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The Electron Transmitter System of Brown Adipose Tissue*

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The components of the electron transmitter system of interscapular brown adipose tissue of male rats have been examined. The presence of an abundance of cytochromes $a + a_3$, b, c, and c_1 was demonstrated spectrophotometrically in particulate matter derived from this tissue. The cytochrome c of the tissue was isolated chromatographically and quantitatively determined, and a content of 2.30 ± 0.22 mg per g of lipid-free dry weight of tissue was found, a value apparently as high as in any other tissue previously described in the rat. The particulate matter derived from the tissue was found to have a content of ubiquinone (coenzyme Q) equivalent on the average to 6 moles per mole of cytochrome c. An additional and approximately equal amount of ubiquinone was found to be present in the nearly colorless fat layer which separates during preparation of the particulate fraction. The ubiquinone was shown to be predominantly the form having a side-chain with 45 carbon atoms (coenzyme Q_9). The high cytochrome content of the tissue is discussed in relation to the tissue's characteristic color, high mitochondrial content, and unusually high rate of oxygen consumption under hormone stimulation.

Brown adipose tissue is most abundant in hibernating animals but also occurs in other animals. It is distinguished, as its name indicates, from the more plentiful white adipose tissue by its color. The nature of the pigmentation as well as the physiologic function of the tissue has been the subject of much speculation, especially in relation to its possible role in the hibernating animal (*cf.* Johansson, 1959). More recently interest in this tissue has been aroused by reports of its preferential invasion by a variety of viruses and the fact that the tissue may serve as a storage and multiplication site for such viruses (*cf.* Sulkin *et al.*, 1959). Data are presented herein which indicate that brown adipose tissue is extremely rich in the cytochromes and ubiquinone and that the cytochromes contribute importantly to the characteristic color of this tissue.

Experimental Procedures

Determination of Cytochrome Pattern.—Male Holtzman rats of weights to be specified were maintained on an *ad libitum* diet of Purina laboratory chow. The animals were sacrificed by decapitation. The interscapular brown adipose tissue was removed immediately; trimmed free of adhering muscle, connective tissue, and white adipose tissue; weighed on a torsion balance; and placed into an ice-cold isotonic sodium chloride solution. This process required approximately 10 minutes for each rat, and sufficient tissue (4 to 12 rats) for each experiment was accumulated in this manner before proceeding to the next step. One piece which was judged to be the nearest to the group average with respect to tissue weight, animal weight, and intensity of tissue color was set aside for determination of its total lipid content and lipid-free dry weight. The values obtained upon this piece of tissue were then used as representative of the total batch for later calculations. The remaining tissues were combined and homogenized in 10 to 15 ml water per g of fresh tissue. This and all subsequent steps in the preparation of the tissue for spectral studies were carried out either in a cold room at 5° or in a refrigerated centrifuge at 2°. Glass homogenizers of the Potter-Elvehjem type were used throughout. The resulting crude homogenate was centrifuged for 1 hour at 25,000 × g, to yield a brown pellet and a slightly turbid supernatant fluid which had a nearly white layer of fat on the surface. The fat layer was removed and in some instances saved for determination of its ubiquinone content. The supernatant fluid was discarded. It showed absorption bands with the hand spectroscope in the regions of 542 and 578 mμ, characteristic of oxygenated hemoglobin (e.g., Drabkin, 1946). Addition of

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0.1 to 0.2 mg sodium dithionite per ml to this fluid produced no trace of cytochrome absorption bands. The pellet, to be called the "initial particulate fraction," was now carried through one or more of the procedures outlined below.

A preparation of the initial particulate fraction was freed of remaining traces of hemoglobin by homogenization with 10 to 15 ml of 0.05 M glycylglycine buffer (*p*H 7.4) per g of original intact tissue and subsequent centrifugation at 25,000 × *g* for 30 to 45 minutes. The resulting supernatant fluid, which was discarded, occasionally showed a very faint band at 550 mμ upon addition of sodium dithionite, presumably due to a trace of cytochrome *c*. The resulting pellet was then homogenized with solution made by the addition of 2 g sucrose to each ml of 0.25 M sodium phosphate buffer (final *p*H = 7.4) (Keilin and Hartree, 1955). The resulting suspension, each ml of which contained the material derived from 123 to 169 mg of the original intact tissue, was used for spectral studies. Various modifications of this procedure, including attempts to separate intact mitochondria, were evaluated before adoption of that described above as the most suitable for the purposes at hand.

