THE QUANTITATIVE DETERMINATION OF CYTOCHROME C*

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In the course of a quantitative study of the distribution of cytochrome c in cancer tissue a consideration of the available methods of analysis led to the development of an improved method which has been applied to this particular study. The method has been used under such a variety of conditions that it is believed that it will give satisfactory results if applied to other problems involving the concentration of cytochrome c in animal tissues.

Both manometric and spectrophotometric techniques have been applied to the determination of cytochrome c by previous investigators. Von Euler and Hellström (2) reported the cytochrome c content of Jensen rat sarcoma on the basis of a qualitative spectrophotometric method. Junowicz-Kocholaty and Hogness (3) have presented a method for cytochrome c determination which is essentially Keilin and Hartree's method for the preparation of pure cytochrome c (4) placed on a quantitative basis. The method requires the use of 100 gm. of tissue, a quantity too large for experiments in which rats and mice are used as experimental animals. Fujita, Hata, and Numata (5) presented a spectrophotometric method for the determination of cytochrome c in which an attempt was made to isolate the cytochrome in pure form. Attempts to recover added cytochrome c by this method gave unsatisfactory results. It is believed that an incomplete precipitation of cytochrome c occurred during the acetone treatment.

By far the most satisfactory method in the literature thus far is

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1 A preliminary report of the results of this study has appeared (1).

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that of Stotz (6) whose method is based on the catalytic function of cytochrome. The determination is made by comparing the rate of oxygen uptake obtained with an unknown solution and that obtained with a standard cytochrome c solution. However, since extraneous factors present a constant danger in the case of analyses based on rate measurements, we attempted to devise an absolute measurement of the cytochrome c concentration. This can be accomplished spectrophotometrically by employing the absorption coefficients for reduced and oxidized cytochrome c. Potter (7) recently suggested that it should be possible to determine cytochrome c spectrophotometrically by means of the enzymatic oxidation and subsequent reduction of the compound, since he obtained 100 per cent recovery of added cytochrome c when the reduction was carried out in the presence of 0.001 M cyanide added after succinate. It seemed that a specific enzymatic spectrophotometric measurement such as this would eliminate difficulties encountered in rate measurement and would also eliminate the interference of hemoglobin (3), thus eliminating the necessity for a hemoglobin-free extract.

The method which we are presenting for the determination of cytochrome c in animal tissues is based on the following three facts: (a) The absorption of reduced cytochrome c is much greater than the absorption of oxidized cytochrome c at a wave-length of 550 mμ. (b) The total absorption of a solution containing several solutes is equal to the sum of their individual absorptions in the absence of interaction. (c) Cytochrome c can be specifically oxidized and reduced by enzymatic means without altering the absorption of other components of the test solution.

Method

Extraction and Concentration—Weighed tissues are homogenized in 5 to 20 volumes of distilled water according to the Potter and Elvehjem technique (8) with a stainless steel pestle. The homogenate is quantitatively transferred to a beaker and the pH is adjusted to 3.5 by addition of 3 per cent trichloroacetic acid with a glass electrode to measure the acidity. The mixture is allowed to stand for 1½ hours to extract the cytochrome c (3, 4, 6). The mixture is then transferred to graduated conical tubes and centrifuged for 10 minutes at 3000 r.p.m. Since the cytochrome is dis-
tributed between the residue and the supernatant, the volumes of both are recorded for subsequent calculations. The supernatant fluid is brought to pH 7.0 with 1 per cent sodium hydroxide, and after standing 5 to 10 minutes the mixture is again centrifuged at 3000 R.P.M. for 10 minutes. The precipitate should be relatively small at this point, and is discarded. The supernatant fluid is poured into graduated centrifuge tubes and a quantity of 100 per cent trichloroacetic acid solution equal to 8 per cent of the volume of supernatant fluid is added. The mixture is allowed to stand for 20 minutes and is then centrifuged for 10 minutes at 3000 R.P.M. The supernatant liquid is carefully removed and discarded. The precipitate containing the cytochrome c, which usually occupies about 0.20 ml. when packed, is taken up in a quantity of water less than 2 ml. and dissolved in a drop of 2 N sodium hydroxide, plus further additions of 0.1 N alkali as needed. Any excess sodium hydroxide is neutralized by addition of 0.1 N hydrochloric acid. Here an external indicator, phenol red, is used to determine when the solution is neutral. The unknown solution is finally diluted to a volume of 2.5 ml. with distilled water.

