquinone at the present time before we have studied, as we plan, its reactivity in various photochemical processes. It seems obvious that this substance is involved somewhere in the electron and hydrogen transport of the Hill reaction like Coenzyme Q₁₀, whose function in electron transport in mitochondrial systems has been clearly established. The question is where exactly it fits into the pattern of normal photosynthesis, i.e., in the transport of hydrogen or electrons preceding the reduction of carbon dioxide. The hypothesis that it may be a specific intermediate in the course of oxygen production cannot be decided on the basis of our experiments on the Hill reaction alone. Purple bacteria which do not evolve oxygen in the light also contain large amounts of such a benzoquinone. This, however, is again not sufficient to prove that Q-255 is not specifically involved in oxygen evolution since the bacterial quinone is different and is characterized by an absorption peak at 272 m μ . From a purely theoretical viewpoint, Q-255 should be capable of most of the reactions tentatively assigned to vitamin K in the photosynthetic process by Wessels.¹⁵ The experiments reported here and the abundance of this compound in the chloroplasts of higher plants and in green algae make it extremely probable that it is a catalyst truly involved in the photosynthetic process. Furthermore, Lester and Crane¹³ have shown recently that Q-255 is also contained in two representatives of marine algae: the red alga *Polysiphonia* sp. and the brown alga, *Fucus* sp. These observations strengthen the notion that this particular natural quinone is confined to photosynthetic tissue.

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