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A COMPARATIVE STUDY OF CYTOCHROME RATIOS IN MITOCHONDRIA FROM ORGANS OF THE RAT, CHICKEN, AND GUINEA PIG

J. N. WILLIAMS, JR.

Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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SUMMARY

The concentrations of mitochondrial cytochromes aa_3, b, c_1, and c have been studied in liver, kidney, brain, heart, and intestine of the rat, guinea pig, and chicken, as well as in beef heart. The ratios of concentrations of cytochromes b, c_1, and c concentrations to aa_3 have also been calculated. Although in rat liver and beef heart mitochondria the ratios of cytochromes aa_3:b:c_1:c lie near the values of 2:2:1:2, in all other organs of the species studied this pattern is not followed. However, the ratios for the same organ from any one species are reproducible. It is concluded that mitochondrial cytochrome ratios cannot in general be expressed in simple whole numbers and that different organs differ markedly with respect to these ratios. The ratios for all organs of the species studied are of the same order of magnitude, the greatest variation being 7.7:1:1 for rat intestinal cytochromes aa_3:c_1:c. In no case are the ratios for cytochromes b, c_1, or c to aa_3 significantly greater than 1.

INTRODUCTION

In a preceding paper[1] it was shown that the ratios of cytochromes aa_3:b:c_1:c in normal rat liver mitochondria are 1:0.95:0.59:0.87. Recalculation of these values using the probably more nearly correct extinction coefficients for cytochromes aa_3 and c reported by VAN GELDER AND SLATER[2,3] gives the ratios of 1:1:0.64:0.8 for rat liver mitochondria. Earlier CHANCE AND WILLIAMS[4] had reported, using different procedures, that the corresponding ratios are 1:0.9:(1.7), where (1.7) represents the combination cytochromes $c + c_1$. Thus the values by the two different methods are quite similar. It is also possible to interpret the ratios as being combinations of the whole numbers 3, 3, 2, and 3, or perhaps 2, 2, 1, and 2, if possible errors in the methods are accepted. In preliminary studies of the ratios of the cytochromes in intact beef heart mitochondria, the author has found ratios of 1:0.87:0.54:0.82, which are fairly close to those for rat liver. VANNESTE[5] has reported ratios of 2:2:2:1:2 for cytochromes a:a_3:b:c_1:c from beef heart mitochondria. The reason for the discrepancy between his results and the present author's is not clear. He did not use deoxycholate in his assay; and in his calculations he used the extinction coefficient of ZAUGG AND RIESKE[6] for cytochrome b, which is twice that employed by the author in his calculations.

SCHOLLMEYER AND KLINGENBERG[7] and KLINGENBERG[8] have reported that the ratio for cytochromes $c + c_1$ to aa_3 in a variety of tissues varies from 1.1 (for rat liver, pigeon heart muscle, and rat heart muscle) to 1.7 (for rat kidney cortex). He concluded that the cytochromes can be regarded as a 'constant proportion group' of enzymes since the ratios of the cytochromes c to aa_3 vary between such narrow limits.

Several submitochondrial products isolated from beef heart mitochondria, such as the 'unit of electron transfer' of BLAIR *et al.*[9], succinate–cytochrome c reductase (Complex II-III) of TISDALE[10], KING's succinate–cytochrome c reductase and b–c_1 particle[11,12], RIESKE's Complex III (ref. 13), and heart mitochondrial electron transporting particles (inner membrane) as reported by KOPACZYK[14], display cytochrome b to c_1 ratios ranging from 2:1 to 3:1. In the case of BLAIR's unit of electron transfer[9] and KOPACZYK's inner membrane[14], in which cytochrome aa_3 is also present, the ratios of cytochromes aa_3:b:c_1 approximate 6:3:1 and 6:2:1, respectively. Thus, though there are discrepancies in the cytochrome ratios among the various submitochondrial particles listed above, there are sufficient similarities to suggest an integral relationship which may be obscured by artifacts of isolation or analysis of the submitochondrial products.

