Spectrophotometric Studies on Intact Muscle

I. *Components of the respiratory chain*

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ABSTRACT The concentrations of the components of the respiratory chain were determined in a variety of intact skeletal muscles by a method of spectrophotometric observation of the transmitted light. In the case of the toad sartorius, these measurements were checked against isolated mitochondrial suspensions prepared from toad skeletal muscles. The relative concentrations of the respiratory components were found to be in reasonable agreement with those of various mitochondrial preparations of mammalian tissues and of the ones from toad skeletal muscle. The rather low cytochrome *b* and pyridine nucleotide levels in the anoxic minus oxygenated difference spectra were shown to be caused to a certain degree by a partial reduction during the resting steady state. Upon treatment with a strong, reducing agent or after long anoxia some absorption bands appeared with maxima at 591, 562 to 564, and 432 to 434 mμ both in the intact and in the mitochondrial fractions of muscle tissue; they do not appear to be associated with the respiratory chain.

INTRODUCTION

Optic, optical, spectroscopic, and spectrophotometric observations on contracting muscles have received the careful attention of physiologists over the course of the years. These investigations can be divided roughly into two classes. In the first of these the aim was the determination of the optical properties of muscle and conclusions were sought about the physical events occurring during the contraction phase. In many the specific goal was insight into the structural changes taking place, especially the rearrangement of the striations in skeletal muscle. Recent progress in this field has been made especially by the studies of Sandow (1936), Buchthal and Knappeis (1940), D. K. Hill (1949, 1953), and Huxley and Taylor (1958).

The second class includes investigations employing optical or spectrophotometric techniques for the determination of changes in some indicator sub-

stance within the muscle. These studies were aimed at providing information about the chemical events taking place in the tissue. The present work falls in this category. It is specifically aimed at providing new information about the energetics of muscular contraction from observation of the kinetics of the recovery metabolism.

For convenience, the published work can be subdivided according to the part of the spectrum used: infrared, visible, or ultraviolet. The infrared was explored by Wood (1951), who observed some differences between resting muscles and muscles recuperating from extensive contractile activity. The identity of the substances undergoing changes in concentration, as witnessed by changes in absorption at various wavelengths, was not ascertained.

Extensive pioneering work in the ultraviolet region was performed by D. K. Hill. The problems of instrumentation, interpretation, and reproducibility of the results were, however, of such a nature that publication appeared to him to be unwarranted (personal communication). Wajzer, Weber, Lerique, and Nekhorocheff (1956) published records without scales for the ordinates and abscissae, which makes it hazardous to draw conclusions from them at the present time.

The spectroscopy of the visible region has received more attention and has yielded information on the occurrence and participation in metabolism of various substances, especially hemoproteins. The rediscovery and the first explanation of the function of the cytochromes by Keilin (1925) rested, as a matter of fact, for a good part on observations on the flight muscles of insects. Millikan (1937, 1939) was the first to employ the absorption changes of myoglobin as an indicator of the moment of incipient hypoxia and of the subsequent rates of oxygen consumption in muscles at rest, during the contractile activity, and during recovery. Urban and Peugnet (1938) reported changes in absorption at approximately 550 mμ in contracting muscles. Their interpretation of these data as responses of cytochrome *c* appears, however, to be doubtful in view of the band width of their filters and the necessarily limited electronic instrumentation at their disposal.

The substances naturally occurring in muscle tissue which are useful as indicators for the monitoring of metabolic activities can be divided into three categories: those that can be used as an index to the O_2 concentration in the tissue (myoglobin and hemoglobin), those that provide an index for the rate of oxidative activity (members of the respiratory chain), and diphosphopyridine nucleotide (DPN) which under certain circumstances also reflects the activity of the glycolytic chain. Observations on all these indicator substances in excised muscle tissue have been made in recent years at this laboratory (Connelly and Chance, 1954; Chance and Connelly, 1957; Jöbsis and Chance, 1957; Weber, 1957; Lübbers and Jöbsis, 1957; Jöbsis, Ramirez, and

Lübbers, 1958; Jöbsis, 1959; Ramirez, 1959; for reviews see Chance, 1959; Chance, Mauriello, and Aubert, 1962).

The present work was undertaken to study the energy restitution processes of intact muscles mainly by the observation of the kinetics of the respiratory chain during and after contractile activity. In this, the first paper, it will be attempted to lay the foundation for this study by determining the concentrations and steady-state reduction-oxidation levels during resting metabolism of the members of the respiratory chain. Some observations on isolated mitochondria, low temperature spectra of muscle tissue, and the occurrence of unidentified absorption peaks are added. Although most of the work on kinetics was performed on toad and frog muscle, some data on cytochrome concentrations in a variety of other muscles are included for comparative purposes.

METHODS

I. *Instrumentation*

The spectrophotometric equipment used is of two types: the "split beam" and the "double beam." Both instruments have been described before (for the split beam see Yang and Legallais, 1954, and Yang, 1954; for the double beam see Chance, 1951, 1954). A detailed description is, therefore, not necessary, but the essential features of the first instrument, which was used in this part of the study, are the following:

In the split beam the light from one monochromator falls upon a vibrating mirror, which deflects the light alternately to two samples. The light emerging from the two samples falls on the same photomultiplier and the difference in the intensities of the two pulses is recorded as a function of the wave length, either in per cent absorption or in optical density (OD). The resulting graph is a spectrum of the differences in absorption between the two samples. Keeping one sample as a reference, the other is subjected to various experimental treatment and the differences in absorption are recorded.

