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A CONVENIENT MICROMETHOD FOR THE ESTIMATION OF MITOCHONDRIAL CYTOCHROME *C*

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SUMMARY

1. A simple method is described for the extraction and estimation of mitochondrial cytochrome *c*; the assay is based on differences in the Soret band of cytochrome *c* in acid and alkaline solutions.

2. The millimolar absorption index for the acid/base shift at 392.5–409.0 nm is 200.4, as compared with 25.1 for the corresponding oxidation/reduction shift at long wavelengths (535–550 nm); the sensitivity of the present method is such that reliable estimates of cytochrome *c* can be obtained with mitochondria isolated from as little as 5 mg fresh weight of insect flight muscle.

3. The ratio of cytochrome *c* to protein in mitochondria isolated from flight muscles of the tsetse fly (*Glossina morsitans*) is 0.95 μmoles/g and of the blowfly (*Sarcophaga nodosa*) 1.14 μmoles/g; in both species the ratio remains constant during adult life.

4. The proportion of cytochromes *c*, *c'*, *b* and aa_3 in mitochondria of the tsetse fly is 1.00:0.51:0.69:0.98 and of the blowfly 1.00:0.46:0.54:0.52.

INTRODUCTION

Estimation of mitochondrial concentration is usually based on the determination of mitochondrial protein[1], and therefore presupposes the preparation of suspensions that are free from contamination by extramitochondrial protein[2]. With many tissues it is difficult to achieve acceptable purification without serious loss of material or of functional integrity. The present work represents an attempt to overcome this difficulty by developing a method based on the estimation of cytochrome rather than of protein. Advantage was taken of the fact that cytochrome *c* can be separated from other mitochondrial cytochromes by mild extraction procedures[3], and that there is a substantial difference in the position of the Soret band between acid and neutral compounds of cytochrome *c*[4]. This acid/base shift provides a more sensitive index of cytochrome concentration than the corresponding oxidation/reduction shift of the Soret peak, and avoids complications associated with the fact that commonly used oxidizing and reducing agents, like ferricyanide and sodium hydrosulphite, show substantial absorption in the region of the Soret band.

MATERIALS AND METHODS

Mitochondria were isolated from flight muscles of the tsetse fly (*Glossina morsitans* Westwood) and the blowfly (*Sarcophaga nodosa* Engel) in a medium containing 0.30 M sucrose, 5 mM Tris (pH 7.4), 1 mM EGTA and 1 % bovine serum albumin (Sigma Chemical Co.). Flight muscles were squeezed from the thorax and stirred in isolation medium at 0° with a small magnetic stirrer for 5 min, filtered through gauze and spun at 1500 × *g* for 7 min. The mitochondrial pellet was resuspended in isolation medium by stirring for 1 min, re-filtered and centrifuged for final re-suspension in medium containing 0.30 M sucrose and 5 mM Tris. For determination of cytochrome/protein ratios the suspension was filtered again through a tight plug of gauze. Examination by phase-contrast microscopy of material prepared in this way showed negligible contamination with myofibrils or cellular debris.

Estimates of cytochrome *c* obtained by the method to be described were checked against estimates obtained by a modification of the method of WILLIAMS[5,6], using revised values for the absorption indices of cytochromes *c*, aa_3 and *b*[7–9]. Spectrophotometric cells were closed with loosely fitting perspex stoppers to provide deep liquid seals and so prevent reoxidation of cytochromes; the attainment of full reduction was checked by the injection of 5 µl of a saturated sodium hydrosulphite solution through small holes in the stoppers. In order to ensure maximal reduction of cytochrome *b* the concentration of sodium deoxycholate was kept below 1 mg per mg of mitochondrial protein. The concentration of standard solutions of cytochrome *c* (Type VI, Sigma Chemical Co.) was estimated by the same method using a millimolar absorption index of 25.1 for the 535–550 nm wavelength pair (21.1 for the reduced – oxidised peak at 550 nm[7]).

