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AN ABNORMAL RATIO OF CYTOCHROMES IN THE RESPIRATORY CHAIN OF MOUSE AND HUMAN MYELOMAS

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SUMMARY

Mouse myeloma cells and mitochondria had the same kinds of cytochrome components in the respiratory chain as the normal ones. Their constitution, however, was abnormally different from that found in normal cells and mitochondria. The cytochrome aa_3 concentration was especially low in the myeloma as compared with cytochrome *c* concentration, and the resulting cytochrome aa_3/c ratio was 0.25, which was the lowest ever reported in animal mitochondria. Normal lymph node cells, producing the immunoglobulin similar to the myeloma cells, had a ratio of 1.1. Human myeloma mitochondria had the same characteristics as the mouse myeloma. Ascite form myeloma originated from mouse solid form myeloma grew faster, and yet aa_3/c ratio in the ascites myeloma cells was larger than that of solid form myeloma. The ratio aa_3/c of 0.5 in the ascites myeloma was found to be quite similar to that observed in various ascites tumor cells such as hepatomas, Ehrlich and sarcoma 180. A significant part of the cytochromes in the respiratory chain of the mouse myeloma remained in the oxidized form in the cyanide-inhibited or anaerobic states, and was reduced only by the addition of dithionite. The properties of the *b* cytochromes in mouse myeloma mitochondria are also described and discussed in the context of multiple forms of the *b* cytochromes in the respiratory chain.

INTRODUCTION

The ratios of cytochromes in the respiratory chain of animal tissue mitochondria have been reported by many investigators [1–26]. Though the actual findings did not agree with the ratio of cytochromes $a_3 : a : b : c : c_1$ being 1 : 1 : 1 : 1 : 1 as claimed by Chance and Williams [1, 2], the ratios aa_3/c and aa_3/b were found to be rather constant in normal tissue mitochondria. Williams [12] has reported that the ratios for cytochromes aa_3 to *c*, c_1 or *b* are not significantly lower than 1 in mitochondria of liver, kidney, heart and intestine of the rat, guinea pig and chicken. However, it has been observed that the ratios of cytochromes in mitochondria of

malignant tumors significantly differ from the normal [15, 18, 20, 22, 25, 26]. The ratio aa_3/c was much lower than 1 in a wide variety of tumors, and the extent of the decrease of this ratio seemed to depend on the growth rate of the tumor [15, 18, 23], although cytochromes *c* and oxidase concentrations and activities are in some tumors not lower or even higher than in normal tissues of the same kinds [4, 5, 11, 15, 19, 20, 24–31].

Myeloma has been widely investigated in the field of immunology, genetics and molecular biology with a view to clarify the mechanism of antibody formation in animals. However, very few studies have been made so far on the energy metabolism of this tumor, which has abnormally active production of immunoglobulin. In the present report we describe the cytochrome composition in this tumor as being extremely different from those observed in normal tissues, and we also describe some properties of the *b* cytochromes in the myeloma mitochondria.

MATERIALS AND METHODS

Myelomas. Transmissible mouse myeloma of ileocaecal origin (X5563), producing IgG_{2a} immunoglobulin, was obtained from the National Institute of Health, Bethesda, Md. and maintained in our laboratory (Institute for Cancer Research, Osaka University Medical School). The tumors ($0.5–1.0 \cdot 10^6$ cells) were aseptically transferred subcutaneously into the backs of C3H/He mice weighing 15–20 g. Tumors from 5 mice, 10–14 days after innoculation, were excised out and finely sliced with scissors in Eagle's solution at 0–4 °C. The sliced tumor was suspended by squeezing a rubber bag gently with the fingers and filtering through a 200 mesh sieve. The filtrate was centrifuged at $50 \times g$ for 5 min and the sedimented cells were resuspended. This procedure was repeated two or three times to remove hemoglobin contamination. The supernatant was then centrifuged at $600 \times g$ for 10 min. The resulting packed cells were suspended in 5 vol. of isotonic sucrose solution containing 0.2 mM EDTA and 5 mM phosphate, pH 7.4. About 70 % of the cells were still alive as determined by the uptake of trypan blue into the cells. The hemolysis procedure carried out according to Chance and Hess [4], led to cell damage of about 30 % as determined by the absorption of trypan blue into the cells.

Another transmissible mouse plasma cell tumor was obtained as ascites cells by transplanting the myeloma cells, X5563, intraperitoneally to C3H/He mice. The cells were harvested in 7–10 days after innoculation and used as described previously [15]. A cell concentration of approx. $1 \cdot 10^5$ per mm^3 proved convenient for our spectrophotometric analysis.

A human myeloma was obtained from the femur of a 60 year old male patient who died of multiple myeloma, producing IgG. The tumor, frozen for about 12 months at −80 °C, was gradually thawed and cut into small pieces, then homogenized by the method described in the following section.

Difference spectra. The difference absorption spectra between the "reduced" and "oxidized" state of respiratory components in the intact cells, mitochondria and microsomes, were obtained at room and liquid nitrogen temperatures as described in previous papers [15, 18]. In order to reduce the respiratory pigments in the intact cells, anaerobiosis by the respiration of the endogenous substrates was usually used. Inhibition with cyanide or antimycin was sometimes used too. In the case of mito-

chondria, they were reduced by respiration with 5 mM succinate and 5 mM glutamate as substrates.

