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The redox-cycling assay is not suited for the detection of pyrroquinoline quinone in biological samples

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Based on the results of the so-called redox-cycling assay it has been claimed that various common foods and beverages as well as mammalian body fluids and tissues contain substantial quantities (μM) of free PQQ [M. Paz et al. (1989) in: PQQ and Quinoproteins (J.A. Jongejan and J.A. Duine, eds.) Kluwer Academic Publishers, Dordrecht, pp. 131–143 and J. Killgore et al. (1989) Science 245, 850–852]. However, by investigating samples from such sources with a biological assay of nM sensitivity, we could not confirm these claims. Analysis of the samples with procedures that proved adequate for the detection of PQQ adducts and conjugates gave equally negative results. To account for the positive response in the redox-cycling assay, as opposed to the negative results obtained by other methods, a search was made for those substances in these samples that caused the false-positive reactions. It was found that a number of commonly occuring biochemicals like ascorbic and dehydroascorbic acid, riboflavin and to a lesser extent pyridoxal phosphate, gave a positive response in the redox-cycling assay. The amounts of these interfering substances that were determined in the samples by independent methods could well explain the response. In separate experiments it was found that the effect of PQQ added to biological samples was obscured over an appreciable range of concentrations. For these reasons it must be concluded that the redox-cycling assay is not suited for the detection of PQQ in these samples. Any claims that are based on the results of this method should be disregarded.

Pyrroloquinoline quinone; Colorimetric redox-cycling assay; Quinoprotein; Distribution of pyrroloquinoline quinone

1. INTRODUCTION

Several methods have been developed for the detection and quantification of pyrroloquinoline quinone (PQQ). Published procedures include characterization of PQQ or a suitable derivative by physical techniques either directly or in combination with chromatographic methods like HPLC and GC (see [1–3]). Highly sensitive biological assays are based on the reconstitution of apoquinoprotein glucose dehydrogenases [1,4]. An immunological assay has also been reported [5].

Recently Flueckiger et al. [6] introduced a chemical test that is claimed to combine the sensitivity of a biological assay with the rapidity and simplicity of a colorimetric measurement. This so-called redox-cycling mehod [7,8] is based on the well-established reactivity of ketones (especially α-diketones, c.f. o-quinones) and aldehydes in the Strecker degradation of α-amino acids [9]. Reaction of PQQ with certain amino acids leads to the formation of reduced PQQ [10]. Reoxidation in the presence of nitroblue tetrazolium salts (NBT) gives rise to the formation of highly colored formazans. The observation that PQQ reacts fast with glycine while it is only slowly reduced by valine has been advocated as a means to correct for the color formation by interfering substances. It has been reported [7] that ascorbic and dehydroascorbic acid do not undergo redox-cycling with glycine.

Using this method, Gallop and coworkers [6–8], and Killgore et al. [11] claimed the presence of free PQQ in various biological samples including citrus fruits, milk and eggs. In view of the documented presence of PQQ-related compounds in several enzymes of mammalian origin [12], these claims could have far-reaching consequences for a possible vitamin character of PQQ. However, as it is doubtful whether PQQ will retain its chemical integrity when it is exposed for a certain length of time to solutions containing appreciable amounts of nucleophiles, we decided to investigate the presence of PQQ in these and similar samples by methods that proved to be adequate for the detection of PQQ in culture media and proteins. Except for the already known presence of PQQ in vinegar and, as reported here, the low levels occurring in some brands of beer, crucial samples mentioned by Paz et al. [7] and Killgore et al. [11] appeared not to contain PQQ. In an attempt to clarify this discrepancy, possible causes for the false-positive results obtained by the redox-cycling assay were investigated.

2. MATERIALS AND METHODS

Milk, eggs, beer (three different brands), vinegar (from red wine), and citrus fruits were obtained from a local grocery. Yeast extract

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was from DIFCO Laboratories, USA. Pig heart and pig serum were obtained from a local slaughter house. Dopamine β-hydroxylase was purified as described [13].

Redox-cycling was performed as described [6–8], using glycinate- (viz. valinate-) NBT solutions (pH 10.0) in a final volume of 1.5 ml. Serum albumin was reduced and dialyzed.

Free PQQ was determined with a biological assay [1] based on reconstitution of glucose dehydrogenase apo-enzyme from *Acinetobacter calcoaceticus* (sensitivity > 1 nM). The occurrence of protein-bound and derivatized PQQ was tested with the hexanol extraction procedure (sensitivity > 1 nmol PQQ in the sample [1,12]). Concentration of PQQ (25–250-fold) was effected by passing the acidified samples (pH 2.0 with 1 M HCl) through a Sep-Pak C_{18} cartridge previously washed with 10 mM HCl. Loosely bound material was removed with generous volumes of 10 mM HCl and water. Any PQQ present was eluted with 1 ml methanol.

Dehydroascorbic acid and L-ascorbic acid were determined colorimetrically [14].