In the present investigation, cytochrome ratios in mitochondria from a variety of organs from several species were studied to see if a reproducible pattern of ratios occurs in a certain organ of any one species and to see whether any relationship exists among the same organs of different species. The results of these studies should also indicate whether mitochondrial cytochrome ratios occur generally as combinations of small integers.

MATERIALS AND METHODS

Adult Sprague–Dawley male rats, adult male Hartley strain guinea pigs, and 6-week-old male chickens (Arbor Farms, Glastonbury, Conn.) were used as experimental animals. All were fed complete diets for several weeks before use.

Purified cytochrome oxidase was a gift of Dr. DAVID WHARTON, Enzyme Institute, Madison, Wisc. Horse heart cytochrome c (Type VI), obtained from Sigma Chemical Company, was assayed spectrophotometrically and found to be 99 % pure.

Mitochondria were isolated immediately after sacrifice of the animals from liver, kidney, brain, heart, washed small intestine (jejunum) by the standard procedure of SCHNEIDER AND HOGEBOOM[15]. The cytochromes were assayed at once, after isolation of the mitochondria by the method of WILLIAMS[1]. The calculations have been modified to include the extinction coefficients of VAN GELDER AND SLATER[2,3] for cytochromes aa_3 and c as proposed by VANNESTE[5]. However, the modification by VANNESTE to employ the cytochrome-b extinction coefficient of ZAUGG AND RIESKE[6] has not been followed since the present author feels that value is probably too high. Protein was determined by the procedure of LOWRY *et al.*[16].

The validity of the cytochrome method when applied to mitochondria from organs other than rat liver was checked in the following way. First, the cytochrome spectrum of the mitochondrial preparation was taken, and the cytochrome concentrations were calculated. Then purified cytochromes aa_3 and c equal to the amounts found in the mitochondrial preparation were mixed and the difference spectrum (reduced—oxidized) was obtained. This spectrum was then subtracted from the mitochondrial spectrum, giving by difference a spectrum presumably exactly lacking cytochromes aa_3 and c but leaving cytochromes b and c_1. The resulting spectrum was then subjected to the same calculation procedure[1] employed for the whole (unquenched) mitochondrial spectrum. Thus if no cytochrome aa_3 or c is obtained after this calculation, the original values for cytochromes aa_3 and c can be assumed to be correct. Also if after quenching cytochromes aa_3 and c from the mitochondrial spectrum the same values for cytochromes b and c_1 are obtained as from the original mitochondrial spectrum, it can be assumed that the values for cytochromes b and c_1 are correct. Underlying these assumptions, however, is the premise that the original qualifications employed in the development of the method[1] are valid; *i.e.*, that the only hemoproteins present which are reducible with dithionite under the conditions employed are the mitochondrial cytochromes and that the cytochromes have not been denatured so that they react abnormally with dithionite. In no case were spectra displaying anomalous peaks obtained either before or after quenching with cytochromes aa_3 and c.

Enough values were obtained from each organ from each species to allow statistical analysis of the results. Student's t-test was applied to all combinations of the means for the ratios of cytochromes $aa_3:b:c_1:c$ to test for significance of differences among the means. The differences were not listed as significant unless $P < 0.01$.