Determination of cytochrome contents and ratios. Quantitative analysis of the respiratory cytochromes aa_3, b and $c+c_1$ was carried out by the difference spectra between the anaerobic and aerobic states at room temperature. The cell suspensions in the reference cuvets were made aerobic by passing O_2 or shaking with air in the presence of 4 μM rotenone, and that in the sample cuvette was allowed to become anaerobic by the endogenous respiration at room temperature. The calculation was carried out according to the procedure of Chance [4] with some modification. The concentration of each cytochrome is expressed in terms of its heme content, using the following extinction coefficients; cytochrome aa_3, $\Delta E_{605-630} = 16.5\ mM^{-1} \cdot cm^{-1}$ (32), cytochrome b, $\Delta E_{562-575} = 17.9\ mM^{-1} \cdot cm^{-1}$ [33], and cytochrome $c+c_1$, $\Delta E_{550-540} = 19.0\ mM^{-1} \cdot cm^{-1}$ [2]. The ratios of contents of cytochrome aa_3 : b-type cytochromes : cytochrome c_1 : cytochrome c were determined from the low temperature difference spectra according to the following calculation; aa_3 : b-type : c_1 : $c = (\Delta E_{601-625})/16.5 : (\Delta E_{559-570} - 0.2\ \Delta E_{554-535})/17.9 : (\Delta E_{554-535} - 0.3\ \Delta E_{559-570})/19.0 : (\Delta E_{548-535} - 0.9\ \Delta E_{554-535} + 0.27\ \Delta E_{559-570})/19.0$. For the calculation of b-type cytochromes and cytochromes c and c_1, interference of absorbance of each cytochrome was corrected based in the low temperature spectrum of each cytochrome reported by Hagihara et al. [34]. Although in the present investigation the ratios of cytochrome contents were operationally determined using the above equations, the method could well be applicable to the low temperature spectra of cytochromes of many tissues.

Respiration. Respiration of the cells and mitochondria in the presence and absence of inhibitors was measured polarographically using a rotating platinum electrode in a closed cell [35].

Preparation of subcellular fraction. Both the tumor cells and fine slices were suspended in 5 vol. of the above isotonic sucrose medium and homogenized with a mill type glass homogenizer [36]. The homogenates were centrifuged again under the same conditions. The homogenates thus obtained were spun at $8000 \times g$ for 8 min. The mitochondrial pellet was suspended in 4 vol. of the above medium and the suspension was centrifuged at $12000 \times g$ for 5 min. The washed mitochondria were suspended in the medium at concentration of 4–5 mg protein/ml. To obtain microsomes, the above supernatant was centrifuged at $15000 \times g$ for 15 min to remove light mitochondria and centrifuged again at $105000 \times g$ for 60 min. The microsomal pellet was suspended in the same medium at a concentration of 3–5 mg protein/ml.

RESULTS

Room temperature spectra of mouse myeloma cells

Fig. 1 shows the difference spectra between various reduced states and an oxidized state of cell suspension obtained from solid myeloma (X5563) of C3H/He mouse. In Curve B, most of the respiratory pigments in the cell suspension in the "measure" cuvette were reduced by anaerobiosis (20 min) due to respiration which was performed with the endogenous substrates. In Curves C and D, reduction of a large part of the respiratory enzymes was achieved by the respiration in the presence

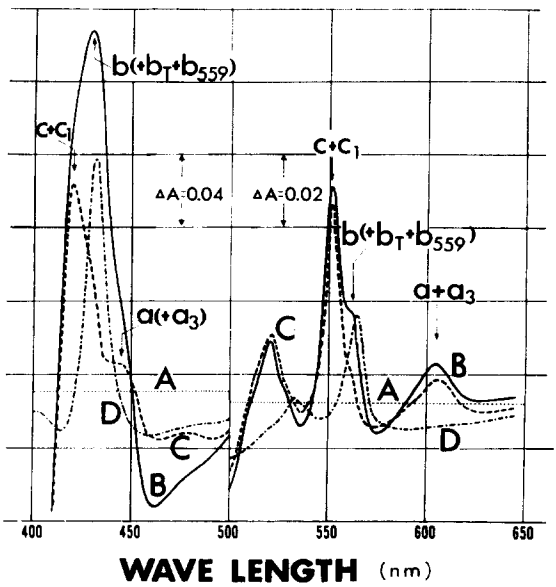

Fig. 1. Room-temperature difference spectra between various reduced states and an oxidized state of mouse myeloma (solid-form) cells. The cell suspension obtained from myeloma (X5563) from the backs of C3H/He mice was suspended in the sucrose/EDTA/phosphate medium to the concentration of 14 mg protein/ml. In all cases, oxidation of the cell suspension in the "reference" cuvet was achieved by keeping it in aerobic state more than 20 min in the presence of 5 μM rotenone without any external substrate. Curve A (–·–·–·–), base line ("aerobe" minus "aerobe" difference spectrum). Curve B (———), "anaerobe" minus "aerobe" difference spectrum. The anaerobiosis of the cell suspension in the presence of 5 mM succinate and kept for 20 min before recording the spectrum. Curve C (– – – – –), "cyanide, aerobe" minus "aerobe" difference spectrum. Reduction of a large part of the respiratory pigments in the "measure" cells was performed by respiration in the presence of 5 mM succinate and 1 mM KCN. Curve D (dotted-broken line), "antimycin, aerobe" minus "aerobe" difference spectrum. Reduction of a part of the respiratory pigments in the "measure" cells was performed by respiration in the presence of 5 mM succinate and 1 μM antimycin. The optical path was 10 mm.

of cyanide or antimycin, respectively. Oxidation of the cell suspension in the "reference" cuvette was achieved by aeration in the presence of rotenone, although almost complete oxidation of cytochromes was observed without added rotenone. These spectra clearly show the presence of all typical respiratory components in the myeloma cells. The myeloma cells obtained as an ascites form in BALB/C mice showed similar characteristics.

