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Laboratory Exercises

Using Cytochome *c* to Monitor Electron Transport and Inhibition in Beef Heart Submitochondrial Particles*

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We present a two-part undergraduate laboratory exercise. In the first part, electron transport in bovine heart submitochondrial particles causing reduction of cytochrome *c* is monitored at 550 nm. Redox-active dyes have historically been used in most previous undergraduate laboratory exercises of this sort but do not demonstrate respiratory inhibition by antimycin A and rotenone. By using cytochrome *c* instead of redox-active dyes, it is possible to observe inhibition of electron transport in the presence of the aforementioned respiratory inhibitors. In the second part, students are asked to design a soluble redox chain between NADH and cytochrome *c* using catalytic amounts of redox-active dyes. The students are also responsible for designing the assays and control. The entire experiment can be performed in 3 h with single-beam spectrophotometers that are currently used in most undergraduate teaching laboratories. This exercise is suitable for large undergraduate classes of over 200 students and can be performed either by a single student or a student pair.

Keywords: Submitochondrial particles, cytochrome *c*, electron transport.

Demonstrating electron transport (ET)[1] and ET inhibition is useful for students learning about oxidative phosphorylation in undergraduate introductory biochemistry courses because it demonstrates central concepts of cellular respiration. Previously, for our large undergraduate biochemistry class an experiment was used (adapted from Ref. 1) whereby ET in bovine heart submitochondrial particles (SMPs; also referred to as Keilin-Hartree heart particles) was monitored by the reduction of the redox dye 2,6-dichlorophenolindophenol (DCPIP). The artificial electron acceptor DCPIP is reported to have a redox potential (+0.217 V), similar to cytochrome c_1 [2]. Although DCPIP was reduced when SMPs were treated with the electron donors NADH and succinate, students were never able to observe inhibition of ET when the respiratory inhibitors rotenone and antimycin A were added to the SMP assays. Recently, we attempted to revise this laboratory by searching for other redox dyes to replace DCPIP. In separate experiments, we tried phenazine methanolsulfate (PMS), ferricyanide (FCN), and oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). In no case were we successful in observing electron transport inhibition in the presence of the aforementioned respiratory inhibitors. We concluded that redox dyes can nonspecifically accept electrons from reduced respiratory coenzymes (cytochromes, flavins, or iron-sulfur groups), thus making it difficult to demonstrate ET concepts to students.

We then decided to try cytochrome *c* (cyt *c*) as a replacement for redox dyes in our assays. Cyt *c* is a natural component of the respiratory chain of mammalian mitochondria, is water soluble, relatively inexpensive to purchase in purified form, and specifically shuttles electrons between complex III (ubiquinol:cyt *c* oxidoreductase) and IV (cyt *c* oxidase) of the respiratory chain. Cyt *c* reduction can be monitored by an increase in absorbance at a wavelength of 550 nm. The experimental data we obtained, which is presented in "Part A," clearly illustrates that cyt *c* is suitable for monitoring both electron transport and ET inhibition in assays with SMPs.

In addition to the experimental data mentioned above, we also present a novel inquiry-based module ("Part B"), which asks students to construct a soluble redox chain between NADH and cyt *c* by adding catalytic amounts of redox-active dyes. The students are responsible for setting up the assays and designing the necessary controls.

Cautionary Note—Students should be warned by the teaching instructor about the necessity of wearing gloves to handle the respiratory inhibitors cyanide, rotenone, and antimycin in Part A as well as the redox dyes in Part B.

MATERIALS AND METHODS

The following information pertains to the preparation of SMPs and solutions by a trained technician in advance of the laboratory.

* The Department of Biological Sciences, University of Calgary, provided financial assistance to A. D. M. during the time in which this laboratory was developed.

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[1] The abbreviations used are: ET, electron transport; SMP, submitochondrial particle; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methanolsulfate; FCN, ferricyanide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; cyt *c*, cytochrome *c*; NADH, nicotinamide adenine dinucleotide (reduced).

This material should be stored at 4 °C until needed. All chemicals are obtained from Sigma (St. Louis, MO) unless otherwise stated. Volumes required per student pair are given in parentheses after the solutions. In order to estimate the total amount needed for the experiment, we suggest multiplying the parenthetical number by total number of students (or student pairs) and then doubling this volume, to compensate for student error.