3. RESULTS AND DISCUSSION

3.1. *Free PQQ*

Application of the redox-cycling assay to standard solutions of PQQ gave results (fig.1) in agreement with those reported [7]. Samples of fresh citrus fruit promoted a coloration (table 1) comparable to that reported by Killgore et al. [11]. As these authors ascribe this activity to the presence of 0.5 to 20 μg of PQQ per gram of citrus fruit, we added these amounts of genuine PQQ to the samples. Surprisingly, no stimulation of colour formation was observed. In order to observe an increased response at least 10 to 100 times the amount of PQQ that was claimed to be present initially, had to be added (fig.2).

Application of the biological assay to crude samples of citrus fruit as well as of 100-fold concentrated samples gave negative results (table 1). In addition, concentrated samples showed an appreciably decreased response in the redox-cycling assay. Apparently, factors contributing to the colour formation that could be observed for untreated samples were not retained during the concentration step. Concentration of samples to which low amounts of PQQ had been added gave rise to the expected response in the biological assay. Colour formation in the redox-cycling assay was again raised only for those samples that had been supplemented with large amounts of PQQ. It is clear from these results that free PQQ is not responsible for the colour formation that is observed in the redox-cycling assay of samples obtained from citrus fruit. Possible causes for the false-positive results were considered.

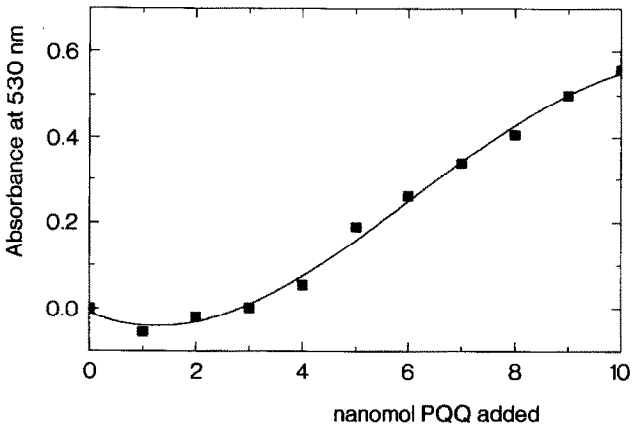

Fig.2. The effect of PQQ addition on the response of grapefruit juice, as determined with the redox-cycling assay. All values were corrected by subtracting that of the original sample (without PQQ addition).

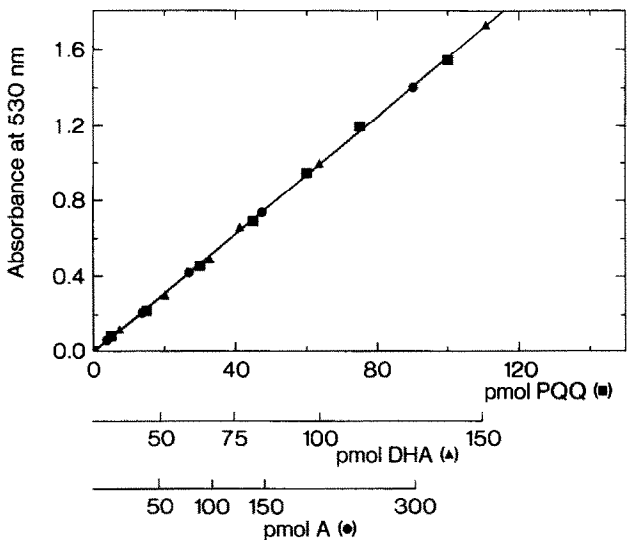

Fig.1. The effect of PQQ (■), ascorbic acid (A, ●) and dehydroascorbic acid (DHA, ▲) in the redox cycling assay [5–7].

According to a review on the Strecker degradation of amino acids [9], conversion can be effected by a large number of agents, including a variety of common biochemicals. As both ascorbic and dehydroascorbic acid are present in citrus fruit, the activity of these compounds in the redox-cycling assay was measured. Contrary to the statement made by Paz et al. [7], both compounds promote colour formation to the extent of PQQ on a molar basis (fig.1). Moreover, they exhibit the same selectivity towards reaction with glycine and valine as does PQQ. As the reason for the poor reduction by valine is probably related to its bulky side chain, it must be expected that the lower rate of reaction of PQQ with valine is the rule rather than the exception. An observation related to this phenomenon has been described by O'Donnell et al. [15]. Primary condensation products of benzophenone with glycine and alanine respectively show different acidities of the C-α-proton. As this will effect the tautomerization rate, subsequent formation of the reduced species will occur at a different rate.