As shown in Curve C of Fig. 1, the addition of cyanide to aerobic cells induced reduction of various respiratory components. However, the extents of *b*-type cytochromes and flavin enzymes reduction were largely diminished as compared to anaerobic reduction cases. It is assumed that only a part (presumably almost half) of cytochrome $b_{(K)}(b_{562})$ is reduced, with both cytochromes b_T (b_{566}) [38–41] and b_{559} [41–43] being reduced slightly or not at all under this condition.

As shown in Curve D of Fig. 1, the addition of antimycin to the aerobic cells induced reduction of flavin enzymes and b-type cytochromes with an α maximum

at 564 nm. Under this condition, most of cytochromes b_T and $b_{(K)}$ are probably reduced, leaving most of cytochrome b_{559} in the oxidized form.

Low-temperature spectra of mouse myeloma

Fig. 2 shows the difference spectra of mouse myeloma cells measured at 77 °K. Oxidation and reduction were achieved before cooling under the same condition as in Fig. 1. In these low temperature spectra, the absorption peaks of each cytochrome component were observed more clearly, and accompanied with shifts in the position, than in the case of room-temperature spectra. The α band of cytochrome aa_3 is seen at 601 nm in the anaerobic minus aerobic difference spectrum as shown in Curve A in Fig. 2. A peak at 559 nm in the Curve A is due to the α band of b-type cytochromes. Peaks at 554 nm and 548 nm represent the α_1 peak of cytochromes c_1 and c, respectively.

In the case of the "cyanide, aerobe" minus "aerobe" difference spectrum shown in Curve B, two large differences are noticed when compared to the "anaerobe"

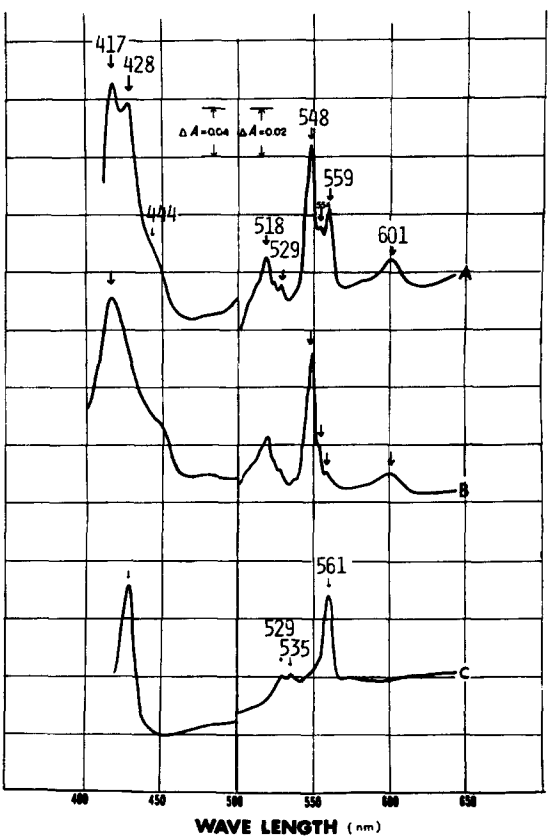

Fig. 2. Low-temperature difference spectra between various reduced states and an oxidized state of mouse myeloma (solid-form) cells. The material is the same as in Fig. 1, except the concentration of cell suspension was 6.8 mg protein/ml. Reductions were carried out as in the cases of Fig. 1 and the spectra were measured at 77 °K. The optical path was 3 mm.

minus "aerobe" spectrum (Curve A). In the cytochrome aa_3 band, the absorption at 601 and 444 nm decreased somewhat and a slight shoulder appeared around 586 nm. These changes are due to formation of a cytochrome a_3 · cyanide complex. The second one is due to lowered reduction of cytochrome *b*. Absorption at 559 nm remarkably decreased and the peak at 428 nm disappeared almost completely. These changes are due to a considerably lower reduction of *b*-type cytochromes in cyanide-treated cells as compared to that in anaerobic cells.

In the "antimycin, aerobe" minus "aerobe" difference spectrum (Curve C), absorption of *b*-type cytochromes increased again to the level of "anaerobe" minus "aerobe" difference spectrum (Curve A), however, the position of the peak shifted to 561 nm. The following assumption can be made from the peak positions, peak height and shapes of spectra around 560 nm in these spectra; (1) By anaerobiosis for more than 20 min (Curve A), most of cytochrome $b_{(K)}$ (α peak, 559 nm at 77 °K [38, 41]) and a smaller percentage of cytochrome b_T (α_1 562 probably with α_2 at 555.5 nm at 77 °K [38, 41]) are in the reduced form. (2) In the cyanide inhibited aerobic state, only cytochrome $b_{(K)}$ is largely reduced, and both cytochromes b_{559} and b_T are mostly oxidized. (3) In the antimycin inhibited state, cytochrome b_T is completely reduced and cytochrome $b_{(K)}$ is nearly so, while cytochrome b_{559} seems not to be reduced.