Assay reagents for Part A are as follows:

- Succinate, 0.2 M, pH 7.0 (0.7 ml) (obtained from BDH Chemicals, Poole, United Kingdom)
- Malonate, 0.2 M, pH 7.0 (0.2 ml)
- Cyt *c*, 0.3 mg/ml in 0.3 M KPO_4 buffer, pH 7.4 (34.6 ml)
- NADH, 2.5 mg/ml (0.6 ml), made fresh the day of the laboratory
- Potassium cyanide (KCN), 0.05 M (1.2 ml) (obtained from Fisher Scientific, Pittsburgh, PA)
- Antimycin, 0.1 mg/ml in 95% ethanol (0.2 ml)
- Rotenone, 0.01 M in 95% ethanol (0.2 ml)
- SMPs (1.35 ml), prepared as described below

Note—Antimycin A, cyanide, and rotenone are toxic chemicals! Handle with care.

Assay reagents for Part B are as follows:

- Cyt *c*, 0.3 mg/ml in 0.3 M KPO_4 buffer, pH 7.4 (22.4 ml)
- PMS, 0.4 mM (0.1 ml), 0.05 mg/ml (0.1 ml)
- FCN, 0.4 mM (0.1 ml) (obtained from Aldrich, Milwaukee, WI)
- TMPD, 0.4 mM (0.1 ml)
- DCPIP, 0.4 mM
- NADH, 0.05 mg/ml (0.8 ml), made fresh the day of the laboratory

Note—PMS, FCN, TMPD, and DCPIP are toxic chemicals! Handle with care.

For preparing the SMPs from bovine heart, all steps are to be performed at 4 °C unless otherwise stated. The following protocol was adapted from Ref. 1. It is also possible to purify SMPs from isolated mitochondria by a variety of other methods [3].

A beef heart (fresh) is freed of visible fat and fibrous tissue and then passed twice through a meat grinder (fine grid plate). The muscle mince is then washed six to eight times over a 4-h time period in 10 volumes of 15 °C water containing 1 mM EDTA adjusted to pH 7.4. During each wash, the material is filtered and the filtrate discarded. Then the muscle mince is squeezed through four layers of cheesecloth to remove excess fluid, and may be frozen at this point in time for up to a year. Usually, 200 g of muscle mince is required to process SMPs for large classes of 220 students (10 laboratory sections of 22 students each). Smaller aliquots may also be processed if class sizes are smaller, but volumes below this need to be scaled down accordingly.

Muscle mince (200 g) is blended at high speed for 7 min in a Waring blender with 500 ml of 20 mM phosphate buffer, pH 7.4, containing 1 mM EDTA. The blended preparation is centrifuged at 5,000 × *g* for 15–20 min to remove larger fragments of muscle residue. The pellet may be re-extracted if desired as above. The pH of the combined supernatants containing the SMPs is adjusted to 5.6 with the careful addition of 1 M acetic acid. The acidified solution is then centrifuged at 13,000 × *g* for 20 min. Finally, the pellet is resuspended with hand homogenization in 0.25 M phosphate buffer, pH 7.4, containing 1 mM EDTA. This solution containing SMPs remains active for up to a week if stored at 4 °C.

The spectrophotometers required in order for the students to perform this laboratory needs to have a slit width no larger than 10 nm. The reason behind this requirement is that the α-band of cyt *c* that is monitored spectroscopically at 500 nm in these experiments is around 8 nm wide. The data presented in this manuscript was obtained with an inexpensive single-beam spectrophotometer (GENESYS 20 model; Thermo Spectronic, Madison, WI), and the results are comparable to those obtained with dual-beam instruments (*i.e.* Shimadzu UV 160 U; Shimadzu Scientific Instruments, Columbia, MD). Older Spectronic 20 instruments commonly used in undergraduate laboratories have relatively wide slit widths, and the cyt *c* absorbance values are decreased because the α-band absorbance is averaged by the spectrophotometer with incoming light. If necessary, data can be obtained with these older instruments by blanking cyt *c* to an absorbance of 0.4, and in this manner the slit width may be manually restricted.