Protein was determined with the Folin–Ciocalteu reagent following 15 h digestion in 1.0 M NaOH to ensure complete solubilisation of mitochondrial protein; bovine serum albumin was used as a standard.

RESULTS

(1) *The acid/base absorption index of cytochrome c*

Cytochrome *c* dissolved in 0.03 M HCl has an absorption peak at 395 nm[4]; when the solution is made alkaline by the addition of NaOH the peak shifts to 406 nm. The corresponding difference spectrum shows a trough at 392.5 nm and a peak at 409 nm, as illustrated in the inset of Fig. 1, and this wavelength pair has been used to provide the absorption index on which the present method is based.

The absorption at 395 nm is not substantially affected by acid strength in the range between 0.02 and 0.04 M, because the effect of changes in hydrogen ion concentration are balanced by opposite and approximately equal effects of changes in chloride concentration[4]. Beyond the point of neutrality, effects of increasing NaOH are also small, so that consistent values for the absorption index can be obtained without critical adjustment of acid/base concentrations. For routine purposes 2.5 ml of cytochrome *c* solution in 0.03 M HCl were pipetted into a spectrophotometric cuvette (1 cm light path) and the absorption read at 392.5 and 409.0 nm. The solution was brought to 0.015 M NaOH, without significant dilution, by the addition of 24 µl of 5.0 M NaOH, and the absorption read again at 392.5 and 409.0 the sum of dif-

ferences between acid and alkaline absorptions providing a measure of the absorption index. Curve a of Fig.1 shows that the absorption index is linearly related to cytochrome *c* concentration over a convenient range of optical densities. The index was standardized with reference to solutions of known cytochrome concentration and the millimolar absorption index was estimated to be 200.4 (S.E. = 1.4; n = 10).

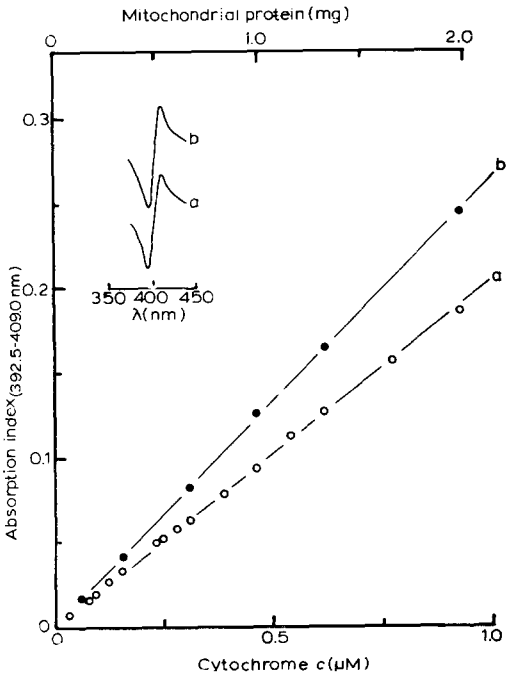

Fig. 1. The acid/base absorption index of cytochrome *c*. Curve a (○—○) shows the relation between the absorption index and cytochrome concentration of a solution containing cytochrome *c* standard (μM, bottom scale); Curve b (●—●) shows the relation between the absorption index of acid-extracted cytochrome *c* and mitochondrial concentration (mg mitochondrial protein, top scale) for the blowfly. Insets show details of the acid/base difference spectrum for cytochrome *c* standard (Curve a) and for a mitochondrial extract (Curve b) between 370 and 440 nm. The absorption index represents the sum of differences in absorbance between acid (0.03 M HCl) and alkaline (0.015 M NaOH) solutions at 392. 5 and 409.0 nm, using a 1-cm light path.