It should be emphasized that the absorption of *b*-type cytochromes of the present myeloma (solid form) is remarkably different between anaerobic state and cyanide-inhibited state, while this difference is usually much smaller in the case of normal tissues as well as Morris hepatomas and some ascites cells [15, 18]. This large difference is probably due to the incompleteness of cyanide inhibition in the respiratory system of the present tumor cells. In fact, polarographic studies have revealed that significant percent of the tumor cells endogenous respiration still remained (approx. 10 %), even in the presence of 5 mM KCN. On the contrary, more than 95–98 % inhibition of the respiration was achieved by 1 μM antimycin A.

Characteristic composition of respiratory components in myeloma cells

Although the present myeloma cells have the same kinds of cytochrome components in the respiratory chain as the normal cells, it is easily noticed from the shape of these spectra that the ratio of the component's contents is extremely abnormal in this tumor. As shown by remarkably low height of the 605 nm peak in Fig. 1, the content of cytochrome aa_3 is extremely low as compared to other cytochromes. The content of each cytochrome in the myeloma cells, calculated using both room- and low-temperature spectra shown in Figs. 1 and 2, is presented in Table I. In the case of "anaerobe" minus "aerobe" difference spectra, the ratio of aa_3 : *b*-type : c_1 : *c* is 0.24 : 0.57 : 0.47 : 1 (0.28 : 0.63 : 0.45 : 1 when calculated from low temperature spectrum). In any of the above cases, the aa_3 : *c* ratio lies between 0.24 and 0.28, and these values are extremely low as compared to other mammalian tissues or cells which show the values from 0.5 to 1.6 [3, 6–13, 15, 18, 20] (cf. Table III). These exceptional characteristics of the myeloma cells are confirmed by the low temperature spectra shown in Fig. 3, but it was found that this is limited to solid form myeloma and is not the case in the ascites form myeloma.

Fig. 3 shows the "cyanide, aerobe" minus "aerobe" difference spectra obtained at 77 °K with the cell suspension of a solid form myeloma which is different from the

TABLE I

CONCENTRATION OF RESPIRATORY CYTOCHROMES IN WHOLE CELLS OF MYELOMAS, LYMPH NODE, LIVER AND ASCITES TUMORS

Material	Reduction	Concentration (nmol/mg protein) of cytochromes[a]				Relative concentrations[b]			
		aa_3	*b*-type	c_1	c	aa_3:	*b*-type:	c_1:	c
Mouse myeloma	Anaerobe	0.044	0.102	0.085	0.180	0.24:	0.57:	0.47:	1
(solid-form)	(Cyanide)	(0.039)	(0.045)	(0.055)	(0.150)	(0.26:	0.30:	0.37:	1)
Mouse myeloma	Anaerobe	0.051	0.061	0.045	0.097	0.53:	0.63:	0.46:	1
(ascites-form)	(Cyanide)	(0.042)	(0.038)	(0.035)	(0.077)	(0.53:	0.49:	0.45:	1)
Lymph node	Anaerobe	0.078	0.076	0.038	0.070	1.11:	0.56:	0.54:	1
Liver[c]	Anaerobe	0.130	0.076	0.075	0.105	1.24:	0.72:	0.71:	1
Ascites tumors[c]									
Ehrlich	Anaerobe	0.051	0.072	0.053	0.098	0.52:	0.22:	0.54:	1
MH134	Anaerobe	0.046	0.022	0.039	0.080	0.59:	0.28:	0.49:	1

[a] Concentration of cytochrome aa_3, *b*-type cytochromes and cytochrome ($c+c_1$) were obtained from room-temperature reduced minus oxidized difference spectra. Oxidation of cytochromes was achieved by aeration in the presence of rotenone. The separate values for c and c_1 were calculated from the $c+c_1$ concentration and c_1/c ratio obtained from the low temperature-spectra according to the Materials and Methods. These concentrations are mean values obtained from 3 (Lymphnode) to 5 (Solid-form myeloma) experiments.

[b] Relative concentrations are expressed by taking cytochrome c content as 1.

[c] [15].

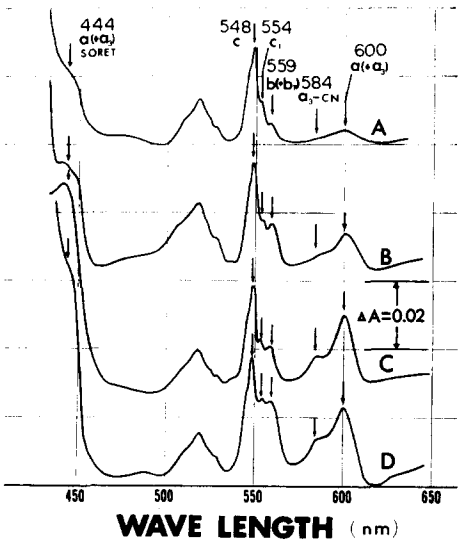

Fig. 3. Low-temperature difference spectra between the presence and absence of cyanide in the aerobic state of the suspension of the two different forms of myelomas, lymph node and liver cells. The cell suspension both in the reference and sample cuvet contained 25 % glycerol. However, the suspensions were aerated for 2 min at room temperature before cooling to 77 °K. The optical path was 3 mm. A, Cells from mouse myeloma (solid-form), 3.5 mg protein/ml; B, Cells from mouse myeloma (ascites-form), 5.7 mg protein/ml; C, Lymph node cells, 6.3 mg protein/ml; D, Normal liver cells, 6.0 mg protein/ml.