For the examination of intact muscle a holder was designed for the recording of differential absorption spectra (Fig. 1). It accommodates two muscles, each in its own separate chamber. Essentially it constitutes an improvement over the one used by Chance and Connelly (1957) and by Weber (1957).

The holder is constructed in two parts, one providing for the attachment of the muscles, the other forming the two cavities of the separate chambers. This second part contains channels for the bubbling of gases and the circulation of the Ringer's solution and others for the circulation of cooling water. The two parts are placed together to form the separate, closed chambers.

The muscles are held in a vertical position against a gently curved surface of nylon netting. They pass under two lucite beams at the ends of the curved netting. This arrangement permits relatively little movement of the muscles in the optical field, while it insures their return nearly to the same position after contraction. Each of

the beams contains a cathode for stimulation, the anode lying on the other side of the muscle in the second part of the cell. The space behind the netting is open to the main part of the chamber to allow diffusion into the muscle from the Ringer's solution on both sides.

The muscles are securely attached at the bottom; the thread at the other end can either be tied to the frame or passed to an external device for the recording of mechanical events.

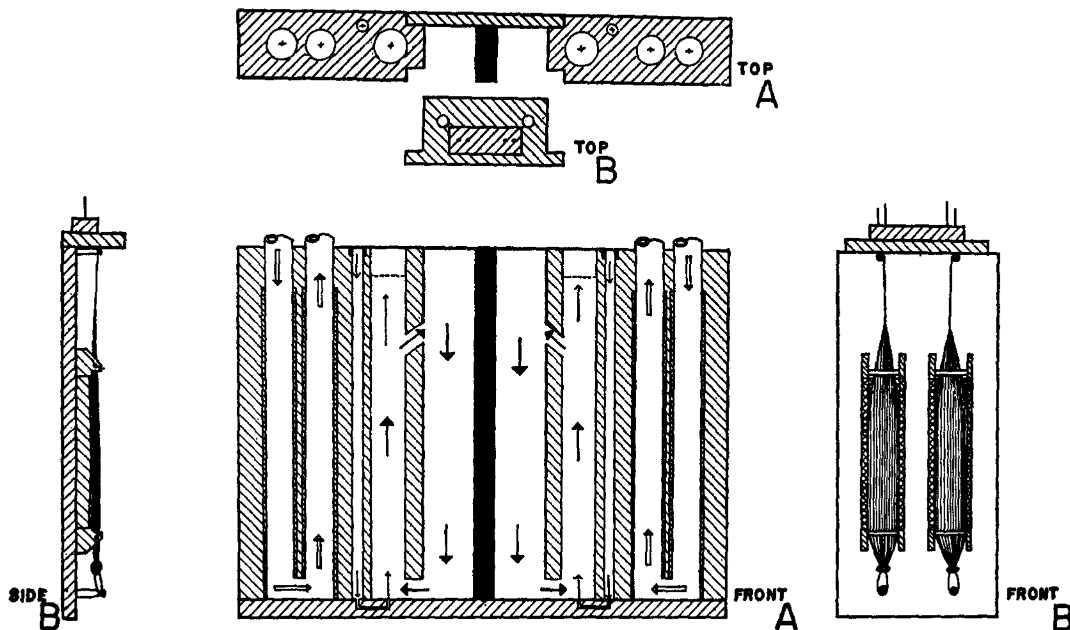


FIGURE 1. The muscle holder for use with the split beam, recording spectrophotometer. For description, see text. Heavily hatched, black acrylic plastic; lightly hatched, clear plastic (plexiglas). Solid arrows show the stream pattern of the Ringer's, line arrows, the gas flow and double line arrows, the flow of the cooling water.

For the measurements at low temperatures, muscles of appropriate thickness were introduced in cuvettes with a 1 mm optical path. The samples were frozen by immersion in liquid N_2 and the spectra taken after withdrawal from nitrogen (see Estabrook, 1956).

II. *Biological Materials*

Sartorius muscles of the frog (*Rana pipiens*), the bullfrog (*Rana catesbiana*), and the tropical toad (*Bufo marinus*), the coraco-hyoideus and the retractor capitis collique muscles of red-eared and painted turtles (*Pseudemys elegans* and *Chrysemys picta*), and the serratus ventralis and gracilis of the rabbit (New Zealand white) were used.

The anuran sartorius preparation is well known and does not need further discussion. The turtle's coraco-hyoideus muscle originates on the coracoid process of

the scapula, and inserts on the hyoid bone. In the larger turtles it is up to 5 cm long and 4 mm wide with a thickness of about 1.5 mm. It can rather easily be dissected out in intact form with small pieces of bone on each end which serve as mechanical attachments. The main direction of the fibers is parallel, but they do not run the full length of the preparation.

