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APPENDIX

Spectrum of Horse-Heart Cytochrome *c*

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As the only published complete spectrum of cytochrome *c* is that given by Theorell (1936) for a preparation containing 0·34% of iron, and therefore impure to the extent of about 28%, it was thought worthwhile to give in detail numerical data of the spectrum of the purest preparation of the protein so far obtained, containing 0·456% of iron. A figure of this spectrum had been published in the review by Keilin & Slater (1953).

MATERIALS AND METHODS

Preparation of cytochrome c. Chromatographic fraction I from horse-heart cytochrome *c* was prepared by the method of Margoliash (1954), by elution from a column of Amberlite IRC-50 with 0·25 M-ammonium acetate, pH 7·0; the eluate was concentrated on a second similar column, eluted with aq. 0·2 M-NH_3 soln. and freeze-dried over H_2SO_4. The preparation thus obtained was rechromatographed under the above conditions and only the material coming off the column at concentrations varying no more than 10% from that at the peak, during elution with 0·25 M-ammonium acetate, was used for the spectral study. The final preparation contained 0·456% of iron and was fully enzymically active in the cytochrome oxidase and succinic oxidase systems. It contained no detectable chromatographic fraction II.

Estimation of iron. This was determined by the method of Keilin & Hartree (1945) and served as the standard for the calculation of the extinction coefficients. Repeated iron determinations agreed to within 1%.

Measurement of extinction. The cytochrome *c* was dissolved in Sørensen 0·1 M-phosphate buffer, pH 6·8. The spectrum was determined in 1 cm. cells with a Hilger Uvispek spectrophotometer, calibrated for wavelength and extinction coefficient with standard filters provided by the National Physical Laboratory.

For the visible part of the spectrum the cytochrome *c* solutions were reduced with buffered (pH 6·8) cysteine solution, and for the ultraviolet spectrum the reduction was effected by platinum black/H_2 gas, the platinum black being centrifuged at the end of the reaction. The completeness of the reduction was tested at 550 mμ by the addition of solid sodium dithionite. In the original solution the cytochrome *c* was fully oxidized, the com-

pletness of the oxidation being tested by the addition of solid $K_3Fe(CN)_6$.

All volumetric glassware was calibrated with mercury, and the micropipettes used were calibrated with pyridine, the ultraviolet-absorption maximum of pyridine, after suitable dilution, being used as a measure of the pyridine concentration.

SPECTRUM

Table 1 gives the millimolar extinction coefficients for cytochrome *c* in the reduced and oxidized forms from 600 to 220 mμ; 1 mole of cytochrome *c* is assumed to contain 1 g. atom of Fe.

Table 1. *Spectrum of horse-heart cytochrome* c *containing* 0·456 % *of iron in the reduced and oxidized forms*

M indicates the position of a maximum in the absorption spectrum, *m* a minimum and *I* an isosbestic point between the reduced and oxidized forms. 1 mole of cytochrome is assumed to contain 1 g. atom of Fe.

Wavelength (mμ)	$10^3 \epsilon$ Reduced	$10^3 \epsilon$ Oxidized	Wavelength (mμ)	$10^3 \epsilon$ Reduced	$10^3 \epsilon$ Oxidized
220	160·7	168·3	430	37·3	30·1
225	130·3	138·8	434	22·7 (*I*)	22·7 (*I*)
230	97·3	103·1	435	20·0	21·5
235	65·7 (*I*)	65·7 (*I*)	440	10·9	17·8
240	45·7	40·8	445	6·5	15·5
245	38·1	28·7	450	4·6	13·6
250	34·8	23·5	455	3·8	11·8
255	32·6	22·1	460	3·5	10·2
260	31·3	21·1	465	3·4	9·2
262	30·5	21·0 (*m*)	470	3·4	8·3
264	29·9 (*m*)	21·1	475	3·3	7·6
268	31·8	21·5	480	3·2	6·9
270	31·8 (*M*)	21·8	485	3·0	6·6
272	31·8	22·1	486	3·0 (*m*)	6·5
275	31·6	22·7	490	3·1	6·4
280	31·0	23·2 (*M*)	495	3·5	6·3
282	30·4	23·0	497	3·9	6·2 (*m*)
285	29·0	22·1	500	4·6	6·4
290	24·1	19·5	504	6·6 (*I*)	6·6 (*I*)
295·5	19·8 (*m*)	15·2	505	7·2	6·8
298	20·4	13·6	508	9·3	7·1
300	21·3	13·0	510	10·4	7·6
302	22·7	12·9 (*m*)	512	11·2	8·1
305	25·7	13·3	515	12·6	8·9
310	31·0	14·3	518	14·5	9·6
315·5	33·6 (*M*)	15·3	520·5	15·9 (*M*)	10·2
320	31·6	16·3	522	15·4	10·4
325	28·1	17·4	523	14·3	10·5
330	24·7	18·6	525	11·9	10·8
335	22·5	19·9	526·5	11·0 (*I*)	11·0 (*I*)
339	20·9 (*I*)	20·9 (*I*)	528	10·1	11·2 (*M*)
340	20·6	21·4	530	9·5	11·1
345	19·0	23·3	533	7·9	11·0
350	17·5	25·4	535·25	7·2 (*m*)	10·9
355	16·1	27·6	540	8·8	10·2
360	14·7	28·5 (*M*)	541·75	9·9 (*I*)	9·9 (*I*)
362	14·4	28·5	543·5	11·4	9·6
365	14·0	28·4	545	14·3	9·4
367	13·9 (*m*)	28·2	547	20·6	9·3
369	14·2	28·0 (*m*)	548	23·9	9·2
370	14·5	28·0	549	26·3	9·2
375	16·0	29·6	550	27·6	9·1
380	18·7	32·7	550·25	27·7 (*M*)	9·0
385	22·8	38·5	551	27·1	8·9
390	29·1	46·6	552	24·2	8·7
395	38·5	59·8	553	17·5	8·5
400	49·3	74·3	555	11·8	8·1
405	74·1	93·6	556·5	7·8 (*I*)	7·8 (*I*)
408	93·7	104·6	558	5·6	7·5
410	106·1 (*I*)	106·1 (*I*) (*M*)	560	3·8	6·9
412	117·6	104·6	565	1·8	6·1
415	128·1	94·4	570	1·0	5·2
416	129·1 (*M*)	88·8	575	0·8	4·2
417	128·0	83·5	580	0·6	3·5
420	111·6	67·3	590	0·4	2·5
425	74·2	43·1	600	0·4	1·7