RESULTS AND DISCUSSION

In Table I are presented the results for the quenching of cytochromes c and aa_3 from the individual mitochondrial spectra. Since studies of this type on rat liver mitochondria have already been reported in an earlier paper[1], only the results for all

TABLE I

QUENCHING OF CYTOCHROMES aa_3 AND c FROM THE MITOCHONDRIAL SPECTRA TO SHOW THAT THE VALUES FOR CYTOCHROMES b AND c_1 REMAINING ARE THE SAME AS IN THE ORIGINAL UNQUENCHED SPECTRA AND THAT THE CYTOCHROMES aa_3 AND c CAN BE ACCOUNTED FOR

Species and organs	*Cytochrome aa_3 quenched* (%)	*Cytochrome c_1 quenched* (%)	*Cytochrome b remaining after cytochromes $aa_3 + c$ quenching* (%)	*Cytochrome c_1 remaining after cytochromes $aa_3 + c$ quenching* (%)
Rat kidney	100	89	98	100
Rat brain	96	85	102	88
Rat heart	97	91	98	111
Rat intestine	97	105	102	102
Guinea pig liver	106	92	113	88
Guinea pig kidney	96	92	100	99
Guinea pig brain	102	92	91	91
Guinea pig heart	88	98	92	96
Guinea pig intestine	107	97	89	98
Chicken liver	96	98	100	99
Chicken kidney	96	97	99	89
Chicken brain	93	98	100	88
Chicken heart	98	101	97	91
Chicken intestine	107	98	101	95
Average	99	95	99	95

other organs of the three species studied are presented in Table I. The results indicate that within 95–99 % of all of the cytochromes aa_3 and c is accounted for, and within the same range of error the cytochromes b and c_1 remaining in the spectra after quenching the cytochromes aa_3 and c are the same as in the original mitochondrial spectra. Thus when considered along with the assumptions made in the development of the original method[1], the results of Table I substantiate the cytochrome values presented in Tables II and III.

TABLE II

CONCENTRATIONS OF MITOCHONDRIAL CYTOCHROMES IN ORGANS OF THE RAT, GUINEA PIG, AND CHICKEN

S.E. is given. The number of determinations on the various animal organs to obtain the averages were as follows: Rat: liver, 101; kidney, 11; brain, 16; heart, 13; intestine, 10. Guinea pig: liver, 12; kidney, 6; brain, 16; heart, 16; intestine, 16. Chicken: liver, 9; kidney, 9; brain, 11; heart, 9; intestine, 13.

Organ and cytochrome		*mμmoles of cytochrome/mg mitochondrial protein*		
		Rat	*Guinea pig*	*Chicken*
Liver	aa_3	0.27 ± 0.005	0.23 ± 0.012	0.36 ± 0.019
	b	0.28 ± 0.005	0.27 ± 0.014	0.35 ± 0.029
	c_1	0.17 ± 0.006	0.18 ± 0.014	0.25 ± 0.019
	c	0.21 ± 0.005	0.13 ± 0.006	0.19 ± 0.014
Kidney	aa_3	0.56 ± 0.041	0.47 ± 0.008	0.35 ± 0.021
	b	0.51 ± 0.051	0.37 ± 0.03	0.40 ± 0.03
	c_1	0.19 ± 0.023	0.19 ± 0.007	0.30 ± 0.022
	c	0.51 ± 0.033	0.24 ± 0.012	0.17 ± 0.010
Brain	aa_3	0.51 ± 0.040	0.61 ± 0.079	0.38 ± 0.046
	b	0.30 ± 0.03	0.32 ± 0.045	0.25 ± 0.043
	c_1	0.10 ± 0.02	0.13 ± 0.028	0.11 ± 0.018
	c	0.17 ± 0.014	0.18 ± 0.026	0.12 ± 0.006
Heart	aa_3	0.98 ± 0.038	0.80 ± 0.076	0.63 ± 0.14
	b	0.76 ± 0.046	0.64 ± 0.068	0.34 ± 0.068
	c_1	0.39 ± 0.032	0.40 ± 0.042	0.22 ± 0.054
	c	0.44 ± 0.028	0.64 ± 0.090	0.27 ± 0.056
Intestine	aa_3	0.55 ± 0.056	0.55 ± 0.025	0.39 ± 0.042
	b	0.40 ± 0.018	0.36 ± 0.021	0.31 ± 0.032
	c_1	0.08 ± 0.013	0.11 ± 0.018	0.13 ± 0.020
	c	0.07 ± 0.007	0.16 ± 0.011	0.08 ± 0.009