The serratus ventralis of the rabbit inserts on the scapula and originates on the ventral surface of the ribs. This muscle consists of several parallel bundles, each originating on one rib. A bundle can be separated from the others and dissected free with a fragment of bone on each side. The various bundles are graded in length and width. Thus one of the proper size can be selected for study. The thickness runs from 0.7 to 2 mm. The gracilis has a broad origin on the pubic symphysis and a narrower insertion on the fascia around the knee. It is trapezoidal in shape, with parallel fibers, and varies in thickness from about 3 mm at the anterior end to about 1 mm posteriorly. Strips of the right length and thickness can also be obtained here.

The physiological salt (Ringer's) solution used for both the amphibian and the reptilian muscles contained NaCl, 113 mM; KCl, 5 mM; $CaCl_2$, 2 mM; phosphate buffer, 2 mM (pH 7.0). The amounts of K^+ and Ca^{++} are slightly higher than those of standard Ringer's. This modification is the result of a long series of experiments on the effect of the ratios and the absolute amounts of these two as well as other ions (Mg^{++}, SO_4^{--}, HCO_3^-) upon the survival of excised muscle, as measured by its tetanic tension (Jöbsis, 1955, unpublished data).

For mammalian tissue either phosphate- or bicarbonate-CO_2-buffered mammalian Ringer's solutions were used. The phosphate solution contained NaCl, 139 mM; KCl, 2.5 mM; $CaCl_2$, 1.0 mM; $MgSO_4$, 1.0 mM, and phosphate buffer, 2 mM (pH 7.4). For the bicarbonate buffer 25 mM $NaHCO_3$ + 5 per cent CO_2 (pH 7.4) was substituted for the phosphate and the NaCl was decreased by 22 mM.

Preparation of the material consisted usually in the perfusion and subsequent excision of the desired muscle. Twenty minutes before killing, the animal was given a subcutaneous injection of heparin (5 mg/kg body weight) to prevent formation of blood clots during the subsequent operations. The animal was killed by pithing (or decapitation in the case of rabbits), and the body cavity was opened. A small cannula was inserted in an appropriate blood vessel and Ringer's solution was run through under a small pressure head for 30 to 45 minutes.

The muscles were subsequently excised and carefully mounted in the muscle holder. They were then left to equilibrate for 2 to 3 hours at room temperature in well oxygenated Ringer's (95 or 100 per cent O_2).

Mitochondrial suspensions were prepared from skeletal muscles of the tropical toad.[1] The animals were killed by decapitation. The muscles of the hindlegs were cut off, finely diced with scissors in ice cold, 0.25 M sucrose, and homogenized in a Waring blendor or ground with sea sand (Merck) in small aliquots. (The sand was first washed 3 times with sucrose solution to remove traces of acid.) The resulting brei was diluted to approximately 10 times its volume, passed through 8 layers of cheese-cloth, and the "filtrate" was spun successively at 500, 5000, and 13000 $\times g$ (10 minutes each) in a refrigerated centrifuge. The main mitochondrial fraction, as

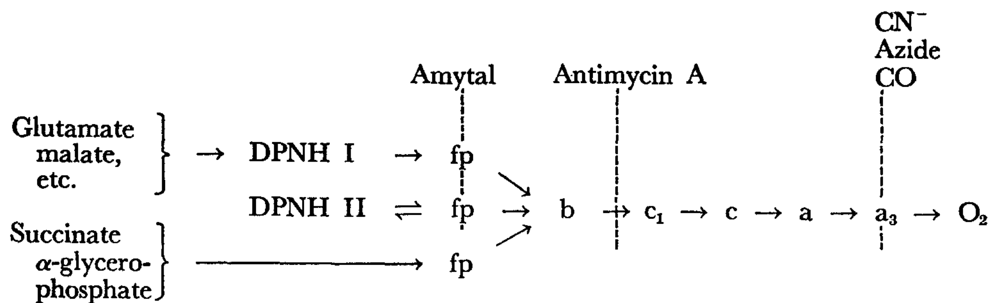
[1] Many thanks are due Mrs. Karen Meadows for assistance in the preparation of the mitochondria.

identified by the cytochrome content and Q_{O_2}, settled at 5000 × g. The resulting pellets were resuspended and washed once, centrifuged again, and suspended in a minimal amount of sucrose solution. At times, 1 mM versene was added to the sucrose solution, without noticeable effect on yield or cytochrome content. For spectrophotometric observations the preparation was diluted with a solution containing 20 mM KCl, 180 mM sucrose, and 14 mM sodium phosphate buffer (pH 7.0).