The slightly lower response of ascorbic acid as compared to dehydroascrobic acid in the redox-cycling assay (fig.1) can be rationalized by recalling that the colour formation is routinely measured after a preset interval. As ascorbic acid has to be oxidized before it

Table 1

PQQ in biological samples

Sample	PQQ content according to the redox cycling method		PQQ content according to the biological assay		PQQ content according to the hexanol extraction procedure		A + DHA[a] content
	Before	After	Before	After	Before	After	
Milk	2.7	0.033	0	0	0	0	60
Egg yolk	38	ND	0	0	0	0	ND
Beer	6.3	0.022	0.005	0.005	ND	ND	ND
Grapefruit	16.8	0.050	0	0	0	0	4000
Lemon	14.2	0.044	0	0	0	0	2300
Orange	8.8	0.041	0	0	0	0	2700
Vinegar	4.2	0.063	0.020	0.020	ND	ND	ND
Yeast extract (10 mg/ml)	ND	ND	0	0	0	0	ND
Urine	ND	ND	0	0	0	0	ND
Pig heart extract	ND	ND	0	0	0	0	ND
Pig serum	ND	ND	0	0	0	0	ND

[a] A and DHA: ascorbic and dehydroascorbic acid, respectively

The concentrations (in μM) refer to the original samples. Before, After: mean before and after concentration for PQQ

becomes active in the Strecker degradation, a lag-time results. The masking of PQQ-related activity in the presence of ascorbic and dehydroascorbic acid can be understood by assuming that a substantial contribution to the reduction of NBT by PQQ results from partially reduced dioxygen species (H_2O_2, $O_2^{\cdot -}$) that are formed during the oxidation of PQQ by O_2. Ascorbic acid will act as a scavenger of such species.

Of course one could put forward that the samples used in this work were exceptional as PQQ could be accidentally derivatized to a product inactive in the biological assay. However, also the hexanol extraction procedure, well suited to determine derivatized PQQ [12], gave negative results (the level of quinoproteins is apparently too low to be detected) (table 1). Therefore, all proven methods for the determination of PQQ did not detect significant levels, while the response in the redox-cycling assay can be ascribed to the presence of ascorbic and dehydroascorbic acid in citrus fruit (table 1). From this, the question can be raised whether the claims for other biological samples are correct.

It has been reported that materials like milk, egg yolk, serum, urine, and biological tissues [6] contain substantial amounts of PQQ. However, results for those materials with the biological assay were negative (table 1). Since high amounts of ascorbic acid cannot be expected in these cases, a search for other interfering substances was carried out. From this it was found that riboflavin, and to a lesser extent pyridoxal phosphate, showed also activity in the redox-cycling assay. Since these compounds are widely distributed, they could be responsible for the false-positive response of the materials mentioned above in the redox-cycling assay. Probably for the same reasons, also in cases like vinegar and beer, where PQQ really occurs, the redox-cycling assay is not quantitative since the amounts found are much higher than those determined with the biological assay (table 1).

Based on gel chromatography and fluorescence detection, it has been claimed that culture media contain PQQ [16]. However, as has been shown already, fluorescence detection is not specific for PQQ [17]. Upon testing yeast extract (10 mg/ml) with the biological assay and the hexanol extraction procedure, no activity was found, neither before nor after concentration (table 1). From the fact that this yeast extract sample did not contain significant amounts of PQQ, it is doubtful whether other culture media extracts do.

3.2. *Other forms of PQQ*

Although the selectivity of the redox-cycling assay is inadequate, its sensitivity is high. Several enzymes have been found which contain covalently bound PQQ or pro-PQQ. Biological assays fail in these cases and the hexanol extraction procedure which is suited for that purpose has a relatively low sensitivity (>1 nmol in the sample). For those reasons one could wonder whether the redox-cycling assay is able to detect and quantify the cofactor in an enzyme preparation without interfering substances. Dopamine β-hydroxylase probably is a quinoprotein as determined with the hydrazine method [13] as well as with the hexanol extraction procedure [12]. A crude preparation has already been examined with the redox-cycling assay by Paz et al. [7]. They found that activity appeared when proteolysis with pronase was carried out. We could confirm this with purified enzyme (results not shown). However, the amounts calculated from these measurements are much

lower than the stoichiometric proportion found with the other methods. On the other hand, if it appears to be possible to discriminate between quinoproteins, flavoproteins, haemoproteins, etc., the redox-cycling assay could be used for the qualitative detection of quinoproteins. Further work on this aspect is in progress.

3.3. *The occurrence of PQQ*

Although the present search for PQQ in biological materials was not extensive, the negative results in a variety of samples (table 1) suggest that the free form may not occur in eukaryotic organisms. Only certain bacteria and their spent culture media and materials probably contaminated with these bacteria during the production process (beer, vinegar) contain free PQQ. Eukaryotic organisms contain quinoproteins with a PQQ-related cofactor [12]. Most probably, biosynthesis of this cofactor does not require free PQQ, in analogy with the case of glutamate decarboxylase in *Escherichia coli* [18]. Therefore, although a positive effect was found upon PQQ administration to test animals [11], the functional role of PQQ in eukaryotes is still unclear.

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