(2) *The extraction of mitochondrial cytochrome c*

A number of different extraction methods (involving phosphate, KCl, distilled water, mild sonication, weak acids and weak alkali) were tested singly or in combination, but none was found as effective as weak HCl for the extraction of cytochrome *c* without detectable contamination by other cytochrome components. The difference spectrum of a mitochondrial extract using 0.03 M HCl is shown in the inset of Fig. 1, and it is seen to conform in detail to that of pure cytochrome *c*. The efficiency of extraction was checked by estimating the amount of cytochrome *c* remaining in the particulate fraction, solubilised by sonication in the presence of deoxycholate and assayed by WILLIAMS[5,6] method. The results showed that 96.8 ± 0.9 % (n = 7) of the mitochondrial cytochrome *c* is extracted by the method described. Recovery was not significantly improved by prolonged or repeated extraction, and for routine

purposes the following procedure was adopted: 0.1 ml of the mitochondrial suspension was extracted with 3.0 ml of 0.03 M HCl; the extract was allowed to stand for 15 min at room temperature, and particulate components were then spun down at 37000 × *g*; 2.5 ml of the supernatant was used for assays as described in the previous section. Curve b of Fig. 1 shows that the absorption index of such extracts is linearly related to the concentration of the mitochondrial suspension. Reliable estimates could be obtained with as little as 200 μg of mitochondrial protein, equivalent to the yield of mitochondria from about 5 mg of insect flight muscle. It should be noted that if ionic, or strongly buffered, suspension media are used it may be necessary to spin down the mitochondria and remove the suspension medium before extracting with acid. Strong buffers could change the pH of the acid extract beyond acceptable limits, and BOERI *et al.*[4] have shown that chloride, and other anions, in concentrations exceeding 0.01 M will cause a substantial shift of the acid peak towards longer wavelengths, so causing a reduction in the absorption index.

To determine whether results obtained by the present method were substantially affected by loss of cytochrome *c* during mitochondrial isolation, supernatant fractions were tested for the presence of cytochrome *c*. The results showed that the cytochrome *c* content of these fractions amounted to no more than 3.14 ± 0.65 % ($n = 7$) of the mitochondrial cytochrome *c*.

The recovery of cytochrome *c* in the acid extract was checked against estimates of total cytochrome *c* in the mitochondrial suspension as determined by the method of WILLIAMS[5]. The results for tsetse flies and blowflies did not differ significantly, and values from a total of 7 assays have been pooled. Recovery averaged 92.9 ± 1.5 % of the expected value, which is within the range of error reported for the method of WILLIAMS[5,6], and it may be concluded that the present method provides a satisfactory basis for the estimation of cytochrome *c*. Such errors as have been shown to be involved would cause a slight underestimate of absolute cytochrome *c* levels, but would not affect the value of the method as a means of estimating mitochondrial concentration.

(3) *The cytochrome c content of mitochondria from insect flight muscle*

The adult stage of the tsetse fly is characterised by substantial development of flight musculature[11], and in the blowfly also, there is a marked increase in the content of mitochondrial substance during the first week of adult life[12]. To establish whether these developments are associated with changes in the ratio of cytochrome *c* to protein, estimates were made of the cytochrome/protein ratio for insects of different age, and the results are set out in Table I. They show that the ratio does not change significantly in the course of adult development, and that the ratio is considerably higher in the blowfly than in the tsetse fly. The constancy of the ratio within species means that where estimates of mitochondrial concentration are based on present methods, they can be converted to the more widely used standard of mitochondrial protein by use of the average values given at the bottom of the table.

The cytochrome and protein determinations on which the results of Table I are based were carried out in triplicate, enabling estimates to be made of the replicability of the method by analysis of variance. The standard deviations for protein estimates, calculated on the basis of the within group variance and expressed as a percentage of the mean, were 1.67 % for blowfly determinations and 1.77 % for tsetse

TABLE I

THE CYTOCHROME *c*/PROTEIN RATIO AT DIFFERENT STAGES DURING THE ADULT LIFE OF TSETSE FLIES AND BLOWFLIES

Cytochrome *c* was estimated by determination of the acid/base absorption index following acid extraction; protein was estimated with the Folin–Ciocalteu reagent. Values are given as the mean ± the standard error, and *n* denotes the number of values on which estimates are based.