Spectrophotometric Measurement The estimation of the cytochrome c content of the unknown solution is carried out with the use of a photoelectric spectrophotometer (7, 9). To each sample 0.3 ml. of 0.25 M phosphate buffer, pH 7.4, and 0.2 ml. of a kidney enzyme preparation containing both succinic dehydrogenase and cytochrome oxidase are added, giving a total volume of 3.0 ml. The unknown solution is next placed in a 1 cm. spectrophotometric cell and the absorption is measured at 550 m\(\mu\) with a 2.5 m\(\mu\) exit slit. The action of cytochrome oxidase in the absence of substrates which reduce cytochrome c converts the latter to the oxidized form. The extinction observed is due to oxidized cytochrome plus other colored substances present, chiefly flavin and hemin derivatives. Next 0.01 ml. of 0.5 M succinate is added to the cell. After mixing, 0.03 ml. of 0.1 M neutralized cyanide is added. This concentration of cyanide stops the action of the oxidase but does not combine with the cytochrome c under these conditions (7). After mixing, the cytochrome c is converted to the reduced form within 30 seconds by the action of succinic dehydrogenase, and a second spectrophotometric reading is taken. From the two extinction values obtained the concentration of cytochrome c may be
calculated. If $C_t$ equals the total cytochrome $c$ in moles per ml., $E_r$ the observed extinction of reduced cytochrome $c$, $E_o$ the observed extinction of oxidized cytochrome $c$, and $\alpha$ for $E_r - E_o$ is $1.91 \times 10^7$ (see (7)), then,

$$C_t = \frac{E_r - E_o}{1.91} \times 10^{-7}$$

Since the final solution has a volume of 3 ml., it is necessary to multiply the result by 3 and, in addition, to correct for the volume of the residue after extraction in order to obtain the amount of cytochrome in the sample.

**Comments on Method**

*Extraction*—Since the extraction of the cytochrome $c$ from the tissue is of primary importance, careful consideration was given to the factors involved in this procedure. In studying the optimum pH for extraction, rat liver and kidney tissues were extracted at acidities varying from pH 3.0 to 4.5. In agreement with the finding of Stotz (6) we found that maximum extraction was obtained at pH 3.5, with an optimum range of about pH 3.2 to 3.8.

Since the effectiveness of extraction cannot be tested by recovery experiments, other lines of evidence must be advanced to test the procedure. That homogenization *per se* disrupts the cells and disperses the cytochrome into the medium is clearly demonstrated by the fact that a dilution effect which can be overcome by the addition of pure cytochrome $c$ can be demonstrated in the case of the succinoxidase system in liver (10). This result is obtained in other tissues as well. Further evidence that extraction is complete was shown by reextracting the first residue obtained in the preparation of pure cytochrome $c$ from beef heart muscle. By again homogenizing the acid-extracted residue, adjusting to pH 3.5 with trichloroacetic acid, and extracting for 1½ hours it was found that the first extraction was complete; i.e., it was found that as much cytochrome $c$ remained in the precipitate as was present in an equal volume of supernatant fluid. A third test was made by increasing the volume of the extraction mixture and holding the amount of tissue constant. It was found that a given volume

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2 The value of the constant should be determined for the particular spectrophotometric conditions used.
of precipitate contained as much cytochrome c as an equal volume of supernatant liquid. It is apparent, therefore, that the extraction procedure is effective but that the precipitate volume must always be recorded and considered in calculating the quantity of cytochrome c present in any sample.

Neutralization of Acid Extract—In a study of the neutralization of the acid extract it was concluded that this is an essential step in the procedure but evidence was obtained that adsorption of cytochrome c on the precipitate took place if the mixture was allowed to stand for longer than the recommended time before centrifugation.

In the special case of rat skeletal muscle poor recovery of added cytochrome was found. However, by adjusting the pH to 5.0 instead of 7.0, 93 per cent recovery of added cytochrome was obtained. The volume of precipitate obtained at this point was decreased by the modification. This suggested that adsorption of cytochrome c on the unusually voluminous precipitate obtained in the case of skeletal muscle was the cause of the loss of cytochrome c. Homogenizing tissues so thoroughly that the particles are too finely dispersed to be precipitated during the first centrifuging process is to be avoided, since in such a case the precipitate volume after neutralization to pH 7.0 is greater and adsorption of cytochrome c may occur. If a tissue is encountered which gives a large precipitate at this step, it is very important that recovery experiments be carried out to test this particular step.