The cytochrome values expressed as mμmoles of cytochromes aa_3, b, c_1, and c per mg mitochondrial protein are presented in Table II; and the ratios of the values for cytochromes b, c_1, and c to aa_3, in Table III. From Table II it is evident that the highest cytochrome-aa_3 concentrations were found in heart mitochondria. This is generally also true for cytochromes b, c_1, and c in the mammalian species. In the chicken, however, no particular pattern exists, the concentration of cytochrome b being approximately equal in all organs studied while cytochromes c_1 and c follow random patterns. However, in each organ of the chicken, the concentrations of cytochromes c_1 and c tend to equal each other. The lowest concentrations of cytochromes c_1 and c occur in brain and intestine of all three species.

KLINGENBERG[8] has reported a value for cytochromes $c + c_1$ in rat brain of 0.16 mμmole per mg protein. This is fairly close to the value of 0.27 for the same combination as shown in Table II. For rat brain he found a value of 0.27 mμmole

TABLE III

RATIOS OF MITOCHONDRIAL CYTOCHROMES b, c_1, AND c TO aa_3 IN ORGANS OF THE RAT, GUINEA PIG, AND CHICKEN; AND SIGNIFICANCES OF DIFFERENCES

Organ and cytochrome		*Ratio of cytochromes b, c_1 and c to aa_3*		
		Rat	*Guinea pig*	*Chicken*
Liver	b	1.0 $(c_1\ c)^{*}$	1.2 $(c_1\ c)$	0.97 $(c_1\ c)$
	c_1	0.63^{**} $(b\ c)$	0.87 (b)	0.70^{**} (b)
	c	0.78^{**} $(b\ c_1)$	0.57^{**} (b)	0.53^{**} (b)
Kidney	b	0.91 (c_1)	0.78^{**} $(c_1\ c)$	1.1 (c)
	c_1	0.34^{**} $(b\ c)$	0.41^{**} (b)	0.86 (c)
	c	0.91 (c_1)	0.51^{**} (b)	0.49^{**} $(b\ c_1)$
Brain	b	0.65^{**} $(c_1\ c)$	0.62^{**} $(c_1\ c)$	0.75^{**} $(c_1\ c)$
	c_1	0.22^{**} (b)	0.27^{**} (b)	0.38^{**} (b)
	c	0.37^{**} (b)	0.25^{**} (b)	0.35^{**} (b)
Heart	b	0.78^{**} $(c_1\ c)$	0.82^{**}	0.64^{**} $(c_1\ c)$
	c_1	0.40^{**} (b)	0.64^{**}	0.37^{**} (b)
	c	0.45^{**} (b)	0.82	0.45^{**} (b)
Intestine	b	0.77^{**} $(c_1\ c)$	0.66^{**} $(c_1\ c)$	0.84 $(c_1\ c)$
	c_1	0.16^{**} (b)	0.20^{**} (b)	0.43^{**} (b)
	c	0.13^{**} (b)	0.29^{**} (b)	0.24^{**} (b)

* Student's t-test indicates that the ratio value listed for the individual cytochrome is significantly different from the values for the cytochromes in parentheses ($P < 0.01$).

** The values marked with the double asterisk are significantly different at the 1% level from 1.0, the value, of course, for cytochrome $aa_3:aa_3$.

cytochromes $c + c_1$ per mg protein; in Table II, a value of 0.38 for this combination is observed. The major discrepancy between his values and the present values occurs with rat heart: KLINGENBERG's, 0.45; Table II, 0.83. For cytochrome aa_3, KLINGENBERG reported values of 0.42 and 0.24 mμmole per mg protein for rat heart and rat liver, respectively. In Table II are reported values for cytochrome aa_3 of 0.98 and 0.27 for the same organs. Thus for both cytochromes $c + c_1$ and aa_3 the present values are roughly double those of KLINGENBERG for rat heart but are very nearly the same for the other organs which can be compared.