RESULTS

I. *Spectra of Intact Muscles at Room Temperature*

Satisfactory anoxic – oxygenated difference spectra of the respiratory chain can only be obtained from muscles devoid of myoglobin and from which the hemoglobin has been carefully washed out. In Fig. 2 such a spectrum of two toad sartorii is given. The various peaks are identified as follows: cytochromes $(a + a_3)_\alpha$ 605 mμ, cytochrome b_α 564 mμ, cytochromes $(c + c_1)_\alpha$ 550 mμ, cytochrome $(c + c_1 + b)_\beta$ 520 mμ, cytochrome $(a + a_3)_\gamma$ 445 mμ, cytochrome c_γ 419 mμ, DPNH 340 mμ. The slight trough with a minimum at 465 mμ is attributed to reduction of flavoproteins (which absorb less in the reduced than in the oxidized form), and the one at 405 is caused by the absorption of the oxidized cytochromes in the reference muscle (see Chance and Williams, 1955). The sequence of these respiratory components is the following (Keilin, 1925; see Chance and Williams, 1956):



The arrows indicate the flow of electrons from the substrates to oxygen, their direction is reversible under specific conditions. The second DPNH fraction appears to receive reducing equivalents only *via* the flavoproteins, rather than directly from the substrates (Chance and Hollunger, 1961). In the present work both fractions probably become reduced during anoxia. (The points of inhibition by cyanide, azide, CO, antimycin A, and amytal (amobarbital) are indicated by the broken lines.)

Several features of this difference spectrum stand out in comparison with similar ones of mammalian liver mitochondria or heart mitochondria (Chance

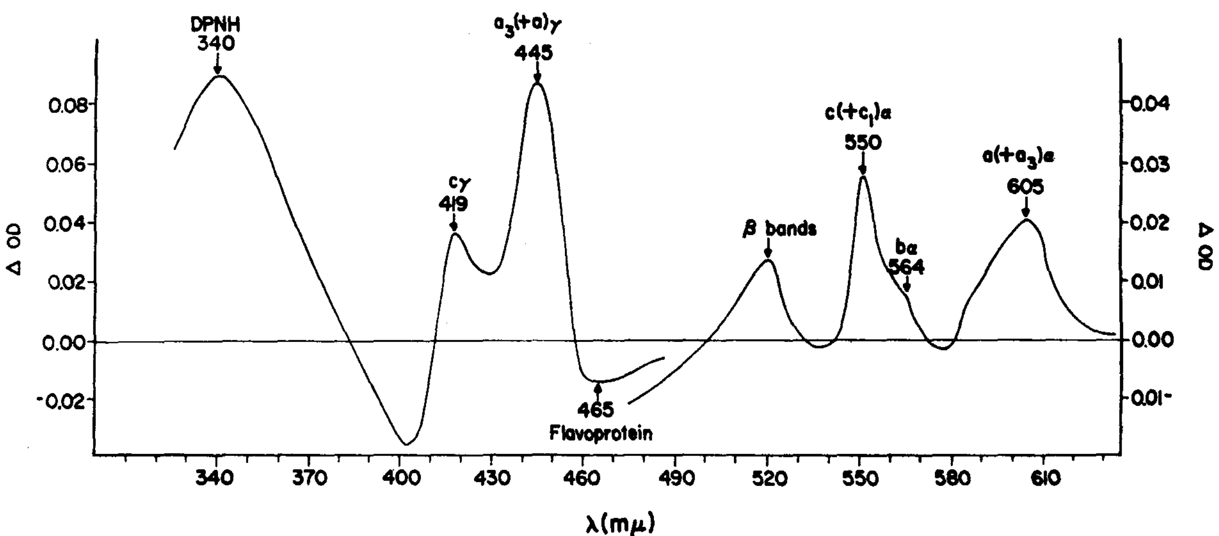


FIGURE 2. Spectrum of the differences between an anoxic and an oxygenated toad sartorius muscle. Anoxia caused by bubbling N_2 through the sample compartment and aided by increasing the respiration of the sample muscle by a few contractions. Reference muscle is oxygenated (100 per cent O_2) and in the resting state. Temperature 20°C, both muscles in circulating Ringer's solution. Ordinate, optical density increment (note separate scales for right and left parts of the curve), abscissa, wave length in mμ.

and Williams, 1955; Chance and Baltscheffsky, 1958). These are the relatively small size of the cytochrome b_α and DPNH peaks and of the flavoprotein trough, the absence of a discernible cytochrome b_γ peak at 430 mμ, and the sharpness of the cytochrome $(c + c_1)_\alpha$ band which occurs at 550 rather than at 551 mμ. These differences were also noted in similar spectra of other muscles.

To get a quantitative expression of the concentrations of the components and of these observed differences, the amounts of the various cytochromes reduced by cyanide, anoxic conditions, or sodium hydrosulfite ($Na_2S_2O_4$) were calculated from the spectra. This was done by measuring the optical density changes at selected wave length pairs (one pair for each component)

TABLE I

EXTINCTION COEFFICIENT (E) IN mMOLE $L^{-1}CM^{-1}$ AT SELECTED WAVE LENGTH PAIRS FOR MEMBERS OF THE RESPIRATORY CHAIN

(Reference muscle aerobic)

Component	Condition	$\lambda_1 - \lambda_2$	E	Reference
		mμ		
Cyt a_3	Anoxia	445–455	60	Chance and Williams, 1955
Cyt a_3	Anoxia	445–465	91	Chance and Williams, 1955
Cyt a	Anoxia	605–630	16	Chance and Williams, 1955
Cyt a	Anoxia	605–590	9.3	Yonetani, 1960
Cyt a	CN^- inhibition	605–630	11.6	Yonetani, 1960
Cyt a	CN^- inhibition	605–590	7.1	Yonetani, 1960
Cyt $c + c_1$	Anoxia; CN^-	550–540	19	Chance and Williams, 1955
Cyt b	Anoxia; CN^-	564–575	20	Chance and Williams, 1955
Flavoprotein	Anoxia; CN^-	465–510	11	Chance and Williams, 1955
DPNH	Anoxia; CN^-	340–374	6	Chance and Williams, 1955

and applying the appropriate extinction coefficient (Chance and Williams, 1955). These sets of wave lengths are given in Table I with the extinction coefficient per millimole per liter per centimeter optical path.