REMARKS

The spectrum shows, in addition to the visually recorded α-, β- and γ-bands, a distinct δ-band, which takes the shape of a shoulder in the oxidized cytochrome *c*, and has a sharp maximum in the reduced form. This band has an isosbestic point for the ferri- and ferro-forms of cytochrome *c* near to 340 mμ, which may be useful in the spectrophotometric study of oxidation and reduction of coenzyme I in systems also containing cytochrome *c*.

There is a distinct difference in the so-called 'protein' band between the reduced and oxidized forms of cytochrome *c* around 280 mμ. Since the spectra of the amino acids that absorb in this region (tyrosine, tryptophan and phenylalanine) were probably not affected by the state of oxidation or reduction of the haem, it must be assumed that the difference between the two spectra was due to a relatively large absorption of the haem in this region, which differed in the ferri- and ferro-states.

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Effect of Fluoropyruvate on the Swelling, Phosphorylative Activity and Respiration of Guinea-Pig Liver Mitochondria

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The great difference in activity between fluoropyruvate and fluoroacetate both *in vivo* (Mager & Blank, 1954) and *in vitro* (Gal, Peters & Wakelin, 1956; Avi-Dor & Mager, 1956*a*) indicates that no fluoroacetyl fragment is formed from fluoropyruvate. The finding that fluoropyruvate alkylates thiol compounds (Avi-Dor & Mager, 1956*b*; Peters & Hall, 1957) explains adequately the inhibition, by this compound, of the reactions (utilizing coenzyme A, 6:8-thioctic acid) which control the entry of pyruvate and therefore possibly of fluoropyruvate into the citric acid cycle.

The effect of fluoropyruvate on the respiration of mitochondrial preparations, on the other hand, seems to be connected more directly with a primary damage to the mitochondrial structure than with an inhibition of a particular enzyme of the respiratory chain (Avi-Dor & Mager, 1956*a*). Whether such a pattern of inhibition is characteristic of fluoropyruvate only, or is also encountered with other thiol-alkylating agents, such as iodoacetate, was therefore investigated.

A comparison has been made of the effects of fluoropyruvate and iodoacetate on the swelling, phosphorylative activity and respiration of liver mitochondria.

EXPERIMENTAL

Materials. Fluoropyruvic acid was synthesized as described by Blank, Mager & Bergmann (1955). Adenosine triphosphate (ATP) was a product of Pabst Laboratories (Milwaukee, Wis., U.S.A.); diphosphopyridine nucleotide (DPN) (95% purity) was a product of Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of A.R. grade.

Preparation of mitochondria. Guinea-pig liver mitochondria were prepared according to the method of Schneider (1948), as modified by Swanson (1956). Mitochondria were suspended in 0·25 M-sucrose. The final suspension (4 mg. of N/ml.) had an extinction of 0·25, when it was diluted 40 times with 0·25 M-sucrose, in a Coleman spectrophotometer at 500 mμ. Unless otherwise stated in the legends, 0·5 ml. of the final suspension was used for each of the reported experiments, except for the spectrophotometric measurements, where 0·04 ml. was employed.

Methods

The extinction of the mitochondrial suspension was determined at 600 mμ in the Beckman spectrophotometer, model DU, with Corex cells of 1 cm. light path. Manometric experiments were carried out by the conventional Warburg technique. Adenosine triphosphatase (ATPase) activity was measured according to the method of Lardy & Wellman (1953). Oxidative phosphorylation was determined by the procedure described by Hunter (1955). The ATP–^{32}P exchange reaction was estimated as described by Swanson (1956). The counts were made in a thin mica-window Geiger counter tube. Corrections were applied for self-absorption of the charcoal layer. The average specific activity of ^{32}P during the experiment was used to calculate the exchange rates, since the initial specific activity changes owing to ATPase activity. For quantitative estimation of phosphorus the method of Fiske & Subbarow (1925) was adopted. The determination of fluoropyruvate was based on the procedure described by Avi-Dor & Mager (1956*b*): the sample was incubated at 58° for 15 min. with a large excess of cysteine. The light-absorption was measured at