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RE-EVALUATION OF CYTOCHROME *c* CONCENTRATIONS IN RAT ORGANS USING A NEW METHOD FOR CYTOCHROME *c*

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SUMMARY

Using a new method for analysis, the cytochrome *c* concentrations in rat liver, heart, brain, lung, kidney, and muscle have been determined and compared with older values reported in the literature. The validity of the procedure has been established by studying the optimal conditions for precipitation of cytochrome *c*, the influence of residual hemin on the calculated values for cytochrome *c*, recoveries of cytochrome *c* added before extraction from various organs and the completeness of extraction of cytochrome *c* from the organs. The concentrations of cytochrome *c* reported here for liver, brain and lung are considerably higher than those reported by earlier workers, while the values for heart, kidney and muscle are nearly the same as those reported earlier.

INTRODUCTION

A wide range of values has been reported in the literature for total cytochrome *c* concentration in various organs of the rat. For example, values from 5 to 17 nmoles per g liver (average = 10), 7–12 nmoles/g muscle (average = 9), 3–6 nmoles/g brain (average = 5), 27–40 nmoles/g heart (average = 33), 17–27 nmoles/g kidney (average = 23) and 0.5–2 nmoles/g lung (average = 1.3) have been reported[1–7]. In the course of studying cytochrome concentrations in mitochondria from various animal organs, a new procedure for estimating total cytochrome *c* has been developed and has been applied to evaluating the concentrations of cytochrome *c* in rat liver, brain, lung, kidney, muscle and heart.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats were used as experimental animals. All were fed a commercial pellet ration for several weeks before use and were not fasted before sacrifice at 8 a.m.

For total cytochrome *c* analysis, 1–4-g portions of the organs to be studied were homogenized in 0.3% $Al_2(SO_4)_3 \cdot 18H_2O$ using a Virtis "23" homogenizer at full speed for 4 min. After centrifuging at 25000 × *g* for 10 min (3°), the supernatant solution was decanted and made 6% in trichloroacetic acid using 100% trichloro-

acetic acid (w/v). After 10 min in an ice bath, the protein precipitate was collected at 25000 × *g* for 10 min (3°). The tubes were drained, and the precipitate was broken up and successively sonicated in three 25-ml portions of cold acetone–2.4 M HCl (1:0.005, v/v) for 1 min and centrifuged for 5 min at 25000 × *g* (3°). The precipitate was then blended in a Virtis homogenizer in an appropriate volume of pyridine–0.2 M KOH (1:1, v/v) (5–20 ml /2 g organ). (Cloudy solutions indicate that not enough pyridine–KOH solution was used.) The difference spectrum (reduced with $Na_2S_2O_4$ *versus* oxidized with 0.05 ml 0.01 M $K_3Fe(CN)_6$) was read from 580 to 520 mμ. The readings should be taken at once after dissolving the precipitate in pyridine–KOH solution.

Since a residual amount of ferriprotoporphyrin IX invariably remains in most preparations even after up to eight extractions with acetone–HCl, the contribution of this ferriprotoporphyrin to the cytochrome *c* spectrum was accounted for by solving two simultaneous equations based on the extinction coefficients of the pyridine hemochromogens of cytochrome *c* and ferriprotoporphyrin IX at the wavelength pairs 550–535 and 557–540 mμ. The absorbance indices (reduced–oxidized) for ferriprotoporphyrin IX pyridine hemochromogen were determined experimentally using pure bovine hemin. These were found to be 18.1 and 6.34 $mM^{-1} \cdot cm^{-1}$ for the wavelength pairs 557–540 mμ and 550–535 mμ, respectively. The absorbance indices (reduced–oxidized) for cytochrome *c* pyridine hemochromogen were determined from cytochrome *c* (Sigma, horse heart, Type VI) standardized using the extintion coefficient reported by VAN GELDER AND SLATER[8]. These were found to be 25.1 and 1.49 $mM^{-1} \cdot cm^{-1}$ for the wavelength pairs 550–535 mμ and 557–540 mμ, respectively.

VALIDITY OF THE METHOD

Concentration of trichloroacetic acid for complete precipitation of cytochrome c. The recovery of 36 nmoles of cytochrome *c* added to 20 ml 0.3 % $Al_2(SO_4)_3 \cdot 18\ H_2O$ or to the $Al_2(SO_4)_3$ extract of rat liver equivalent to 1.5 g of liver was not complete until the final concentration of trichloroacetic acid was 5 % and leveled off thereafter. 6 % trichloroacetic acid was chosen as the best concentration.

Length of time for precipitation of cytochrome c. Complete precipitation was obtained after 5 min with no further change for 30 min. A 10-min precipitation time was chosen for the procedure.