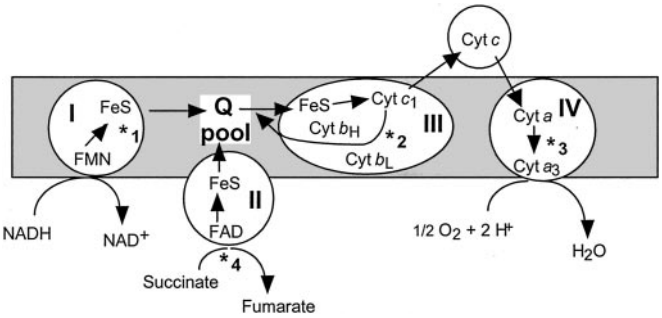


FIG. 1. **The respiratory chain of mammalian mitochondria.** FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; FeS, iron sulfur centers. The *b*, *c*, and *a* cytochromes are designated as cyt *b*, *c*, or *a* (with appropriate subscripts). The roman numerals correspond to the four respiratory complexes: I, NADH: ubiquinone oxidoreductase; II, succinate:ubiquinone oxidoreductase; III, ubiquinol:cyt *c* oxidoreductase; IV, cyt *c* oxidase. The respiratory inhibitor rotenone inhibits at the asterisked sites. Rotenone inhibits at site 1, antimycin A inhibits at site 2, and cyanide inhibits at site 3. Malonate resembles succinate and competitively inhibits complex II at asterisked site 4.

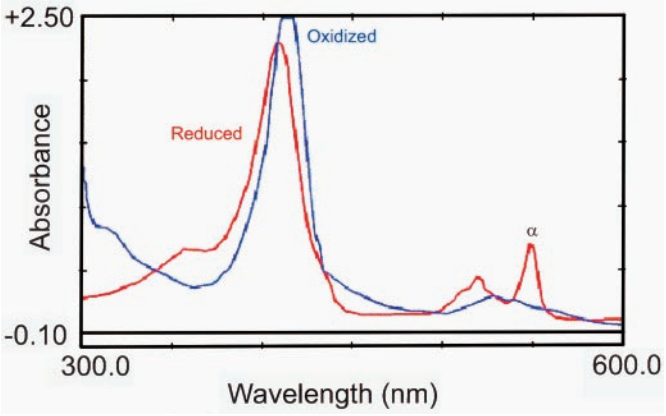


FIG. 2. **Absorption spectra of cyt *c*.** Oxidized cyt *c*, 0.3 mg/ml (*blue trace*), was artificially reduced with 1 mM ascorbate (*red trace*).

RESULTS AND DISCUSSION

Part A

A simplified version of the redox chain of beef heart SMPs showing only the major electron transporting cofactors is illustrated in Fig. 1. About 50% of bovine heart SMPs in a given preparation are inside-out [4], and in this population the NADH:ubiquinol oxido-reductase (complex I) should be directly assessable to the electron donor NADH. The other electron-donating substance used in this experiment is succinate. Succinate is transported across the SMP membrane and should thus be capable of donating electrons directly to complex II (succinate:ubiquinol oxidoreductase) in both right-side and inside-out vesicles.

TABLE I
Assay tube set up for Part A

	Tube										
	1	2	3	4	5	6	7	8	9	10	11
Cyt *c* in buffer	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
SMPs	0.1	0.1	0.05	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Water	0.2	0.1	0.15				0.2	0.1			
KCN		0.1	0.1	0.1	0.1	0.1		0.1	0.1	0.1	0.1
Malonate				0.1					0.1		
Antimycin					0.1					0.1	
Rotenone						0.1					0.1
Succinate	0.1	0.1	0.1	0.1	0.1	0.1					
NADH							0.1	0.1	0.1	0.1	0.1

TABLE II
Raw data showing reduction of cytochrome c by succinate and NADH (550 nm)

Column numbers correspond to assay tubes in Table I.