In the presence of myoglobin (in this study in the turtle coraco-hyoideus and the two rabbit muscles) or hemoglobin the oxidized respiratory chain components can be reduced for assay by treatment with cyanide or azide. The exception is cytochrome a_3 which forms a complex with these inhibitors without a distinct absorption maximum (Yonetani, 1960) making it impossible to determine its concentration in this manner.

An alternative method for the circumvention of the interference of strong myoglobin bands would, in principle, be treatment with sodium nitrite, which converts myoglobin into metmyoglobin. This compound is unreactive with oxygen and, therefore, does not interfere with reduced – oxidized difference spectra. After washing out the nitrite, the respiratory chain components should then be assayed under anoxic conditions (see Lemberg and Legge, 1949; Chance, 1952). But during attempts at the anoxic reduction of the

respiratory chain after the elution of the nitrite, it was found in the turtle coraco-hyoideus that formation of myoglobin from metmyoglobin was taking place at a sufficiently rapid rate to interfere with these measurements. A similar observation was reported by Colpa-Boonstra (1959) and by Colpa-Boonstra and Minnaert (1959) for Keilin and Hartree heart muscle preparations in the presence of DPNH.

In Table II the concentrations of components reducible by treatment with cyanide (generally 2 mM; a concentration range of 1 to 6 mM was found to give identical results), by anoxia (exclusion of O_2 aided by an increased respiratory rate produced by a few contractions), and by reduction with $Na_2S_2O_4$, are listed for a number of muscles. The length of the optical path through the tissue was found from the weight, width, and length of the muscle in the optical field, assuming a density of 1.05. The concentrations were then calculated by application of the proper extinction coefficient.

The variability among specimens is shown in the column of the ranges of the cytochrome *a* contractions. The variation is maximally ± 28 per cent and usually larger than 20 per cent. The range of the concentrations of the other components relative to cytochrome *a* is much smaller except for DPNH. This last component varies typically ± 10 to 20 per cent, while the other four members of the respiratory chain show a range of variations usually less than 10 per cent. Thus a considerable amount of biological variation is evident in the absolute concentrations of the complement of respiratory enzymes. The composition of the chain, however, appears to be more rigidly regulated. The greater variability of the DPNH values may well originate in varying levels of available substrate (Jöbsis, 1963).

These spectra of intact muscles show only that amount of each component that was in the oxidized state in the resting muscle. For the accurate assessment of the entire concentration it would be necessary to have the chain completely oxidized in one sample and entirely reduced in the other. When working with isolated mitochondria the totally oxidized condition can be attained by the addition of ADP or an uncoupler to one sample in the absence of substrates. In the intact tissue, however, it is impossible to deplete the substrate stores reliably.

The blocking of electron transfer from DPNH to the flavoproteins by amytal, however, will effectively cut off the remainder of the chain from DPN-dependent substrates. Amytal does not interrupt the flow of electrons from succinate to cytochrome *b*. With its use it is only possible to calculate the concentration of cytochrome *b* not reduced by the electron flow from succinate. However, the assumption that the succinate concentration is low is probably justified, as amytal will inhibit α-ketoglutarate formation.

The lower curve of Fig. 3 shows the differences in absorption between an amytal-treated muscle and an untreated reference muscle. Amytal treatment

TABLE II

CONCENTRATION OF CYTOCHROME *a* AND OF OTHER RESPIRATORY CHAIN COMPONENTS RELATIVE TO CYTOCHROME *a* IN VARIOUS MUSCLES

Amounts reduced over the steady state in resting, aerobic muscles

Animal	Muscle	Treatment	No. of determinations	Cytochrome *a*		Concentration relative to cytochrome *a*				
				μM/kg	Range	a_3	$c + c_1$	*b*	fp	DPNH
Toad (*Bufo marinus*)	Sartorius	NaCN*	5	13.5	11.7–16.3	‡	0.86	0.21	0.76	7.0
		Anoxia§	3	12.0	10.4–13.5	0.87	1.1	0.36	1.5	9.2
		$Na_2S_2O_4$‖	4	13.7	10.8–15.0	0.91	1.2	0.45	1.6	‡
Leopard frog (*Rana pipiens*)	Sartorius	NaCN	3	3.2	2.9–3.7	‡	1.2	0.31	0.73	8.7
		Anoxia	3	3.4	3.0–4.2	0.89	1.5	0.65	1.3	12.5
Bullfrog (*Rana catesbiana*)	Sartorius	Anoxia	2	1.3	1.2–1.4	0.87	0.99	0.37	1.2	8.9
		$Na_2S_2O_4$	2	1.4	1.2–1.5	0.86	1.1	0.41	1.8	‡
Turtle (*Pseudemys elegans*)	Coraco-hyoideus	NaCN	4	6.5	4.1–7.6	‡	0.91	0.31	0.75	7.5
	Retractor capitis collique	NaCN	2	0.54	0.49–0.58	‡	1.2	?¶	?¶	8.1
Rabbit (New Zealand white)	Serratus ventralis	NaCN	3	3.0	3.5–2.8	‡	1.0	0.28	0.78	8.1
	Gracilis	NaCN	3	1.8	1.4–2.3	‡	0.93	0.25	0.74	8.3

* Usually 2×10^{-3} M.