The quantitative measurements of absorption spectra were carried out at room temperature with a Beckman model DU spectrophotometer which had been calibrated at several wave lengths with a mercury lamp and at 550 mμ with reduced cytochrome *c*. The tissue preparations were contained in cuvets of 1-cm path length which were attached to a Thunberg-type tube (Ball *et al.*, 1951). Absorption spectra of the suspensions in concentrated sucrose solution were read against a blank of homogenized milk which had been diluted on the order of 200-fold with water. The turbidity of such a blank can conveniently be adjusted to approximate that of the tissue suspension. The optical density of such a milk blank read against water increased steadily with decreasing wave length and showed no trace of selective absorption in any part of the wave length region employed. The absorption spectrum of the tissue particulate suspension was first determined under aerobic conditions. The preparation was then rendered anaerobic by repeated evacuation of the Thunberg-type tube and filling with oxygen-free nitrogen. Either 0.1 ml of a neutralized cysteine solution or solid ascorbic acid was then tipped from the side-arm into the contents of the main chamber to yield a final concentration of 3×10^{-3} M or 2×10^{-3} M, respectively, and the absorption spectrum was again measured. The spectrum was next determined under anaerobic conditions after addition of DPNH to a final concentration of 1×10^{-3} M. Finally, the spectrum was determined after aerobic addition of sodium dithionite at approximately 0.5 mg per ml.

In order to measure the cytochrome c_1 content of the preparation it was necessary to modify the original procedure in such a way that cytochrome *c* was first removed. This was accomplished by homogenization of the initial particulate fraction with an isotonic sodium chloride solution (10 to 15 ml per g of original intact tissue) instead of the glycylglycine buffer, followed by centrifugation at 25,000 × *g* for 30 minutes (Estabrook, 1958). This extraction and centrifugation procedure was repeated in order to ensure complete removal of cytochrome *c* from the particulate matter. Any remaining hemoglobin was also quantitatively removed by the extraction procedure. The final brown pellet was then homogenized in the concentrated buffered sucrose solution described above and studied spectrally. Each ml of the final suspension contained the material derived from approximately 190 mg fresh tissue.

Chromatographic Separation of Cytochrome c *and Quantitative Determination.*—A modification of the method of Loftfield and Bonnichsen (1956) was employed. The initial particulate fraction was homogenized with water to yield a suspension, each ml of which contained the material derived from 60 to 200 mg of the original intact tissue. The *p*H was then adjusted to 4.1 ± 0.1 with 0.1 N H_2SO_4, and the suspension was allowed to stand for 1 hour with occasional stirring. After centrifugation at 25,000 × *g* for 30 minutes, the resulting supernatant fluid was brought to *p*H 7.2 ± 0.3. A small amount of newly formed precipitate was removed by centrifugation at 1000 × *g* for 5 minutes. The clear supernatant fluid was passed through a 10 mm (length) × 4 mm (diameter) column of Amberlite XE-64 in the ammonium form, and the column was then rinsed with several ml of water. Remaining traces of interfering pigments were eluted from the column with no more than 1.5 ml of 0.25 M ammonium acetate buffer (*p*H 9.5). The cytochrome *c* was eluted by a small volume of 1 M ammonium acetate (*p*H 7.0). Owing to the occasional presence of faint turbidity in the eluate, the cytochrome *c* was calculated from the optical density at the 550 mμ peak minus the optical density at 550 mμ on a baseline drawn between points on the absorption curve at 535 and 565 mμ. The molar extinction coefficients for pure cytochrome *c* of Margoliash and Frohwirt (1959) at these wave lengths were used, as was a molecular weight of 12,250, calculated from their reported value of 0.456% for the iron content.