Time	Tube									
	1	2	3	4	5	6	7	8	9	10
0:00	0.002	0.000	0.000	0.001	0.001	0.002	0.001	0.003	0.003	0.001
0:30	0.009	0.009	0.009	0.003	0.016	0.029	0.012	0.027	0.012	0.048
1:00	0.014	0.013	0.013	0.004	0.024	0.048	0.018	0.032	0.014	0.080
1:30	0.016	0.016	0.016	0.005	0.031	0.062	0.023	0.037	0.015	0.101
2:00	0.017	0.018	0.018	0.005	0.036	0.075	0.026	0.041	0.017	0.114
2:30	0.018	0.019	0.018	0.006	0.040	0.089	0.030	0.044	0.021	0.125
3:00	−0.031	−0.014	−0.020	−0.020	−0.016	0.020	0.021	0.035	0.029	0.131
3:30	−0.011	0.093	0.030	−0.007	−0.023	0.025	0.035	0.102	0.099	0.183
4:00	−0.016	0.181	0.070	−0.009	−0.022	0.028	0.030	0.167	0.173	0.255
4:30	−0.015	0.225	0.112	0.025	−0.020	0.033	0.028	0.226	0.239	0.277
5:00	−0.011	0.235	0.158	0.042	−0.018	0.038	0.027	0.277	0.287	0.285
5:30	−0.007	0.237	0.201	0.058	−0.017	0.041	0.028	0.298	0.296	0.287
6:00	−0.001	0.238	0.229	0.074	−0.015	0.045	0.029	0.298	0.296	0.287

At this point, it is useful to mention the sites where inhibition of respiration can occur (see Fig. 1). Rotenone binds to the ubiquinone (Q)-docking site at complex I (*1), preventing electron flow from NADH via the iron-sulfur clusters (FeS) to the Q pool [5]. Antimycin A binds near cytochrome b_H (*2) in complex III and blocks the forward oxidation of reduced cytochrome b_H and promotes reverse electron transfer from reduced b_H to semi-ubiquinone [6]. Cyanide binds at oxidized cytochrome a_3 in complex IV (*3) and stops electron flow to the terminal electron acceptor, oxygen [7]. Malonate is a compound that structurally resembles succinate and is therefore a competitive inhibitor of complex II (*4) [8].

Fig. 2 shows oxidized (blue) absorbance spectra of 0.3 mg/ml cyt *c*. The red trace shows cyt *c* after reduction with ascorbate. The major difference between the two spectra occurs in the region of the α-band at 550 nm. The micromolar extinction coefficient for reduced horse heart cyt *c* in 0.1 M phosphate buffer, pH 6.8, is 23.94 mM^{-1} cm^{-1} at 550 nm [9].

Table I denotes how the series of assays to be performed by students in this part of the experiment are to be set up. The reagents listed through rotenone in Table I may be pipetted into test tubes ahead of time (the electron-donating substances are not added yet). SMPs are added (one test tube at a time) at time (T) = 0:00 min. The absorbance is then recorded at 550 nm every 30 s for a 3-min time period to determine the endogenous rate of cyt *c* reduction. At T = 2:45 min, the electron-donating substance (either succinate or NADH) is added and the absorbance measured for another 3 min. Actual data obtained by a single undergraduate student (A. Melin) is recorded in Table II.

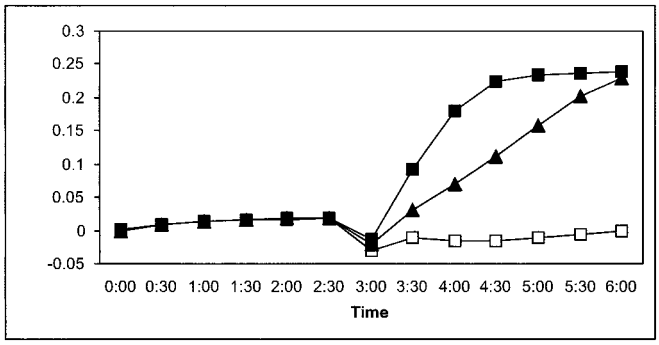


FIG. 3. **Reduction of cyt *c* with succinate-fed SMPs.** □, in the absence of cyanide (CN−); ■, in the presence of CN; ▲, in the presence of CN but using half the SMP concentration.

Fig. 3 shows the results obtained when SMPs were incubated with succinate (Table II; test tubes 1–3). In the absence of cyanide (□), little build up of reduced cyt *c* is observed, because electrons can immediately flow from reduced cyt *c* through complex IV due to the high affinity of cyt *c* for the respiratory complexes. However, when the flow of electrons from cyt *c* to complex IV is blocked with cyanide, the level of the cyt *c* population steadily increases and is observed as an increase in absorbance at 500 nm (■). The slope of the initial linear portion of this reduction curve (initial rate) is used to estimate the rate of ET. It is interesting to note that when half the concentration of

SMPs is added to the assay mixture (▲), the initial rate of cyt *c* reduction decreases by 50%. This illustrates to students the catalytic nature of the respiratory chain (the rate increases proportionally to the amount of SMPs).