‡ Not measurable under these conditions.

§ 10 to 20 minutes after cytochrome *c* had reached its maximal reduction in N_2 and after a few contractions were produced.

‖ 20 to 30 minutes after excess.

¶ No measurable amount.

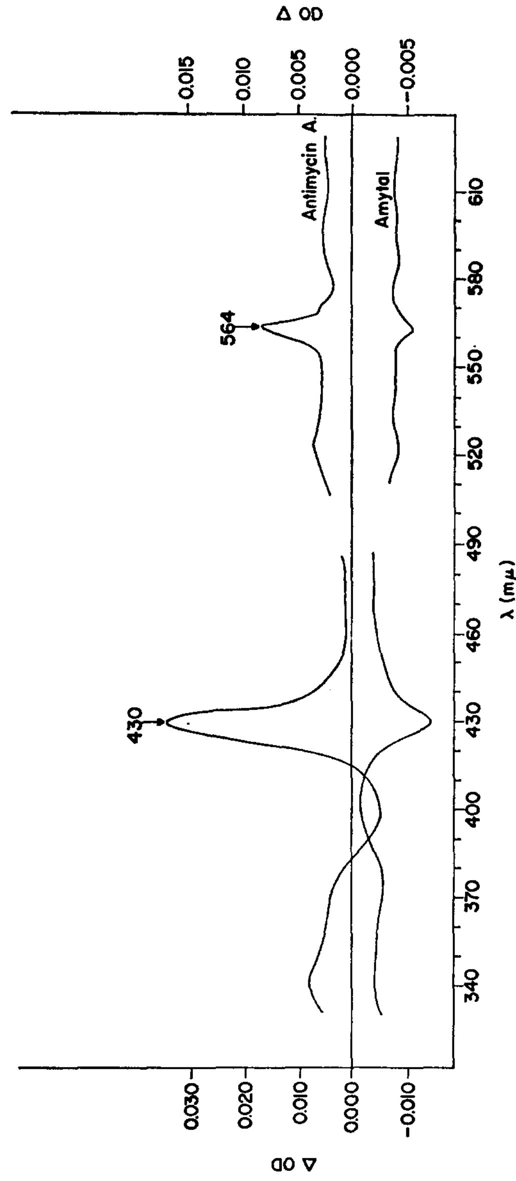


FIGURE 3. Difference spectra of toad sartorius muscles treated with antimycin A (100 μg/ml) or amytal (2 mM). Reference muscles in the oxygenated, resting state. Temperature 22°C.

results in the appearance of troughs at 564 and 430 mμ and sometimes a small extra absorption at 465 mμ, indicating the oxidation of cytochrome *b* and flavoprotein respectively. The average amount of *b* oxidized in this way was 0.80 μmole/kg in the toad sartorius (6 experiments), 0.29 in the frog sartorius (3 experiments), and 0.45 in the turtle coraco-hyoideus (4 experiments). Adding these amounts to those found by the inhibition by NaCN, we find, respectively, 3.8, 1.3, and 2.5 μmoles/kg for the total assessable cytochrome *b* concentrations. It follows that cytochrome *b* is at least 21, 22, and 18 per cent reduced in the tissues at rest, and that the concentrations relative to cytochrome *a* are at least 0.27, 0.40, and 0.38 for the toad sartorius, frog sartorius, and turtle coraco-hyoideus respectively. The amounts of flavoprotein occasionally found to be oxidized by amytal are negligible in calculations of the flavoprotein concentration.

The absence of troughs at 605, 550, 445, or 419 mμ indicates that a, a_3, and $c + c_1$ are not measurably reduced by the electron flow from DPNH during resting metabolism. Otherwise, the use of amytal should have produced a sizable oxidation of these even if the flow of electrons from succinate were considerable.

Antimycin A, which stops the electron flow from b to c_1, also proved to be effective in whole muscle tissue. The typical absorption spectrum of reduced cytochrome *b* appeared after its addition to the sample muscle (Fig. 3, upper curve). The concentrations of *b* assessed in this way were 2.45 μmoles/kg (3 experiments) and 2.31 μmoles/kg (1 experiment) for the toad sartorius and turtle coraco-hyoideus respectively. These amounts are quite in agreement with the concentrations found by inhibition with cyanide.

If we record the difference spectrum anoxic *vs.* amytal, we obtain a spectrum which resembles the anoxic-oxygenated difference spectrum of isolated mitochondria in the absence of substrate. A considerably greater reduction of DPN by anoxia over that by amytal is usually observed and at the moment goes unexplained. The absorption by cytochrome *b* at 430 mμ has now become noticeable, as could be expected after the oxidation of cytochrome *b* in the amytal-treated muscle serving as reference.