Determination of Ubiquinone Content.—The method employed was patterned after that of Crane *et al.* (1959a). The initial particulate fraction from 1.2 to 1.7 g fresh tissue was homogenized with 8 ml water, and an aliquot of one fourth or one half of this homogenate was centrifuged at 25,000 × *g* for 1 hour. The resulting pellet was saponified with 5 ml of 0.89 N KOH in 2:1 ethanol-water containing 60 mg pyrogallol by refluxing under oxygen-free nitrogen for 30 minutes. The unsaponifiable fraction was recovered by three successive extractions with 1 ml heptane each. After acidification of the combined heptane extracts by addition of 0.2 ml of 0.1 N HCl in order to facilitate separation of phases, the heptane phase was washed twice with 1-ml portions of

water. The second water wash always had a *p*H of 5 or higher after separation of phases. The washed heptane extract was evaporated to dryness at 70° under a stream of oxygen-free nitrogen and the last traces of solvent were removed by placing the residue under high vacuum. The residue was dissolved in 3.00 ml absolute ethanol and its absorption spectrum was read in a calibrated 1-cm silica cell against a blank of absolute ethanol. After reduction by addition of 0.02 ml of a freshly prepared 10% aqueous solution of KBH_4, the absorption spectrum was again determined, this time against a blank of absolute ethanol to which 0.02 ml of the KBH_4 solution had been added. The decrease in absorption at 275 mμ following reduction by KBH_4 was taken as a measure of the amount of ubiquinone present, using a value of 158 for the decrease in $E^{1\%}_{1\ cm}$ of ubiquinone-45 (Lester *et al.*, 1959).

The fat layer resulting from centrifugation of the original homogenate of 1.2 to 1.7 g fresh tissue was saponified with 5 ml of 0.89 N KOH in 95% ethanol containing 60 mg pyrogallol under nitrogen for 50 minutes. Following addition of 2.5 ml water, the unsaponifiable fraction was extracted and studied spectrally in the manner described above for the particulate fraction.

Determination of Lipid-Free Dry Weight.—Total dry weight was determined by drying the tissue to constant weight (24 hours) *in vacuo* over P_2O_5 at room temperature. Total lipids were then removed by three successive 10-minute extractions with 2:1 chloroform-methanol (Folch *et al.*, 1957). The lipid-free tissue was again exposed to high vacuum over P_2O_5 for at least 3 hours and was then weighed. The fact that the loss in weight due to extraction by chloroform-methanol was found to be equal to the weight of lipids recovered from the solvents after the extraction indicated that the previous removal of water from the tissue had been quantitative.

Materials

Ubiquinone-50 was a gift of Dr. K. Folkers of Merck, Sharp and Dohme Research Laboratories. DPNH was obtained from Sigma Chemical Company. All solvents used in the determination of ubiquinone contents were redistilled before use. Glass-distilled water was used in all preparations of tissues for spectral measurements of cytochromes.

Results

Cytochrome Pattern.—Figure 1 shows difference spectra between various stages of reduction of a typical suspension of the particulate fraction of brown adipose tissue in buffered concentrated sucrose solution. The absorption spectrum taken under aerobic conditions without addition of reducing agents (not shown) never had any suggestion of a peak or shoulder in the region of 578 mμ, thus indicating that hemoglobin had been completely removed when the particles were washed with