Fig. 4 shows the results obtained in assays 4–6 of Table II (succinate + CN in the presents of inhibitors) with assay 2 included for comparison (succinate + CN only). Here it is possible to observe a significant inhibition of electron flow from succinate to cyt *c* in the presence of the competitive inhibitor malonate (a structural analogue of succinate). The respiratory inhibitor antimycin A strongly inhibits ET in SMPs. An unexpected result, however, was the observation that the complex I inhibitor rotenone also could inhibit electron flow between succinate and cyt *c*. To explain this possibility to students, it is necessary to inform them in the theory section of the laboratory manual of a previous reference [5], which showed antimycin A is capable of binding at the rotenone inhibition site in complex I. These results simply suggest that the reverse may be also be possible (rotenone can bind the antimycin A site in complex III).

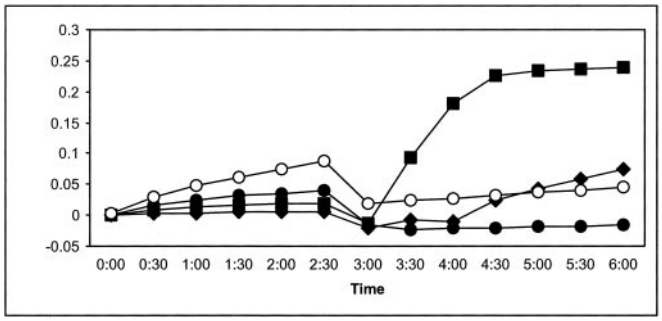


FIG. 4. **Reduction of cyt *c* with succinate-fed SMPs (CN included in all assays).** ■, in the absence of any other inhibitor; ♦, in the presence of malonate; ○, in the presence of rotenone; ●, in the presence of antimycin A.

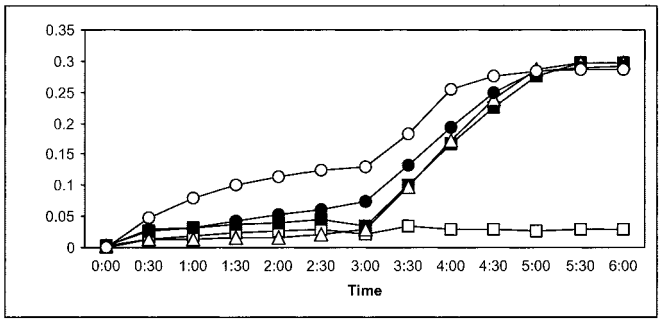
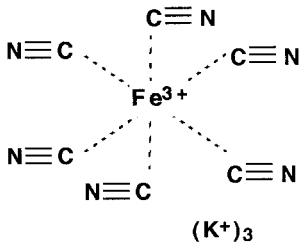


FIG. 5. **Reduction of cyt *c* with NADH-fed SMPs.** □, in the absence of cyanide (CN−); ■, in the presence of CN−; △, in the presence of malonate; ●, in the presence of antimycin; ○, in the presence of rotenone.

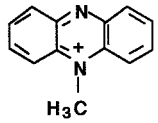
Fig. 5 (Table II; test tubes 7–12) compares the rate of cyt *c* reduction when NADH is used as an electron donor. In the absence of CN (□), this rate is negligible because electrons from reduced cyt *c* are quickly transferred to complex IV (as was also observed in Fig. 3 with succinate). In the presence of CN (■), we get a rapid rate of cyt *c* reduction, again confirming results observed in Fig. 3. Malonate, the competitive inhibitor of complex II, has no effect on the rate of cyt *c* reduction because complex II is no longer part of the electron flow pathway (△). Unexpected results, however, are observed in Fig. 5 with the respiratory inhibitors rotenone and antimycin A (assayed separately) (○, ●). Neither inhibitor had any effect on electron flow between NADH and cyt *c*. Our results suggest that electrons from NADH enter complex I and are then capable of reducing cyt *c* directly instead of indirectly through the ubiquinone pool and complex III. Again, students should be informed in the theory section of their laboratory manual of a report in the literature [10] using bovine heart SMPs that confirm our observation that complex I does display cyt *c* reductase activity. This activity can be inhibited by the compound adriamycin, also known as the cancer drug Doxorubicin [10]. Unfortunately, Doxo-