Upon perusal of Table II it is seen that the three types of treatment listed give comparable results as far as the absolute and relative concentrations of cytochromes a_3 and a are concerned. The other components are found at higher concentrations when reduced by short (20 minutes) anoxia than after CN^- treatment and higher yet after treatment with hydrosulfite. The results with $Na_2S_2O_4$ can be duplicated by long, continued anoxia (4 to 6 hours). The occurrence of the extra amounts of flavoprotein parallels the findings of Chance and Williams (1955) who distinguished three categories of flavoproteins. In our case it is necessary to distinguish four; the following figures refer to the toad sartorius. Antimycin A treatment shows up category I equal to

4.8 μmoles/kg; cyanide inhibition reduces category II equal to 10.2 μmoles/kg; during anoxia (10 to 20 minutes after cytochrome *c* has become reduced) 18.3 μmoles/kg is reduced and is referred to as category III while treatment with $Na_2S_2O_4$ reduces category IV equal to 22.3 μmoles/kg (note that these categories are not separate fractions; IV, for instance, contains all other categories). In both the frog and the bullfrog the difference between categories III and IV is larger than 4 μmoles.

The nature of the extra optical density changes produced by $Na_2S_2O_4$ or long continued anoxia over those caused by cyanide treatment or short anoxia is shown up by the recording of a long (5 hours) *vs.* short (20 minutes) anoxia difference spectrum (Fig. 4). The peaks are located at 591 and 561 mμ, a broad band runs from 432 to about 438 mμ, and a shoulder occurs at about 573 mμ. Occasionally some extra absorption around 550 mμ is also seen. The small peak at 340 mμ indicates some additional DPN reduction, the trough around 570 mμ flavoprotein (category IV to III), and the broad band around 510 mμ might be analogous to the β-bands of cytochromes. The absorption at 561 and 550 mμ could be caused by extra reductions of cytochromes *b* and $c + c_1$, respectively.

The broad peak at 591 and the shoulder around 573 mμ do not correspond to known components of the respiratory chain. If some hemoglobin were left in the muscles and more in one than in the other, this would, of course, be deoxygenated in both cases and show up as an absolute spectrum. In that case, the maxima of deoxygenated toad's hemoglobin should be traceable, with peaks at 555 and 432 mμ. The peaks would, of course, show up as troughs if the extra quantity of hemoglobin were contained in the reference muscle. The present maxima and minima in the visible region differ too much to ascribe them to such an absolute spectrum of hemoglobin; they are also seen in $Na_2S_2O_4$ *vs.* CN^- spectra, and, furthermore, these peaks have always occurred in the sample muscle, making it most implausible to ascribe them to the chance occurrence of a slightly higher amount of hemoglobin.

The possibility still exists that an unknown myoglobin-like substance does occur in toad sartorii (by chance always at a higher concentration in the sample muscle) but at a concentration low enough not to interfere with the anoxic – oxygenated difference spectra, where it could be masked by the large absorption changes of the cytochromes. In order to show this, it should be possible to observe the peaks in a muscle which is slowly becoming anoxic. This experiment would also provide data on the kinetics of the reduction of the various components. It can easily be performed by cutting off the air supply to the sample muscle. As the available O_2 is used up, the muscle slowly becomes anoxic. During this period, spectra are recorded. If the extra absorptions at 561 and 591 mμ are caused by myoglobin, they should appear before the peaks of the members of the respiratory chain.

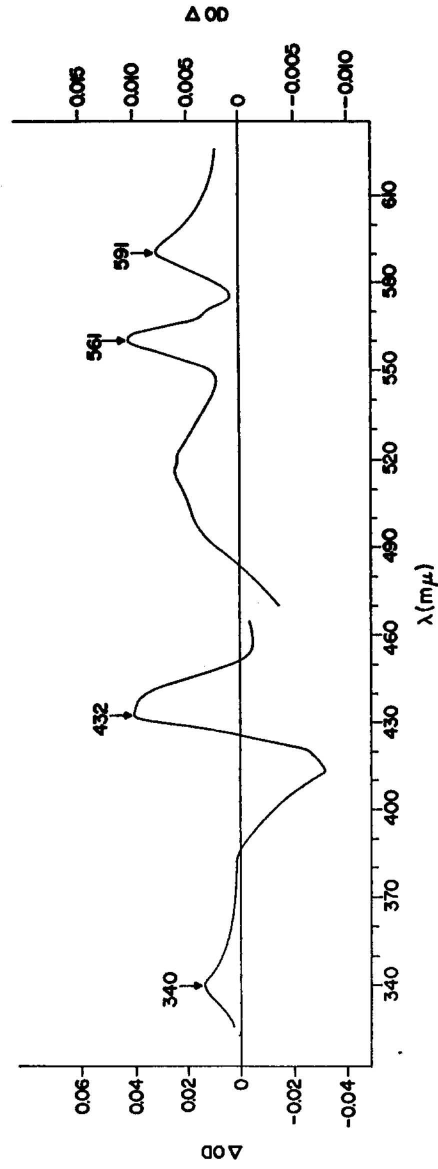


FIGURE 4. Difference spectrum of two toad sartorii anoxic for different periods: sample for 5 hours; reference for 20 minutes. Both muscles stimulated repeatedly to increase the speed of the oxygen disappearance. 20°C.