In the analysis of livers containing tumors induced by dimethylaminoazobenzene a difficulty was encountered in that the presence of a fatty constituent rendered the final solution too turbid. It appeared that extraction of the fat-like substance would be necessary before the analysis was made, but by carefully removing the supernatant liquid from the precipitate after the first centrifuging, the fatty substance adhered to the sides of the centrifuge tube and in that manner could be separated from the supernatant liquid.

Quantitative Precipitation of Cytochrome c—Any method for the analysis of cytochrome c must involve a step in which the cytochrome in a relatively large volume of extract is concentrated to a small volume. Stotz (6) precipitated the cytochrome with phosphotungstic acid, and then removed the latter with barium in the presence of phosphate to avoid an excess of barium. The final
solution obviously contains a saturated solution of barium phosphate and when this is concentrated in vacuo a precipitate of barium phosphate is likely to result. Since cytochrome c is adsorbed on barium phosphate, there is considerable danger of losing cytochrome at this step, and we have been unable to recover small amounts of cytochrome. This procedure is likely to give too low results on samples which are originally low in cytochrome.

Since our proposed method would give valid results even in the case of partial inhibitions of rate, we attempted to concentrate the cytochrome by precipitation with phosphotungstic acid and to make the measurement in the presence of the phosphotungstate. This was not feasible, since the latter completely inhibited the enzyme preparation. However, experiments showed that sodium trichloroacetate did not interfere with the enzymatic oxidation and reduction, and trichloroacetic acid was therefore tested as a cytochrome precipitant, since it is known to be very effective as a protein precipitant. Pure samples of cytochrome were used and the analysis was effected spectrophotometrically by chemical reduction with sodium hydrosulfite. It was found that in all samples in which the final concentration of trichloroacetic acid was greater than 5 per cent the cytochrome was completely precipitated. It was further found that a given amount of cytochrome c could be quantitatively precipitated from a volume of extract several times larger than is ever encountered in practice. It is thus possible to effect the concentration of cytochrome c with trichloroacetic acid and to make the quantitative measurement without removing the precipitant.

Quantity of Tissue Needed—In order to insure a sufficient difference in extinction between the oxidized and reduced cytochrome c when determined spectrophotometrically it was necessary to determine the quantity of tissue necessary for the determination. 1 to 2 gm. of normal rat tissues is sufficient, while 5 to 10 gm. of cancer tissue are required. In studying the accuracy of the method various tissues such as rat liver, spleen, and cancer tissue were used in recovery experiments. In all cases we have recovered greater than 93 per cent of added cytochrome c.

Although the cyanide concentration has been adjusted to a very low concentration, experiments were designed to make certain that cyanide did not combine with part of the cytochrome c, as has been noted (7) in the case of higher concentrations of cyanide.
Small quantities of cytochrome c ranging from $0.025$ to $0.075 \times 10^{-4}$ mole of cytochrome c were added to 1 gm. samples of Flexner-Jobling rat carcinoma, so that the final concentration of cytochrome c in each sample was lower than that present in any actual tissue analysis. Recovery between 96 and 100 per cent was obtained in all samples, thus eliminating the possibility of cyanide combining with cytochrome c in this analysis, as well as insuring the over-all accuracy of the method.

Method of Preparation of Enzyme—A rat kidney is homogenized in 9 volumes of cold m/30 phosphate buffer (pH 7.4), centrifuged for 4 minutes at 1000 R.P.M., and then filtered through coarse filter paper. Although the cytochrome c has not been removed from this preparation, the quantity present in 0.2 ml. cannot be detected spectrophotometrically and thus does not interfere in the determination. The strength of the enzyme must be proved with a sample of pure cytochrome. Reduction should occur within 30 seconds.