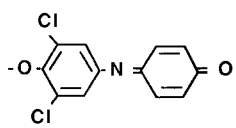
Potassium Ferricyanide (FCN)
Eo' = +0.42 V
n = 1

N,N,N',N',-Tetramethyl-p-phenylene diamine (TMPD)
Eo' = + 0.26 V
n = 2

FIG. 6. **Redox dyes that may be used to shuttle electrons between NADH and cyt *c*.**



Phenazine Methosulfate (PMS)
Eo' = + 0.08 V
n = 2



2,6 Dichlorophenolindophenol (DCPIP)
Eo' = 0.22 V
n = 2

TABLE III

Assay tube set-up for Part B (student pre-laboratory assignment)

All volumes are in milliliters, and NADH was added after 3 min, after endogenous rates are determined.

	Assay					
	Control	DCPIP	PMS	FCN	TMPD	1/10 dye
Cyt *c*	2.8	2.8	2.8	2.8	2.8	2.8
Water	0.1					0.09
DCPIP		0.1				0.01 ?
PMS			0.1			0.01 ?
FCN				0.1		0.01 ?
TMPD					0.1	0.01 ?
NADH	0.1	0.1	0.1	0.1	0.1	0.1

TABLE IV

Raw data of reduction of cyt c by various redox dyes (550 nm)

Time	Control	DCPIP	PMS	FCN	TMPD	1/10 PMS
0:00	0.000	0.000	0.000	0.000	0.000	0.000
0:30	0.000	0.000	0.000	0.000	−0.001	0.000
1:00	−0.001	0.000	0.000	0.000	−0.001	0.000
1:30	0.000	0.001	0.000	0.000	−0.001	0.000
2:00	0.000	0.001	0.000	0.000	−0.001	0.000
2:30	−0.001	0.001	0.000	0.000	−0.001	0.000
3:00	0.000	−0.008	0.339	−0.004	−0.013	0.047
3:30	0.000	0.000	0.341	−0.004	−0.012	0.227
4:00	0.002	0.008	0.341	−0.004	−0.012	0.320
4:30	0.000	0.017	0.341	−0.005	−0.010	0.338
5:00	−0.001	0.026	0.341	−0.005	−0.013	0.343
5:30	0.000	0.033	0.341	−0.005	−0.009	0.345
6:00	0.000	0.040	0.341	−0.005	−0.008	0.345

rubicin is extremely expensive from commercial sources (Sigma quotes $209 U.S./10 mg) and was not tested in this study. It may be possible to purchase Doxorubicin from hospital pharmacies at a cheaper rate. Biochemistry instructors are also welcome to omit the NADH assays if they feel the material adds too many complications to the experiment.

When writing up their reports, students will be asked to first subtract the endogenous rates from the initial velocity values they observe when succinate or NADH is added (slope of the initial linear portion of the cyt *c* reduction curve). From these initial velocities, it is possible to calculate the percentage rates observed in the presence of inhibitors relative to the succinate + CN rate, which is set to a maximum of 100%. Students can also calculate the reduction rates as micromoles of cyt *c* reduced per minute per milliliter using Beer's Law:

$$A = Ebc$$

where A is the absorbance value at 550 nm, E is the extinction coefficient for reduced cyt *c* (reported to be 23.94 mM^{-1} cm^{-1} [9]), b is the path length of the curvette (1 cm), and c is the unknown concentration. For example, if the absorbance rate $A = 0.046$ units/min, $c = A/Eb$, or 0.046/23.94 mM^{-1} cm^{-1} × 1 cm. The value obtained should be divided by 1000 to convert to a molar concentration as the extinction coefficient is mM. From this we get a cyt *c* reduction rate of 1.92 μmol/min.