sample used in Figs. 1 and 2 (Curve A), an ascites form myeloma (Curve B), mouse lymph node (Curve C) and normal mouse liver (Curve D). Lymph node cells were chosen as a control, since they are immunoglobulin-producing cells similar to the myeloma cells, although they are not the original tumor tissue. Mouse liver cells were also compared. The ratios of cytochromes aa_3 : b-type : c_1 : c calculated from these spectra are presented also in Table I. These values were confirmed by a number of experiments (usually 3 to 5 times analysis). The most remarkable difference among the different kinds of cells was shown in the aa_3/c ratio. The value of 0.26 obtained with the solid myeloma is less than 1/5 of the liver's and is exceptionally low, even among ascites tumors which have shown considerably lower ratio than the normal tissues [15]. Although the b-type/c ratio of the solid myeloma was also low, comparison of these b-type cytochromes from the above spectra is not possible since only a part of them is reduced in cyanide-treated cells. The c_1/c ratio of both solid and ascites myeloma forms is average in rapidly growing tumors.

Cytochromes in mitochondria and microsomal fraction

Microsomal fraction of mouse myelomas (solid form) was analyzed at room-temperature by three kinds of difference spectra; difference spectra between the presence and absence of NADH (0.1 mM) in the aerobic suspension, those between the presence and absence of carbon monooxide in the dithionite reduced anaerobic suspension and those between dithionite reduced and the aerobic suspension. No clear evidence substantiated the presence of cytochrome b_5 or cytochrome P-450 in

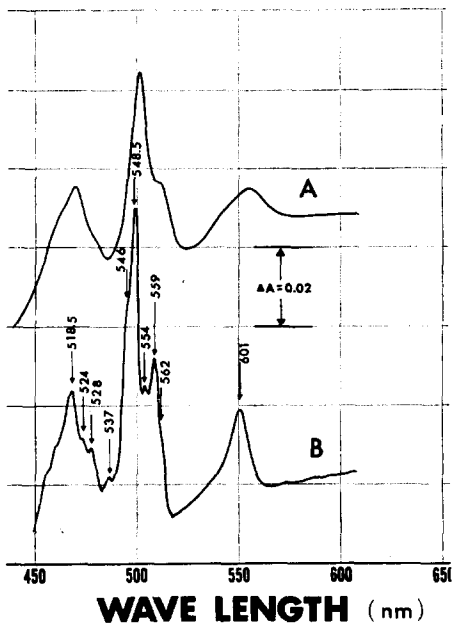

Fig. 4. Anaerobic minus aerobic difference spectra of mouse myeloma (solid-form) mitochondria. Curve A was obtained at room temperature and Curve B, at low-temperature. Mitochondria was suspended in the sucrose/EDTA/phosphate medium at a concentration of 3.9 mg protein/ml (1.9 mg protein/ml in the low-temperature spectrum). Anaerobiosis was attained by the respiration with 5 mM succinate and 5 mM glutamate.

the microsomal fraction. A small amount of cytochrome *c* was obtained which is presumably due to contamination of mitochondrial fragments.

As shown in Fig. 4, the "anaerobe" minus "aerobe" difference spectra of the mouse myeloma mitochondria both at room- and low-temperatures are very similar to the corresponding spectra of the myeloma cells. In the case of mitochondria, the details of absorption peaks and troughs in the low temperature spectrum (Curve B, Fig. 4) are shown more clearly than in the case of cell suspensions, because a narrower slit in the optical system could be used with the more transparent mitochondria.

Table II shows the concentration of cytochromes obtained from these spectra. As supposed from the shape of the spectra, the ratios of cytochrome aa_3, *b*-type and c_1 to *c* in mitochondria are similar to those in intact cells. This similarity suggests that almost all of cytochromes reduced in the anaerobic condition of the myeloma cells are located in mitochondria. Although the spectrum is not shown in the figure, the reduction of cytochromes aa_3, *b*-type, c_1 and *c* of the mitochondria in the presence of cyanide was also fairly similar to those of the cyanide treated cells. As in the case of the cell suspension, the addition of antimycin to mitochondria caused greater reduction among *b*-type cytochromes than the anaerobic condition.

Mitochondria of human multiple myeloma

It would seem interesting to see whether the abnormal composition of the mouse myeloma respiratory system also existed in human tumors of the same origin.

TABLE II

CYTOCHROME CONCENTRATIONS IN MOUSE AND HUMAN MYELOMA MITOCHONDRIA

Concentrations and relative concentrations were determined as described in Table I.
The values are means of 2 (human myeloma) to 5 experiments.