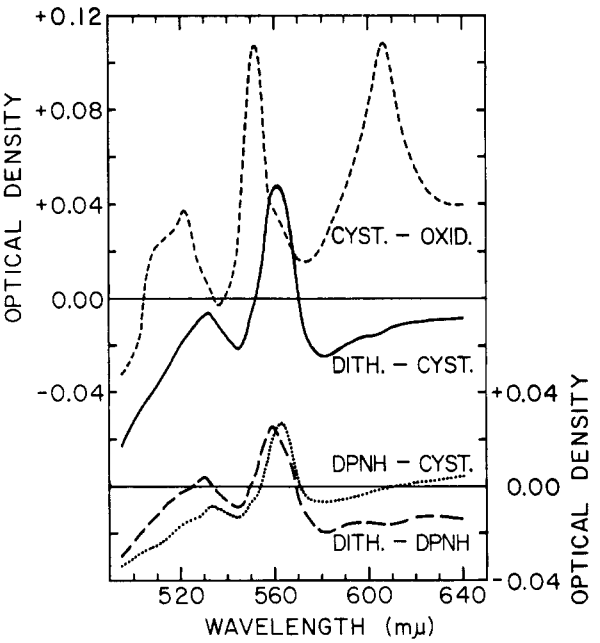

FIG. 1.—Absorption spectra of suspension of particulate fraction of brown adipose tissue in buffered concentrated sucrose solution. All four curves are difference spectra. The pairs of reducing conditions from which each difference spectrum was calculated are indicated next to each curve. The optical density scale on the left side applies to the upper two curves, and that on the right side applies to the lower two curves. In the experiment illustrated, each ml of the final suspension contained the material derived from 162 mg fresh tissue. The rats used for the experiment shown ranged in body weight from 190 to 235 g, with a mean of 224 g. For further details see text.

glycylglycine buffer during their preparation. The upper curve in Figure 1 is the difference between the spectrum of the oxidized state and that obtained after reduction by cysteine under anaerobic conditions. In several preparations this curve had peaks between 603 and 607 mμ, at 551 to 552 mμ, and at 521 to 522 mμ; this fact indicated the presence of cytochromes $a + a_3$ and c and strongly suggested the presence of cytochrome c_1. As indicated in Figure 1, the difference between the spectra of the cysteine-reduced and the dithionite-reduced preparation shows a major peak at 560 to 561 mμ and a smaller peak at 531 mμ. Each of these peaks may be split into two, as is indicated by the lower two spectra in Figure 1. When the difference in spectra between the cysteine-reduced and the DPNH-reduced preparation is plotted, then a major peak at 562 to 563 mμ and a very small peak at approximately 533 mμ appear. Presumably this represents cytochrome *b* which has been reduced enzymatically. Further reduction of the preparation by dithionite reveals a difference spectrum (dithionite-reduced minus DPNH-reduced) which shows peaks at 558 to 560 mμ and at 530 mμ. The Soret region was also investigated with the sucrose preparations, and it behaved in a manner consistent with the presence of cytochromes $a + a_3$, b, and c and possibly c_1.

The content of cytochrome c_1 in brown adipose tissue relative to the amounts of the other cytochromes was measured in the type of experiment the results of which are illustrated in Figure 2. In these experiments an aliquot of a tissue homogenate was taken for chromatographic isolation and determination of cytochrome *c*. The remainder of the homogenate was used for determination of cytochrome c_1. Both determinations were carried out as described under Experimental Procedures. The absorption spectrum of the cytochrome *c* eluted from the chromatographic column and reduced by dithionite is shown in Figure 2. The main peak

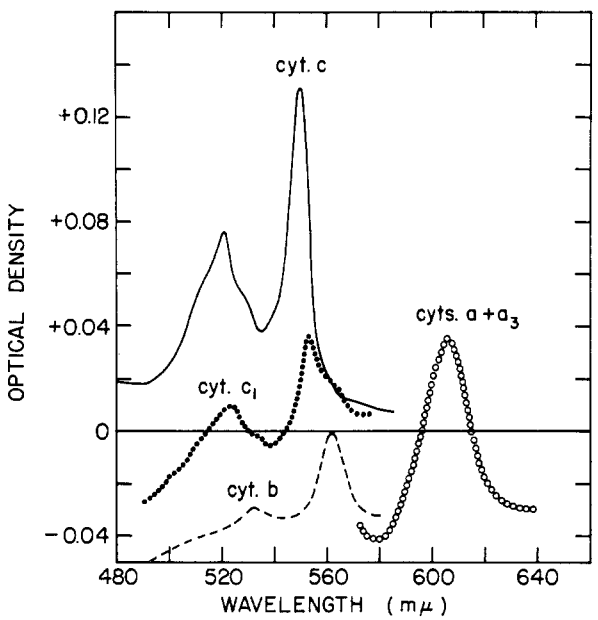

FIG. 2.—Absorption spectra of suspension of cytochrome *c*-free particulate fraction of brown adipose tissue in buffered concentrated sucrose solution and of extracted cytochrome *c*. The spectrum for cytochrome *c* is the absolute absorption spectrum obtained after application of the method described in the text for the chromatographic determination of cytochrome *c*. The spectra corresponding to each of the other cytochromes are the following difference spectra: cytochromes $a + a_3$: dithionite-reduced minus oxidized; cytochrome c_1: ascorbate-reduced minus oxidized; cytochrome *b*: DPNH-reduced minus ascorbate-reduced. In the experiment illustrated, each ml of the final suspension in concentrated sucrose solution contained the material derived from 189 mg fresh tissue, and the curve for cytochrome *c* has been corrected to this concentration of tissue. The rats used for the experiment shown ranged in body weight from 210 to 235 g, with a mean of 225 g. For further details see the text.