Since the determination of cytochrome c by this method depends upon measuring the difference in extinction between oxidized and reduced cytochrome c, it is essential that the cytochrome c in the unknown solution be completely oxidized before the initial reading is taken. There are three steps in this method which insure complete oxidation of cytochrome c, (a) the precipitation of cytochrome c with trichloroacetic acid (3), (b) dissolving the trichloroacetic acid precipitate in sodium hydroxide (3), (c) the action of the cytochrome oxidase in the final solution. In order to prove that the quantity of cytochrome oxidase used was sufficient, even if all of the cytochrome present were in the reduced form, an experiment was carried out in which 11 g of ascorbic acid were added to $2 \times 10^{-8}$ mole of cytochrome c. This resulted in reduction of about 80 per cent of the cytochrome c. Varying quantities of the enzyme preparation were used and as little as 0.01 ml. of the enzyme preparation was found to give complete oxidation in 2 minutes. Since 0.2 ml. of the enzyme preparation is used in actual analyses, assurance of complete oxidation was obtained. The turbidity of the enzyme does not interfere with the method, largely because it is automatically corrected for.

Removal of Hemoglobin—As mentioned previously, the presence

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3 A homogenate of pooled samples was used and the concentration of cytochrome in the tumor was determined on an aliquot equivalent to 5 gm.
of hemoglobin does not ordinarily interfere with the determination. However, in the case of certain tissues such as spleen it is desirable to remove part of the hemoglobin to decrease the total absorption in the spectrophotometric measurement. This can be accomplished by acidifying the final unknown solution to pH 4 to 6 with dilute HCl, which precipitates the hemoglobin. The mixture is centrifuged and the precipitate is washed with 0.1 N HCl. The combined supernatant liquids are again neutralized to pH 7.0.

**Table I**

*Cytochrome c Content of Normal Rat Tissues*

The results are expressed as micrograms of cytochrome c per gm. of fresh tissue.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Heart</th>
<th>Kidney</th>
<th>Skeletal muscle</th>
<th>Brain</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
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</table>

Average.............. 371 247 97 50 90 43 21

" from Stotz (6)........ 530 330 160 75 68 48 29

Recovery of over 90 per cent of the cytochrome c from spleen was obtained.

**Application of Method**

*Distribution of Cytochrome c in Normal Rat Tissues*—This method of analysis has been applied to several tissues from normal adult rats. A wide variation in cytochrome c content of different organs was noted, in substantial agreement with the results of Stotz (6). Table I gives the cytochrome c content of the various tissues analyzed. Duplicate samples check within about 5 per cent of the average value but it should be noted that there is
rather wide variation between animals as was also noted by Stotz. The results in Table I were calculated with 16,500 as the molecular weight of cytochrome c. The average values obtained by Stotz with the same molecular weight are included for comparison.

It is of interest to note that the levels obtained by Stotz are about 50 per cent higher than ours in the case of tissues high in cytochrome c, while they check very closely in the case of lung and spleen, which are low in cytochrome. It is not possible to say whether the differences are in the material or the methods. Since the molecular weight may actually be as low as 11,700 (11) it might be preferable to state the results in moles as given in the method of calculation above.

### Table II

**Cytochrome c Content of Chick Embryo**

The results are expressed as micrograms of cytochrome c per gm. of fresh tissue.

<table>
<thead>
<tr>
<th>Age of embryo</th>
<th>No. of samples</th>
<th>Maximum</th>
<th>Minimum</th>
<th>Average</th>
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<tr>
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</table>

Stotz reported that rat embryos were low in cytochrome c. We have analyzed a number of chick embryos which are also low in cytochrome c, and they show an increase in cytochrome concentration with age, as is shown in Table II. This material is so low in cytochrome c as to make the analysis extremely difficult, as well as of questionable accuracy as far as comparative studies on embryos of a given age are concerned. It would be of considerable interest to determine whether the cytochrome c concentration in very young rats is inversely related to their recently demonstrated ability (12) to withstand short periods of anaerobiosis.

**SUMMARY**

1. A new method for the determination of cytochrome c in small quantities of tissue from experimental animals has been developed.
2. Existing methods of extraction are used in the method but concentration is effected by precipitation with trichloroacetic acid.

3. The analysis is based on the quantitative spectrophotometric measurement of the change in extinction when cytochrome c is oxidized and reduced by specific enzymes.

4. Results obtained with normal rat tissues and chick embryo are given.

**BIBLIOGRAPHY**

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