Reduction	Concentration (nmoles/mg protein)				Relative Concentration			
	aa_3	*b*-type	c_1	*c*	aa_3	*b*-type	c_1	*c*
Mouse myeloma								
Anaerobe	0.113	0.208	0.160	0.440	0.26	0.47	0.36	1
Cyanide	0.108	0.104	0.110	0.360	0.30	0.29	0.31	1
Antimycin	–	0.275	–	–	–	0.76	–	–
Human myeloma								
Cyanide	0.072	0.037	0.120	0.150	0.48	0.25	0.80	1
Antimycin	–	0.170	–	–	–	1.13	–	–

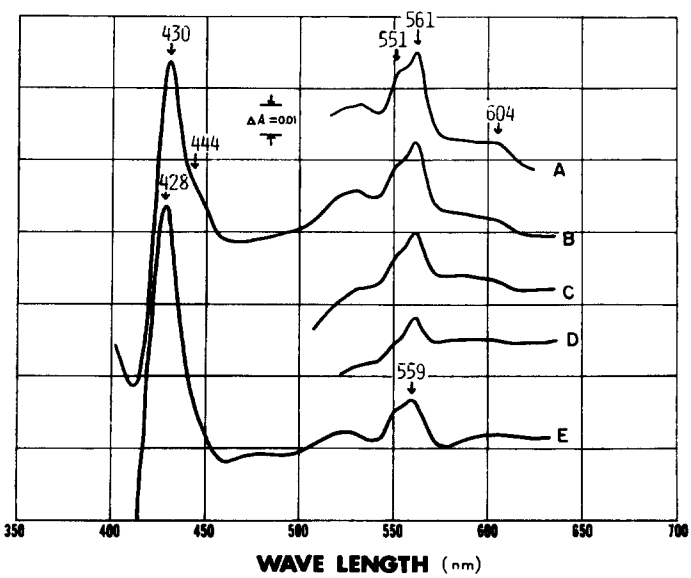

Fig. 5. Further reduction of cytochromes in mouse myeloma (solid-form) cells after anaerobiosis attained. The cell suspension in the "measure" cuvette was saturated with air and allowed to become anaerobic by endogenous respiration and kept in this condition. The cell suspension in the "reference" cuvette was treated exactly in the same way after 20 min. The difference spectra were recorded in a few min intervals. Concentration of the cell suspension was 12.5 mg protein/ml and all procedures were carried out at room temperature. A, spectrum between the cells 20 min after anaerobiosis and those immediately after anaerobiosis; B, spectrum between 23 min and 3 min after anaerobiosis; C, spectrum between 27 and 7 min after anaerobiosis; D, spectrum between 35 min and 15 min after anaerobiosis; E, spectrum between dithionite reduction and 20 min anaerobiosis.

Owing to the hemoglobin interference with the "anaerobe" minus "aerobe" difference spectrum, only the "cyanide, aerobe" minus "aerobe" difference spectrum of cytochromes in mitochondria of multiple myeloma of a 60 year old man was measured, both at room and low temperatures. The contents of each cytochrome in the mitochondria calculated from these spectra are shown in Table II. Although the aa_3/c ratio of the human mitochondria was very low, it was higher than that of the mouse myeloma. This is probably due to a leak of cytochrome *c* during preparation of mitochondria. Exceptionally high c_1/c ratio in these mitochondria suggests the leakage of this cytochrome. If this is the case, human myeloma cytochrome composition is thought to be very similar to mouse myeloma.

Cytochromes remaining in oxidized form after an anaerobiosis

Although most of mitochondrial cytochromes change to the reduced form as soon as the suspension of the cells, or the mitochondria, reaches the anaerobic state, some cytochromes still remain partially or mostly in the oxidized form. A large portion of such cytochromes gradually reduced with time during prolonged anaerobiosis. Fig. 5, Curve A is the difference spectrum of the mouse myeloma cells recorded rapidly (scanning speed, 10 nm/s) between 20 min and 0 min anaerobiosis. This spectrum shows the α bands of the components which are in the oxidized form at the beginning of anaerobiosis and converted to the reduced form after 20 min. These components contain fairly large amounts of *b*-type cytochromes, some *c*-type cytochromes and a smaller quantity of cytochrome aa_3. These cytochromes are reduced gradually with time until they reach a nearly constant state after 20 min.

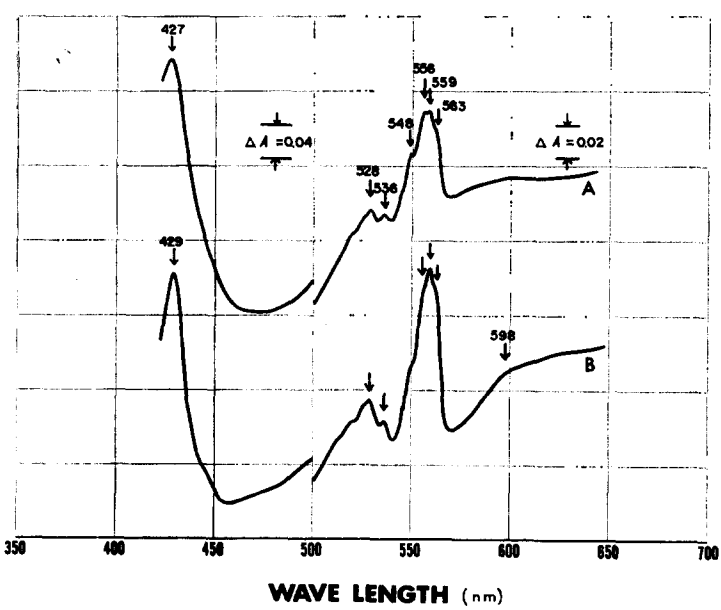

Fig. 6. Low-temperature difference spectra of mouse myeloma (solid-form) cells between dithionite reduced and anaerobic (10 min) state (A), and dithionite reduced and cyanide inhibited aerobic state (B). Concentration of cell suspension, 17 mg protein/ml, temperature, 77 °K.