is at 550 mμ, with a secondary peak at 521 mμ, and the shape of the spectrum closely resembles that given for highly purified cytochrome *c* by Margoliash and Frohwirt (1959). The difference spectrum between the ascorbate-reduced and the oxidized preparations shows the typical cytochrome c_1 peaks at 553 mμ and at 523 to 524 mμ (Ball and Cooper, 1957; Estabrook, 1958; Green *et al.*, 1959). Assuming that the molar extinction coefficient of the 553 mμ peak of cytochrome c_1 (Green *et al.*, 1959) is approximately equal to that of the 550 mμ peak of cytochrome *c* (Margoliash and Frohwirt, 1959), it could be seen from absorption spectra such as that illustrated in Figure 2 that the molar ratio of cytochrome c_1 to cytochrome *c* was between 0.3 and 0.4 in several preparations. A small but negligible amount of interfering absorption, possibly due to cytochrome *b*, in the region of 560 to 565 mμ is also evident. Owing presumably to the nearly complete removal of cytochrome *c* from the preparation, cytochromes $a + a_3$ were only incompletely and very slowly reduced under anaerobic conditions with either ascorbate or DPNH in this type of experiment. A measure of the cytochrome $a + a_3$ content was therefore obtained by plotting the difference in the spectra of the dithionite-reduced and the oxidized preparations. The difference in the spectra of the DPNH-reduced and the ascorbate-reduced preparations shows peaks at 562 mμ and 532 to 533 mμ, characteristic of cytochrome *b*.

Spectral studies were also carried out on preparations in which the total cytochromes of the hemoglobin-free particulate fraction were solubilized by the method of Ball (1956) with 2 or 3% sodium deoxycholate in 0.05 M glycylglycine buffer (final *p*H = 7.4). Both the visible and the Soret regions were investigated. Although the results were not as readily reproducible as in the case of the suspensions in concentrated sucrose solution, the data from both types of studies were in good agreement.

Determination of Cytochrome c Content.—Cytochrome *c* was isolated from the brown adipose tissue of six different batches of rats by the chromatographic procedure described above. Spectrophotometric assay of the eluates yielded a value of 2.30 ± 0.22 (standard deviation) mg of cytochrome *c* per g of lipid-free dry weight of brown adipose tissue. On a lipid-free dry-weight basis, the cytochrome *c* content did not appear to vary appreciably with rat weight over a range of animal weights from 200 to 400 g.

Ubiquinone Content.—Absorption spectra of the unsaponifiable material from the particulate fraction of brown adipose tissue from a representative experiment are shown in Figure 3. The curve for the untreated material, in which the ubiquinone is in the oxidized state, shows a peak at 275 mμ. After reduction by KBH_4 the major peak is found at 288 to 290 mμ. It can be seen that the difference spectrum between the oxidized state and that following reduction by KBH_4 for the unsaponifiable material is almost exactly superimposable upon that calculated from the data of Lester *et al.* (1959) for the same molar concentration of pure ubiquinone-45. Values for ubiquinone contents from three experiments are shown in Table I. Aliquots of the same original tissue homogenates used for the ubiquinone assay were taken for the determination of cytochrome *c* by the procedure described above. The values shown also in Table I indicate that there are approximately 6 moles of ubiquinone per mole of cytochrome *c* in the particu-

