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The replacement of cytochrome c in digitonin fragments from beef-heart sarcosomes

TSOU[1] and others[2–5] using Keilin–Hartree heart muscle particle preparations, have demonstrated that cytochrome *c*-deficient preparations could be made and that exogenous cytochrome *c* has much less enzymatic activity than endogenous. In mitochondria the removal of cytochrome *c* (ref. 6) produces greater reactivity with exogenous cytochrome *c* (ref. 7). This communication deals with the extraction and reinsertion of cytochrome *c* in phosphorylating digitonin submitochondrial fragments.

Digitonin fragments were prepared and stored according to the HAAS AND ELLIOTT[8,9] modifications of the methods of COOPER AND LEHNINGER[10] and DEVLIN AND LEHNINGER[11].

Aliquots of digitonin fragments (2.5 ml) were placed into each of four precooled polypropylene 30 rotor (Spinco) ultracentrifuge tubes and 5.0 ml of 0.10 M phosphate buffer (pH 7.4) were added. All tubes were kept at 2° for 30 min. The tubes were centrifuged for 40 min at 105000 × *g*. The supernatants were collected and stored (—20°). The pellet from one tube was suspended in 0.25 M sucrose *plus* 0.01 M Tris buffer (pH 7.4).

The pellets from the other two tubes were homogenized in 2.5 ml of $2.5 \cdot 10^{-4}$ M cytochrome *c*, held at 2° for 30 min, centrifuged at 105000 × *g* for 40 min, and one pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and was immediately centrifuged as before and resuspended in sucrose–Tris medium (2.5 ml). The other pellet was resuspended in sucrose–Tris medium. Treated digitonin fragments were stored as previously described[8].

Respiratory activity was observed as described by STRICKLAND, ZIEGLER AND ANTHONY[12] using a reaction medium containing 20 ml 0.02 M phosphate buffer (pH 7.4); 20 ml of the 75 mM sucrose–225 mM mannitol–0.1 mM EDTA solution; 4 ml 1 M KCl; and 2 ml 1 M $MgCl_2$ and bovine albumin (1 % final concentration). Protein concentrations were determined by the method of LOWRY *et al.*[13].

Abbreviation: PMS, phenazine methosulphate.

TABLE I

THE RESPIRATORY ACTIVITY AND CYTOCHROME CONCENTRATIONS OF VARIOUSLY TREATED DIGITONIN FRAGMENTS

The volume of the cell of the oxygen electrode on all additions reached 3.50 ml and the temperature of the reaction was 25°. 0.2 ml of digitonin fragments was used. 10 μl 1 M succinate and 10 μl 1 M β-hydroxybutyrate, 5 μl 0.047 M ADP and 15 μl PMS (2%) were added where indicated.

Expt. No.	*Fragments*	*Substrate*	*Addition*	*Oxygen uptake* ($m\mu M$/sec)*	*Respiratory control ratio***	*Cytochrome*	*Concentrations*** (mM)*
1 (a)	Digitonin fragments	Succinate	—	357			
			ADP	412	1.2		
			PMS	652		$a + a_3$	0.0009
						b	0.0008
(b)	Digitonin fragments	β-Hydroxy-	—	179		$c + c_1$	0.0007
		butyrate	ADP	237	1.30		
			PMS	357			
2 (a)	Digitonin fragments after cytochrome c extraction	Succinate	—	113			
			ADP	231	2.05		
			PMS	804		$a + a_3$	0.0011
						b	0.0009
(b)	Digitonin fragments after cytochrome c extraction	β-Hydroxy-	—	231		$c + c_1$	0.0002
		butyrate	ADP	349	1.50		
			PMS	349			
3 (a)	Digitonin fragments treated with cytochrome	Succinate	—	510			
			ADP	707	1.40		
			PMS	1124		$a + a_3$	0.0005
						b	0.0017
(b)	Digitonin fragments treated with cytochrome	β-Hydroxy-	—	203		$c + c_1$	0.0036
		butyrate	ADP	203	1.00		
			PMS	510			
4 (a)	Digitonin fragments treated with cytochrome and washed	Succinate	—	463			
			ADP	625	1.15		
			PMS	1162		$a + a_3$	0.0010
						b	0.0009
(b)	Digitonin fragments treated with cytochrome and washed	β-Hydroxy-	—	113		$c + c_1$	0.0014
		butyrate	ADP	231	2.05		
			PMS	231			

* The oxygen uptake and cytochrome concentrations were calculated for a concentration of digitonin fragments giving 1 mg protein per ml.

** The respiratory control ratio is the ratio of the uptake of oxygen in the presence of ADP to the uptake of oxygen prior to the addition of ADP.