As shown by Curves B (after 3 min), C (7 min) and D (15 min), cytochrome aa_3 is reduced the most rapidly, with *c*-type cytochromes reduced thereafter. Curve E shows that the components still remained in the oxidized form, even after 20 min anaerobiosis. These components are probably of *c*- and *b*-type cytochromes.

The finer spectral analyses of cytochromes remaining in oxidized state after anaerobiosis, as well as after cyanide inhibition in the aerobic state, were carried out by low-temperature difference spectra. Fig. 6, Curves A and B, shows the spectra of the myeloma cells between the presence and absence of dithionite after 10 min anaerobiosis, and in the cyanide treated aerobic condition, respectively. In Curve A a rather broad α band is seen around 556–559 nm with shoulders at about 563 nm and 549 nm, while in Curve B a sharp peak is seen at 559 nm with shoulders at 562 nm, 556 nm and 549 nm. The peaks at 559 nm and 549 nm can be identified as cytochromes *b* and *c* while the band at 563 nm and 556 nm may be due to cytochromes b_T and b_{559}. Judging from the extent of absorption at 559 nm, about a quarter of cytochrome *b* remains in the oxidized state after 10 min anaerobiosis, and about a half after cyanide treatment. A fairly large amount of cytochromes b_T and b_{559} and a smaller amount of cytochrome *c* are also in the oxidized state in the cyanide treated cells, and to a somewhat lesser extent in the anaerobic cells. When the low-temperature difference spectrum of the myeloma mitochondria was taken between the presence and absence of dithionite in the anaerobic state due to succinate respiration, a peak at 559 nm with shoulders at 562, 556 and 549 nm was seen as in the case of the above cells.

DISCUSSION

The present spectrophotometric study has revealed that the respiratory system of these tumor cells contained all cytochrome components (*a*, a_3, *b*, b_T, b_{559}, c_1 and *c*) and flavoproteins in its mitochondria. When cyanide, antimycin and rotenone are added to aerobic cells, they inhibit the endogenous respiration of the cells, hence the behavior of these cytochromes is essentially the same as the normal cells. The respiratory rate of the cells (approx. 2 μM O_2 per s per 10^5 cells per mm^3 at 30 °C) was found to be reasonable (cf. ref. 2). However, the stoichiometry of the cytochrome components analyzed in the mouse myeloma is quite different from those observed in various mammalian tissues. The ratio of cytochromes aa_3 : *b*-type : c_1 : *c* was found to be approx. 0.25 : 0.5–0.75 : 0.5 : 1 in the mouse myeloma cells and mitochondria, whereas many other tissues, including various tumors, showed the values of 0.5–1.4 : 0.4–0.8 : 0.4–0.8 : 1. It may easily be noticed that the cytochrome aa_3/c ratio of the myeloma is exceptionally low, a value lower than any others of animal mitochondria which appeared in the literatures (Table III). As discussed by King et al. [9], the precision of the absolute values of cytochrome contents in mitochondria or cells is not high, and the comparison of molar ratios of individual components seems to be more reliable. Moreover, this aa_3/c ratio might reflect a characterization of the respiratory system of tissues. Ozawa et al. [25] has shown in human liver mitochondria that the change in the ratio $aa_3/c+c_1$ is closely correlated with the degree of phosphorylation rates of the mitochondria. They have also shown a significant decrease of cytochrome $aa_3/c+c_1$ ratio in mitochondria of human hepatoma and other profound pathological disturbance of the liver [25, 26].

In the present investigation, as is the practice in the usual investigation for

TABLE III

THE RATIO OF CYTOCHROME aa_3 TO c IN ANIMAL MITOCHONDRIA AND INTACT CELLS

Material	Animals	aa_3/c	Reference
Liver	Rat	1.3	[2]
Heart	Rat, mouse, cow, rabbit, dog	0.7–0.8*	[6]
Kidney, Liver, Muscle	Rat, pigeon	0.7–0.9*	[6]
Heart	Beef	2.9	[8]
Heart	Beef	1.2*	[9]
Kidney, Liver, Brain, Heart	Rat, guinea pig, chicken	1.0–3.0	[12]
Intestine	Rat	7.7	[12]
Muscle	Rat, dog, human	0.9*	[13]
Liver	Rat, mouse	1.2–1.4	[14, 18]
Liver	Human	0.9–1.1*	[45]
Lymph node	Mouse	1.1	this paper
Morris hepatoma	Rat	0.7–0.8	[18]
DAB-induced hepatoma	Rat	0.5	[22]
Hepatoma	Human	0.4–0.8*	[25, 26]
Ascites hepatoma	Rat, mouse	0.5–0.6	[15]
Ascites tumor (EL_2, ELD)	Mouse	0.4–0.5	[11]
Amelanotic melanoma	Mouse	0.4*	[24]
Ascites form myeloma	Mouse	0.5	this paper
Solid form myeloma	Mouse	0.25	this paper

* The value expressed as $aa_3/c+c_1$.