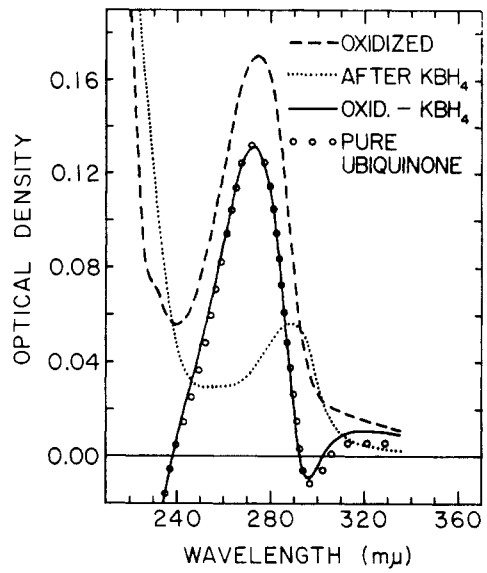

FIG. 3.—Absorption spectra of unsaponifiable material from particulate fraction of brown adipose tissue. The absorption spectrum taken after reduction by KBH_4 has been corrected for absorption due to KBH_4 itself. The difference spectrum between the oxidized state and following reduction by KBH_4 for the unsaponifiable fraction of brown adipose tissue is compared with the same type of difference spectrum calculated for the same molar concentration of pure ubiquinone-45 from the data of Lester *et al.* (1959). The solvent in all cases is absolute ethanol. In the experiment illustrated, each ml of the final preparation contained the material derived from 67 mg fresh tissue. The rats used for the experiment shown ranged in body weight from 305 to 365 g, with a mean of 343 g. For further details see the text.

late fraction of brown adipose tissue. This ratio does not appear to vary markedly with the age and size of the rats over the range studied.

From the data in Table I it can be seen that an additional amount of ubiquinone was found in the nearly colorless fat layer which separates to the top during centrifugation of the original tissue homogenate. In each of the three experiments the total amount of ubiquinone found in the fat layer was slightly greater than that found in the particulate fraction. There was no suggestion of any strong positive correlation between the fat content of the tissue and the fraction of the total ubiquinone of the tissue found in the fat layer.

Chain Length of Ubiquinone.—The number of isoprenoid units in the side-chain of the ubiquinone of the particulate fraction and of the fat layer was determined by the paper chromatographic method of Lester and Ramasarma (1959). The unsaponifiable fraction was prepared as described above and aliquots of it in *n*-heptane solution estimated to contain 30 to 50 μg ubiquinone were applied to Whatman No. 1 filter paper. Spots with a similar quantity of authentic ubiquinone-50 were applied to the same papers. The solvent system for the ascending chromatography was 4:1 (v/v) i-propanol–water. The resulting spots were then made visible by reaction with aqueous $KMnO_4$. The R_F value of the authentic ubiquinone-50 was not altered by subjecting it to the saponification procedure.

An average R_F value of 0.36 was obtained for the authentic sample of ubiquinone-50. The average R_F value for the ubiquinone of either the particulate fraction or the fat layer was 0.45. Thus the ratio of the R_F value of the adipose tissue ubiquinone to that of the authentic ubiquinone-50 was 1.25. Lester and Ramasarma (1959) reported R_F values of 0.27 and 0.36 for ubiquinone-50 and ubiquinone-45, respectively; these values yield a corresponding ratio of 1.33. Since Lester and Ramasarma found even higher R_F values for ubiquinone homoloques having yet shorter isoprenoid side-chains, we conclude that the ubiquinone of brown adipose tissue contains nine isoprenoid units in its side-chain. It has been shown by others (Gloor and Wiss, 1960; Lawson *et al.*, 1960; Olson and Dialameh, 1960) that the rat is unusual among mammals insofar as the ubiquinone of its liver is primarily ubiquinone-45 rather than the usual ubiquinone-50.