Part B

In this inquiry-based portion of the laboratory, students will try to construct a soluble redox chain between NADH

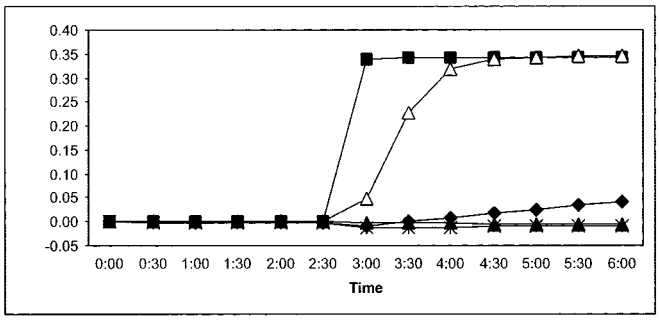


FIG. 7. **Reduction of cyt *c* by various redox dyes.** ■, in the presence of 0.4 mM PMS; △, in the presence of 0.04 mM PMS; ◆, in the presence of DCPIP; [triaf], in the presence of FCN; ∗, in the presence of TMPD.

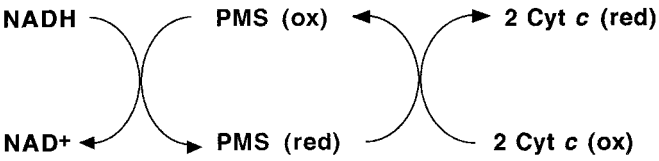


FIG. 8. **A soluble redox chain between NADH and cyt *c* using PMS.**

(−0.32 V) and cyt *c* (+0.25 V). Because NADH is a two-electron donor/acceptor and cyt *c* is only capable of donating or accepting one electron at a time, students will need to try to use redox dyes as electron shuttles to accomplish the task. Students will test the dyes FCN, DCPIP, PMS, and TMPD. The structures of these dyes (FCN (ntp server: niehs.nih.gov), TMPD (11), PMS (11), DCPIP (1)) and their redox potentials (2) can be seen in Fig. 6. Some dyes are capable of accepting and donating only one electron at a time ($n = 1$). These redox chemicals will not function as an intermediary link between NADH and cyt *c* (FCN is an example). Other dyes may be capable of accepting or donating either one or two electrons at a time ($n = 2$), but their redox potential may be below that of cyt *c* (TMPD is an example).

It is up to the students to design the assays (including controls) for this section of the laboratory. A sample of the pre-laboratory assignment is shown in Table III. In the control assay, the absorbance of cyt *c* should be monitored for several minutes to establish an endogenous rate, and NADH should be added in order to see if electron transfer (even a slow rate) is possible. In separate test tubes (assays 2–5), students should test, one at a time, whether or not the redox dyes can reduce cyt *c*. Then, at $T = 3{:}00$ min, NADH is added and the rate monitored again for 3 min to see if the dyes can shuttle electrons between NADH and cyt *c*. The concentration of the redox dyes used in this experiment is 0.4 mM, which is one-tenth that of NADH. The most successful dye should be studied in one-tenth the concentration (0.04 mM). Students can do this dilution themselves. Experimental results that were obtained in this part of the experiment are recorded in Table IV and are illustrated in Fig. 7. Here it can be seen that a slow reduction of cyt *c* is possible with DCPIP but that PMS gives even better results, even when diluted 10-fold (0.04 M concentration).

At the end of this study, students should compare the

structure of PMS with the structures of flavins or ubiquinone (found in the coenzyme section of all biochemistry text books) in order to understand how this redox dye might function as an electon shuttle between NADH and cyt *c*. In the end, they should draw the soluble redox chain constructed in a similar manner as is illustrated in Fig. 8.

CONCLUDING REMARKS

In Part A, we present a novel laboratory experiment using cyt *c* to replace traditional redox dyes as a method for monitoring ET in bovine heart SMPs. This is beneficial because cyt *c* is more specific at transporting electrons and thus inhibition of ET in the respiratory chain can be properly demonstrated. In Part B, we outline a novel inquiry-based experiment that allows students to construct a soluble redox chain between NADH and cyt *c* using redox active dyes. Knowledge of the chemistry of these dyes as well as how their redox potentials fit between those of NADH and cyt *c* is essential for understanding the experimental results. Both parts of this experiment will allow students to gain a deeper understanding of the respiratory complexes. Experimental results and sample calculations are presented to support and explain our findings.

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