Other workers[17–24] have studied cytochrome-c concentrations in various organs of the rat. In the three organs which can be compared with the present work (heart, liver, and brain) other workers[17–19] have found ratios for cytochrome c in these three organs, respectively, to be 1:0.30:0.16. In the present studies the ratios for the same organs are 1:0.48:0.39. While the two sets of results are not strictly comparable, since the earlier workers employed procedures for the total isolation of cytochrome c and from this paper mitochondrial cytochrome-c values are compared, the highest concentration of cytochrome c in these organs was found in the heart. Also the author has found, using a newly developed procedure for isolation and estimation of total organ cytochrome c (unpublished), that the cytochrome-c concentration of rat liver appears to be more than double that found by the earlier workers (approx. 22 mμmoles per g of liver compared to 10 mμmoles per g of liver). Thus the ratio of liver to heart cytochrome c found by the earlier investigators might well be higher than their results indicate and more in line with the present results.

In Table III it can be seen that no simple relationship of whole number ratios of the cytochromes to each other exists. It can only be stated that the ratio of cytochrome b to aa_3 approaches 1:1 in about 50% of the cases and approaches 2:3 in the rest. The ratio of cytochrome c_1 to aa_3 appears to approach four distributions, 1:1 (2 cases, av. = 0.87), 2:3 (3 cases, av. = 0.66), 1:3 (7 cases, av. = 0.37) and 1:5 (3 cases, av. = 0.19). For the cytochrome c to aa_3 ratios the distributions also are complicated. They can be sorted into 1:1 (1 case, 0.91), 4:5 (2 cases, av. = 0.80), 1:2 (6 cases, av. = 0.50), and 1:3 (3 cases, av. = 0.34).

It has been reported[13] that in Complex III, a well-defined particle from beef heart mitochondria which contains cytochromes b and c_1, the ratio of cytochrome b to c_1 is 2. In Table III, however, it can be seen that the ratios of cytochrome b to c_1 vary from 1.3 in guinea pig heart and chicken kidney to as high as 4.7 in rat intestine. In five cases (rat heart, guinea pig kidney and brain, and chicken brain and intestine) the cytochrome b to c_1 ratios approximate 2; but the wide range found among the other organs contradicts the generality that the cytochrome b to c_1 ratio is always 2 as it appears to be for Complex III from beef heart mitochondria.

The greatest similarity in cytochrome ratios for all three species occurs in brain. Beyond this there appears to be no consistent pattern for the cytochrome ratios either organ-wise or species-wise.

ESTABROOK[25] found for guinea pig liver mitochondria a ratio of 0.41 for cytochrome c_1:aa_3 and 0.46 for cytochrome c:aa_3. In Table III the results for this species' liver indicate 0.87 and 0.57 for the same cytochrome ratios, respectively. Thus although the results for cytochrome c are somewhat similar, those for cytochrome c_1 are quite different. The reason for the discrepancy with cytochrome c_1 is not known. It is possible that difference in strain of guinea pig used by ESTABROOK and in the present work may account for the difference in results. This may be an interesting point for study.

The original questions which led to the present study were as follows: (1) do simple whole number relationships in general exist for the cytochrome ratios of animal organs; (2) if so, are these ratios the same for mitochondria from all organs of all species; and (3) are the mitochondrial cytochrome ratios from any one organ of a certain species reproducible? Only the last question can be answered in the positive; the statistical analysis indicates that the mean values for the ratios for any one organ of any one species of a particular strain are highly predictable. It is also of special interest that the ratios of the cytochromes are all of the same order of magnitude in all of the organs studied (although there is as much as an 8-fold variation) and that cytochrome b, c_1, or c concentrations are never significantly higher than the concentration of cytochrome aa_3.

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