DISCUSSION

Ruska and Quast (1935) suggested that the color of brown adipose tissue might be due to the presence of heme-containing compounds, but these workers were unable to demonstrate the presence of iron by microanalysis. A qualitative demonstration of the presence of cytochrome *c* in the brown adipose tissue of the ground squirrel was reported by Hook and Barron (1941). Observations we have made on the intact tissue with a hand spectroscope clearly revealed the absorption bands attributable to the reduced forms of cytochromes $a + a_3$, b, and $c + c_1$. The experiments reported in the present paper provide evidence to indicate that the characteristic brown color is due primarily to a very high content of cytochromes

TABLE I
UBIQUINONE, CYTOCHROME *c*, AND TOTAL LIPID CONTENTS OF BROWN ADIPOSE TISSUE

	Experiment 1	Experiment 2	Experiment 3
Ubiquinone in particles (mμmoles/g intact tissue)	137	155	118
Cytochrome *c* in particles (mμmoles/g intact tissue)	24.0	21.8	21.3
Moles ubiquinone in particles/mole cytochrome *c*	5.7	7.1	5.5
Ubiquinone in fat layer (mμmoles/g intact tissue)	190	170	126
Mg total lipid/100 mg intact tissue	40.5	44.4	50.1
Mg lipid-free dry weight/100 mg intact tissue	13.2	12.6	11.0
Mean and range of rat weights (g)	217 (193–226)	343 (305–365)	432.5 (405–485)
No. of animals	11	8	8

in this tissue. When the tissue is homogenized in 0.25 M sucrose solution and centrifuged in the manner used for sedimentation of mitochondria, the brown color is found to be associated with the sedimented fraction. Examination of a suspension of this particulate fraction in the Beckman spectrophotometer either before or after addition of reducing agents revealed no major absorption peaks in the visible region other than those attributable to cytochromes. The brown color can be extracted from the particulate fraction by a buffered 2 or 3% sodium deoxycholate solution in a manner previously used to solubilize the cytochromes of other tissues (Ball, 1956; Spiro and Ball, 1961a,b). Examination of this extract also reveals that cytochromes comprise the bulk of the pigmented material. A portion of the color of this tissue *in vivo* is undoubtedly attributable to hemoglobin, since this tissue has a rich blood supply (*cf.* Johansson, 1959).

The above conclusions are supported by the finding that the cytochrome *c* content of brown adipose tissue is as high as that found for any other tissue in the rat. Application of the procedure described above for the chromatographic separation and spectrophotometric measurement of cytochrome *c* yielded a value of 2.30 ± 0.22 mg per g of lipid-free dry weight of brown adipose tissue. The tissue of the rat previously thought to be the richest with respect to cytochrome *c* appears to be heart muscle, for which Crandall and Drabkin (1946) reported a content of 1.94 mg per g of total dry weight. The high cytochrome content of brown adipose tissue is in accord with reports of an extremely high content of mitochondria with closely and regularly spaced cristae in this tissue (Lever, 1957; Ekholm, 1957, 1958; Napolitano and Fawcett, 1958).

The high cytochrome content of brown adipose tissue correlates well with the high rates of oxygen consumption observed for this tissue. Hook and Barron (1941) found rates of oxygen uptake ranging from 98 to 350 μl O_2 per 100 mg fresh weight per hour in slices of brown adipose tissue from the ground squirrel, and a rate of 390 μl O_2 per mg fresh weight per hour can be calculated from the data of Beloff-Chain *et al.* (1959) for homogenates of rat brown adipose tissue fortified with adenosine triphosphate, DPN, and glucose. Preliminary experiments carried out in this laboratory in collaboration with S. E. Shackney have shown that a combination of epinephrine and insulin added *in vitro* causes a three- to four-fold stimulation of the rate of oxygen consumption of small pieces of intact brown adipose tissue from rats weighing between 150 and 200 g, the stimulation resulting consistently in values of around 400 to 500 μl O_2 per 100 mg fresh weight per hour (35 to 40 μl O_2 per mg lipid-free dry weight per hour). The respiratory rate of brown adipose tissue is thus as high as that reported for any other tissue in the rat, and is comparable, on a lipid-free dry-weight basis, to the extremely high rates of oxygen consumption observed in white adipose tissue (*e.g.*, Jungas and Ball, 1960). The physiologic significance of this unusually large capacity for energy production exhibited by brown adipose tissue remains to be elucidated.

From a consideration of the relative heights of the peaks due to the various cytochromes (Fig. 1 and 2), it is evident that the ratios of one cytochrome to another in the particulate matter of brown adipose tissue are not strikingly different from such ratios for mitochondria of tissues such as heart muscle or liver (Chance, 1952; Chance and Williams, 1955). The amounts of cytochromes relative to one another observed in brown adipose tissue did not change markedly with age in rats weighing from 200 to 450 g. The cytochrome content expressed per unit weight of intact tissue tended to decrease with increasing age, but on a lipid-free dry-weight basis the cytochrome content did not appear to alter significantly over this weight range.