Age days	*μmoles cytochrome c per g protein*			
	Tsetse fly	*n*	*Blowfly*	*n*
Unfed	0.946 ± 0.017	3	—	—
2–6	0.958 ± 0.023	5	1.135 ± 0.024	6
7–9	0.927 ± 0.014	10	1.152 ± 0.015	14
10–15	0.973 ± 0.008	10	1.126 ± 0.021	3
Average	0.951 ± 0.008	28	1.144 ± 0.011	23

fly determinations: the corresponding values for cytochrome estimates were 1.14 % and 1.48 %, indicating that the replicability of the present method is as good as that of standard methods for the determination of mitochondrial concentration.

Attempts were made to apply present methods to the estimation of cytochrome *c* in whole thoraces, or in whole muscle, of the two species of insect. It was found, however, that the acid/base difference spectrum of extracts of whole tissue differed substantially from that of cytochrome *c*, and that the occurrence of a component with a difference peak at 430 nm interfered at the wavelength pair employed for cytochrome *c* estimation (392.5–409.0 nm) to the extent of about 10 %. A better basis for estimates on whole tissue would be provided by the oxidation/reduction spectrum of acid extracts at long wavelengths, where a spectrum which does not differ significantly from that of cytochrome *c* can be obtained.

(4) *Cytochrome ratios in mitochondria from insect flight muscle*

Analyses of cytochrome *c* by the method of WILLIAMS[5,6], as reported in Section 2 above, provided information on the ratio of different cytochromes in mitcochondria of the two species of insect. In view of the conflicting results obtained by earlier workers[13,14], and of later reassessments of the extinction coefficients of different cytochrome components[6–8], it seemed worthwhile to put the results on record, though they are not strictly pertinent to the present investigation. For mitochondria of the tsetse fly the proportion of cytochrome *c*: *c'*: *b*: aa_3 is 1.00: 0.511 ± 0.010:0.685 ± 0.017:0.982 ± 0.020 (n = 11), and for the blowfly 1.00:0.480 ± 0.033:0.541 ± 0.024: 0.518 ± 0.016 (n = 8). The blowfly values do not differ significantly from a 2:1:1:1 stoichiometric ratio, but the tsetse fly has a relatively greater proportion of cytochromes *b* and aa_3, and the values for this species cannot be fitted to any simple ratio. In view of recent work with mammalian mitochondria, which shows a wide divergence in proportionate composition depending on the organ and the species from which the mitochondria are derived[6], it does not seem profitable to attach any special significance to the apparently simple stochiometry that characterises blowfly mitochondria.

DISCUSSION

Present results have shown that mitochondrial cytochrome *c* can be extracted from insect mitochondria by a single treatment of mitochondrial suspensions with 0.03 M HCl, and that the acid/base absorption index, using the 392.5–409.0 nm wavelength pair provides a reliable index of the cytochrome *c* concentration of acid extracts. This provides a convenient method for the determination of mitochondrial concentration, as accurate and almost as sensitive as standard methods based on protein estimation; it has the great advantage over such methods that it will be unaffected by the inevitable contamination of mitochondrial suspensions with extramitochondrial protein.

The cytochrome/protein ratio is significantly higher in blowfly than in tsetse fly mitochondria, and the general level of about 1 μmole/g protein recorded in the present work is nearly twice as high as that reported for mammalian mitochondria[6]. In view of the high oxidative capacity that characterises mitochondria from insect flight muscle[15], it is possible that a relation may emerge between oxidative capacity and cytochrome *c*/protein ratio, since among vertebrates higher values are found in mitochondria isolated from heart muscle than from less active tissues like intestine or liver.

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