The values of ratio aa_3/c were recalculated on the basis of the millimolar extinction coefficients used in this paper. When the extinction coefficient recommended by Van Gelder is used [46], the values presented should be multiplied by 1.3.

cytochrome analysis, the spectral properties have been equated to cytochrome contents. The spectral method employed, however, measures only heme moieties of heme proteins, and the possibilities remain obscure that the content of cytochrome apoenzyme is changed, or that, as in the case of cytochrome *c* oxidase-less yeast mutant [37] the apoenzyme is synthesized but that all of the subunits have not been assembled into the inner mitochondrial membrane. Further study on these points should be made.

The decline in the cytochrome aa_3 concentration and aa_3/c ratio in tumors seemed to be dependent on the tumor growth rate as previously suggested by Sato et al. [23]. In the present investigation the growth of myeloma after transplantation ($5 \cdot 10^5$ cells) was so fast that the host mouse was usually dead in 19.2 ± 1.2 days (H. Senoh, unpublished data). However, it would be unwise to attribute the high growth rate to the aa_3/c ratio alone, since various ascites hepatomas of similar growth rate showed slightly higher aa_3/c ratio [15]. In another test case the same number of the myeloma cells of the same strain X5563 transplanted as ascites tumors in the host mouse resulted in a higher concentration of cytochrome aa_3 than the solid form myeloma and caused the consequent death of the mouse in 10.2 ± 0.4 days. Most ascites tumors of similar growth rate showed similar aa_3/c ratios, independent of the original tumor such as hepatoma, sarcoma and others [15]. Therefore, this ratio would partly depend upon the life environment of the tumor cells.

It has recently become clear that at least two *b*-type cytochromes exist in all mammalian tissue mitochondrial respiratory chains that have been tested as well as in yeast and plant [38–41]. These are classical cytochrome $b_{(K)}$ (also called cytochrome b_{562}) and a new cytochrome b_T (b_{566}). Sato et al. have also described an additional *b*-type cytochrome, which has been named b_{559} in normal mammalian tissues as well as in many tumors [15, 18, 42, 43]. The α absorption peaks of these cytochromes in the reduced minus oxidized spectrum are as follows; b_{559}, 559 nm (556 nm at 77 °K); $b_{(K)}$, 562 nm (559 nm at 77 °K); b_T, probably double peaks with α_1, 566 and α_2, 558 nm (562 and 555 nm at 77 °K) (cf. e.g. ref. 40). As shown in Figs. 1 and 2, only a small portion of *b*-type cytochromes is in the reduced form in the cyanide-treated aerobic cells. Addition of antimycin to the aerobic cells induced a considerable increase in the reduction of *b*-type cytochromes. The extent of reduction by anaerobiosis in the cells is similar to that of antimycin inhibition, but the peak positions are different (559 nm by anaerobiosis and 561 nm by antimycin at 77 °K). As shown in Fig. 5, some portion of *b*-type cytochromes, together with a small portion of *c*- and *a*-type cytochromes, remained in the oxidized form. These cytochromes were gradually reduced with time under the anaerobic conditions. The anaerobic reduction was nearly completed in 20 min, but a small concentration of *b*- and *c*-type cytochromes still remained in the oxidized state, and which could have been converted to the reduced form by the addition of dithionite (Fig. 5E and Fig. 6A). In the present myeloma cells, considerably large concentrations of cytochromes b_T and b_{559} remained in the oxidized form in the cyanide inhibited state, and also in the early stage of anaerobiosis, suggesting that these cytochromes might not be linked directly to the respiratory chain. Alternatively the inside of the cyanide-treated or anaerobic cells might be in the low energy state so that cytochrome b_T is in the low potential form [38] under these conditions and is difficult to be reduced. The properties of cytochrome b_{559} are not known at present, and its behaviour cannot be discussed based on its nature. The amount of cytochrome b_{559} contained in the myeloma (solid form) appears to be considerably higher than various normal tissues that have been tested.

In the case of mitochondria from normal tissues, the peak for cytochrome *c* (448 nm) was not at all or in some cases, just slightly observable in the "cyanide, aerobe" minus "dithionite", or "anaerobe" minus "dithionite" difference spectra at low temperature. On the other hand, these peaks were clearly noticeable in the above spectra of various ascites tumors [15] as well as the present mouse myeloma of both solid and ascites forms. Reduction of cytochrome *b* by anaerobiosis was also incomplete. The incomplete reduction of these cytochromes by anaerobiosis or cyanide inhibition together with an abnormal cytochrome aa_3/c ratio in tumors suggests a marked derangement of tumor mitochondria.

The addition of rotenone or amytal did not induce any significant cytochrome oxidation in the cells respiring the endogenous substrates (cf. ref. 4), indicating that almost all cytochromes were in the oxidized form in the aerobic cells. These inhibitors inhibited more than 95 % of the endogenous respiration. The result suggests that the mitochondria of the respiring myeloma cells are high in state 3 and very low in the phosphate potential. This may be further supported by the fact that the addition of cyanide to the respiring cells did not induce any reduction of cytochrome b_T. A similar observation was reported in ascites hepatoma cells such as AH 7974 and

MH 134 [15]. These observations may be interestingly compared with the fact that the addition of cyanide to the respiring yeast cells resulted in reduction of not only cytochrome $b_{(K)}$ but also b_T, the latter of which was sensitive to uncoupler [44].

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