Influence of residual hemin on cytochrome c concentration using the simultaneous equation method. The purpose of the following experiments was to show that the simultaneous equation calculation accounts correctly for the amount of hemin usually found in organ extracts after acetone–HCl extraction. When increasing levels of pure bovine hemoglobin (0–1200 nmoles) were extracted several times with acetone–HCl, 2.4 % of the hemin consistently remained in the protein precipitate. Since 500–600 nmoles of hemin are usually present initially in the $Al_2(SO_4)_3$ extract per g of rat liver, then 12–14 nmoles of hemin will be present in the final protein precipitate in which cytochrome *c* will be analyzed. This concentration of hemin is near the concentration of cytochrome *c* found in animal organs. In experiments in which increasing levels of bovine hemoglobin were added to 9 nmoles of cytochrome *c* and pyridine hemochromogens prepared from the mixtures, the following results were obtained: for hemoglobin to cytochrome *c* ratio of 2:1, 9.5 nmoles of cytochrome *c* were recovered;

for a ratio of 5:1, 9.4 nmoles of cytochrome *c* were recovered; for a ratio of 6:1, 8.7 nmoles of cytochrome *c* were recovered; and for a ratio of 8:1, 8.9 nmoles of cytochrome *c* were recovered. Thus the overall recovery of cytochrome *c* was 9.1 nmoles, or 101 %, even with hemin concentrations far in excess of that present after acetone–HCl extraction. In organ extracts, however, it is essential that the acetone–HCl extractions be carried out since the hemin to cytochrome *c* ratios are usually much greater than those studied in the preceding experiments.

Recoveries of cytochrome c added to various rat organs before homogenization. When 8–38 nmoles of cytochrome *c* were added to 1–4-g portions of organs of the rat and when the entire analytical procedure was carried out including homogenization, precipitation with trichloroacetic acid, extractions with acetone–HCl and preparation of the pyridine homochromogens from the final precipitate, the following recoveries were obtained: liver, 103 ± 7 %; heart, 98 ± 5 %; brain, 91 ± 13 %; lung, 98 ± 11 %; kidney, 108 ± 10 %; and large muscles of the hind leg, 89 ± 5 %. Each value represents the average of 5–6 recovery checks with standard deviations.

In the calculations of cytochrome *c* concentration per g of organ, the assumption was made that cytochrome *c* was equally distributed in the supernatant fluid and in the precipitate after the $Al_2(SO_4)_3$ fractionation. This assumption was verified by experiments in which the precipitate was re-extracted with $Al_2(SO_4)_3$ and the residual cytochrome *c* was analyzed in the usual manner. The amount of the cytochrome *c* obtained by the further extraction was the same as that which could be accounted for by assuming equal distribution in the supernatant fluid and the precipitate.

RESULTS AND DISCUSSION

In Table I are presented the concentrations of cytochrome *c* in rat organs. Thus the values for liver and brain are about twice the average values (10 and 5, respectively), and the value for lung is about 6 times the average value (1.3) reported by other workers[1–7]. The values for heart, kidney and muscle are approximately the same as those reported by earlier workers (33, 23 and 9, respectively). The question arises concerning the differences in values obtained by the present procedure and those obtained by others for liver, brain and lung. In each of these cases the present values are higher than the previously reported values. The major difference in the

TABLE I

CYTOCHROME *c* CONCENTRATIONS IN ORGANS OF THE MALE ALBINO RAT

The numbers in parentheses denote the number of animals employed to obtain the average values. Data are expressed as mean ± S.D.

Organ	*nmoles of cytochrome c per g fresh organs*
Liver (15)	20.6 ± 4.1
Heart (6)	35.5 ± 1.8
Brain (6)	7.92 ± 1.3
Lung (5)	8.20 ± 1.8
Kidney (5)	23.6 ± 2.2
Muscle (6)	6.84 ± 1.3

procedures are that most of the earlier methods require a differential precipitation of hemoglobin (and other extraneous proteins) and cytochrome *c*. In our hands it has been found that a considerable portion of the cytochrome *c* precipitates along with the "hemoglobin" fraction when using the earlier methods. Moreover, the degree of coprecipitation of cytochrome *c* would depend to a great extent on the amount of extraneous proteins precipitated. Thus for organs in which the size of this precipitate is quite large, more cytochrome *c* would be lost than if the amount of the precipitate is small.

From a recalculation of the values for mitochondrial cytochrome *c* reported recently by WILLIAMS[9], only 4.2 nmoles of cytochrome *c* from a total of 20.6 nmoles per g of fresh liver are isolated with the mitochondria. Similar calculations for heart, brain and kidney mitochondrial cytochrome *c* yield values of 4.7 nmoles for heart, 1.17 nmoles for brain and 6.63 nmoles for kidney per g of fresh organ. Thus the yield of cytochrome *c* in the mitochondria isolated from the various organs compared to the total cytochrome *c* present in the unfractionated organ is 20 % for liver, 13 % for heart, 15 % for brain and 28 % for kidney. Muscle and lung cannot be compared in this manner, since mitochondrial data for those organs were not obtained in the previous study[9]. Other workers[10–13] have also found that the percentage of total cytochrome *c* found in the mitochondrial fraction from liver is considerably less than 100. This has been attributed to contamination of other fractions by mitochondria, to a redistribution of cytochrome *c* from mitochondria to other cellular fractions during isolation or to a combination of these two factors. Thus from the preceding calculations it appears that this holds true not only for liver but for heart, brain and kidney as well. To what extent the ratios for cytochrome *c* to cytochromes aa_3 reported recently[9] in various organs of different species are valid because of possible redistribution of cytochrome *c* during cellular fractionation is being investigated at present. Certainly, however, the previously reported[9] ratios of cytochromes *b* and c_1 to cytochromes aa_3 should be strictly valid because of the insoluble nature of these cytochromes.

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