The nature of the material (or materials) responsible for the peaks at 530 mμ and 558 to 560 mμ, which appear only after reduction with dithionite (*cf.* Fig. 1), is not clear. The material was not reduced by agents which are known to reduce enzymatically the microsomal cytochrome of liver with a major peak at 557 mμ (Strittmatter and Ball, 1952) or the recently described microsomal cytochrome of the adrenal medulla with a major peak at 559 mμ (Spiro and Ball, 1961a). The absence of an observable microsomal-type cytochrome in brown adipose tissue correlates well with the paucity of endoplasmic reticulum in this tissue as revealed by the electron microscope (Lever, 1957; Ekholm, 1957, 1958; Napolitano and Fawcett, 1958). Other workers (Chance and Williams, 1955; Devlin, 1959; Holton and Colpa-Boonstra, 1960; Spiro and Ball, 1961b) have observed a difference in the intensity of the absorption maximum in the region around 560 mμ depending upon whether succinate or dithionite was used as a reducing agent. These observations along with those presented here raise the possibility that more than one component is responsible for the absorption usually assigned to cytochrome *b* in this region. If this is indeed the case then the rough estimate of −0.04 v for the oxidation-reduction potential of the cytochrome *b* system made by Ball (1938) from observations on a Keilin-Hartree type preparation of heart muscle may be in error. This estimate was based upon the assumption that only cytochrome *b* contributed to the absorption seen in the presence of dithionite. This value may therefore represent roughly the end-point between the cytochrome *b* system and some other component present in nearly equal amounts. Feldman and Wainio (1960) have recently estimated the potential of cytochrome *b* isolated from mammalian sources as lying in the range of +0.060 to +0.078 v, and Holton and Colpa-Boonstra (1960) give values lying between +0.064 and +0.089 v. Since these values are some 0.1 v above the value esti-

mated by Ball, it may be that the second component forms a system with a potential 0.1 v below −0.04 v or −0.14 v. This would be a value lying near that of the potential of the liver microsomal hemochromogen as estimated by Strittmatter and Ball (1952).

Ubiquinone in Brown Adipose Tissue.—The finding of a six-fold molar excess of ubiquinone over cytochrome *c* in the particulate fraction of brown adipose tissue (Table I) is reminiscent of the similar situation earlier pointed out by Joel *et al.* (1958) for the electron transmitter system of beef heart muscle. The other components of the electron transfer chain of the mitochondrial membrane occur, however, in closer to one-to-one molar ratios (*cf.* Ball, 1956; Devlin, 1959; Ball and Joel (1962). The meaning of this high ratio of ubiquinone to the cytochromes is not clear at this time; but certainly any full explanation of the participation of ubiquinone in the electron transmitter system and in oxidative phosphorylation, if it indeed so participates, must be able to account for this large excess of ubiquinone over the other electron carriers.

The significance of the *additional* amount of ubiquinone found in the fat layer which separates to the top during centrifugation of the original tissue homogenate (see Table I) is not clear. Its presence is not explainable merely on the basis of contamination of the fat layer by mitochondria, since the fat layer is nearly colorless, and considerable color would be imparted to it by the amount of mitochondria which would be required in order to account for the amount of ubiquinone present. Two possibilities appear open for the origin of the ubiquinone found in the fat layer: either ubiquinone may occur *in vivo* in the fat droplets of brown adipose tissue, or on the other hand ubiquinone may have been removed from the mitochondria by a solvent action of the fat droplets during homogenization of the tissue, just as ubiquinone can be extracted from the mitochondria of other tissues by common nonpolar solvents (*e.g.*, Crane *et al.*, 1957; Pumphrey *et al.*, 1958; Crane *et al.*, 1959b). If this latter possibility is the case, then it can be seen from the data of Table I that the mitochondria *in vivo* would have had 13 moles of ubiquinone per mole of cytochrome *c*.

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