*** These calculations are according to the wavelength pairs and absorbance indices reported by ESTABROOK AND HOLOWINSKY[15] for difference spectra (reduced/oxidized) recorded in a Cary 11 spectrophotometer using quartz cuvettes (1-ml capacity, 1-cm light path).

Cytochrome *c* present in the digitonin fragments (1 mg protein per ml) was 0.0007 mM. After treatment with 0.1 M phosphate buffer, the cytochrome *c* concentration was 0.0002 mM (Table I). Respiratory activity with succinate was decreased by treatment with 0.1 M phosphate buffer. The respiratory control ratio was increased in the cytochrome *c*-deficient fragments as compared with the untreated digitonin fragments.

The absorption spectrum of cytochrome *c*-deficient digitonin fragments, after exposure to $2.5 \cdot 10^{-4}$ M cytochrome *c* followed by centrifugation and resuspension, showed a 550 mμ absorbance, equivalent to a cytochrome *c* concentration of 0.0036 mM. If centrifuged cytochrome *c*-treated fragments were rapidly suspended in 0.1 M phosphate buffer and immediately centrifuged, the cytochrome *c* trapped between (or loosely bound to) the fragments, was removed, giving a cytochrome *c* concentration of 0.0014 mM, double the original concentration (Table I). Increased cytochrome *c* absorption in the cytochrome *c*-treated fragments and the broadening observed with the wide slit required by highly scattering specimens, increased the apparent cytochrome *b* concentration.

Highest respiratory rates were observed with the fragments treated with cytochrome *c*. Increased respiration cannot be due to bypassing of the cytochrome *c* oxidase by denatured cytochrome *c*, as the respiratory control ratio increased slightly as well. Washing-treated fragments with 0.1 M phosphate buffer caused a slight decrease in respiration and a decrease in respiratory control ratio with succinate as the substrate. No respiratory control of β-hydroxybutyrate-supported respiration was observed in the unwashed cytochrome *c*-treated fragments, and phenazine methosulphate (PMS) stimulation of respiration indicated that succinate dehydrogenase was not destroyed.

Recently LENAZ AND MACLENNEN[14] have noted the non-extractability of cytochrome *c* from sonic fragments. Since sonic fragments differ somewhat from digitonin fragments in properties such as ATPase activity, ATP–ADP exchange, ATP–P_i exchange, relative activity of phosphorylation sites and in stability, we decided to study the removal and reinsertion of cytochrome *c* in the digitonin fragments. When much of the cytochrome *c* has been removed from the respiratory chain, the flow of electrons from the β-hydroxybutyrate system was favored over that from the succinate system. The relative decrease in the respiratory activity of the fragments with succinate, after the removal of the native cytochrome *c*, is clearly indicated by the oxygen uptake data. Since the loss was restored by the reinsertion of cytochrome *c*, it is evident that respiration in the cytochrome *c*-deficient fragment was limited by cytochrome *c*.

The respiratory control ratio, with succinate as substrate, increased during the treatment with 0.1 M phosphate even though the respiration markedly decreased. The respiratory control ratio of 1.40 with extracted fragments after exposure to cytochrome *c* indicates that the respiration must be mediated by the electron-transport chain as confirmed by the washing out of trapped cytochrome *c*, reducing the concentration of cytochrome *c* in the preparation by 60 % and the respiratory rate by 10 %, without significant change in respiratory control.

With β-hydroxybutyrate, the respiratory control ratio was increased by the extraction procedure and, after reinsertion of cytochrome *c* and washing, still showed respiratory control. PMS-mediated respiration with β-hydroxybutyrate was increased by reinsertion and then decreased by washing of the fragments.

The extraction of cytochrome *c* from the fragments by 0.1 M phosphate buffer also removes uncoupling agents. During the further treatment the accessibility of electrons to PMS from succinate dehydrogenase is steadily increased, while the level of respiration with β-hydroxybutyrate remained constant or diminished. TSOU[1] has shown a difference in the two forms of cytochrome *c* based on their complexing capacity with cyanide. He has pointed out that exogenous cytochrome *c* combines with cyanide to form a stable complex, while the endogenous cytochrome *c* is not affected by cyanide even in a prolonged incubation. Behavior of the fragments in the presence of trapped cytochrome *c* indicated that the exogenous cytochrome *c* was in good equilibrium with the endogenous cytochrome *c*, and that the bypass of cytochrome oxidase by denatured cytochrome *c* was not increased over the control.

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The effect of soluble proteins on the fragmented sarcoplasmic reticulum

In an investigation into the nature of the dialyzable cofactor of the fragmented sarcoplasmic reticulum (FSR) (refs. 1–3) it was found that the soluble fraction of a muscle homogenate extended the delay in syneresis of myofibrils produced by FSR (ref. 4). One of the effective substances appeared to be inorganic phosphate and the

Abbreviation: FSR, fragmented sarcoplasmic reticulum.