The results of such an experiment are shown in Fig. 5, in which the amount of absorption of each component in the final spectrum (245 minutes) is called 100 per cent. It is clear that cytochromes a_3, a, c, and flavoprotein have very similar reduction rates, as could be expected from their occurrence in the respiratory chain. DPN appears to react more slowly, perhaps because there is a contribution of cytoplasmic DPN which continues to become more reduced during anoxia (Jöbsis, 1963). The bands at 591 and 561 mμ develop more slowly also. The graph makes it clear that neither the peak at 591 nor the extra absorption at 561 mμ is to be ascribed to myoglobin as they appear much more slowly than do the ones of the respiratory chain members. The peak labeled "b" (561 to 575 mμ) develops faster than "x" (591 to 575),

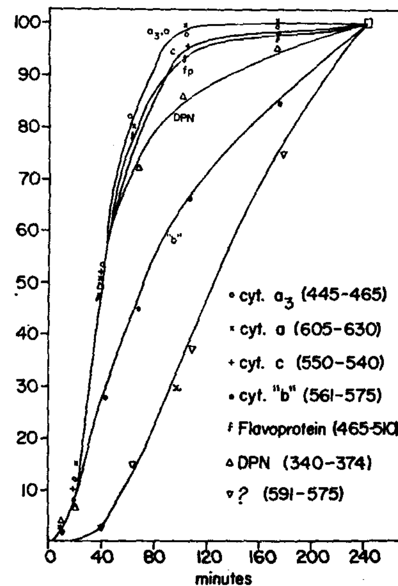


FIGURE 5. Graph of the kinetics of the appearance of the absorption peaks in a resting muscle slowly becoming anoxic. Ordinate, per cent of the final level. Abscissa, minutes after cutting off the air supply. Reference muscle oxygenated. Toad sartorii, 10°C.

partly, of course, because cytochrome c and the true b, associated with the cytochrome chain, also become reduced and contribute to the optical density at 561 mμ. From this experiment, two conclusions can be drawn: (*a*) that the extra absorptions in $Na_2S_2O_4$ *vs.* CN^- reduced difference spectra and in long anoxia *vs.* CN^- (or *vs.* short anoxia) are not caused by hemoglobin- or myoglobin-like pigments, and (*b*) that these peaks are due to pigments not directly included in the classical respiratory chain.

Without the molar extinction coefficients of the unknown components, it is not possible to calculate their concentrations. For comparative purposes they can be related to a component reduced by CN^- or by short anoxia. For this comparison cytochrome c (at 550 to 540 mμ) is preferable to cytochrome a (605 to 630 mμ) because the latter peak differs in intensity after cyanide

treatment and in anoxia (see Table I). The peaks for the unknown components were measured as the Δ OD between 561 and 575 and 591 and 575 mμ. The ratio of the cytochrome *c* peak to these was 2.4 and 2.2, respectively (averages of 3 experiments).

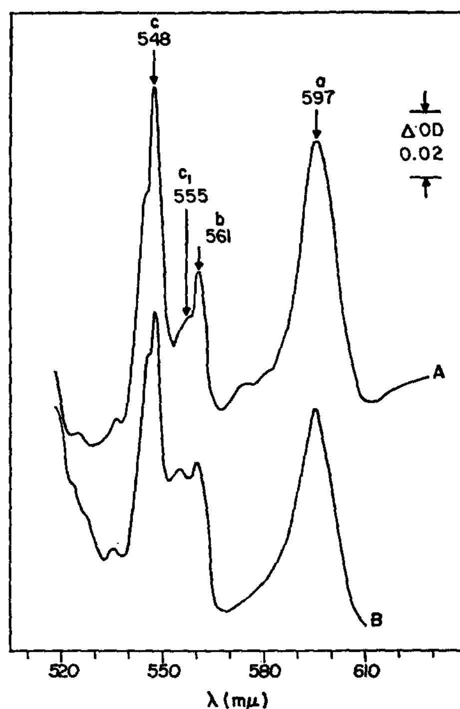


FIGURE 6. Difference spectra of muscles at liquid N_2 temperature. Curve A, sample 2 mM NaCN for 30 minutes; reference oxygenated, resting. Curve B, sample 2 mM NaCN; reference oxygenated, 2 mM amytal for 85 minutes. Two pairs of toad sartorii. The difference in total *c* and *a* levels should be ascribed to a difference in thickness and to individual variation among the pairs.

II. *Low Temperature Spectra of Intact Muscles*

In order to shed more light on the question of the occurrence of cytochrome c_1, and on the identity of the extra absorption developed by the treatment with $Na_2S_2O_4$ and long anoxia, some difference spectra were recorded at the temperature of liquid nitrogen. At this low temperature the absorption bands of the cytochromes are shifted a few mμ to the ultraviolet and become narrower with a consequent increase in extinction at the wave length of the peak (Keilin and Hartree, 1949). This allows one to resolve absorption peaks which are imperfectly resolved at room temperature.

A CN^--inhibited *vs.* aerobic difference spectrum of 2 toad sartorii is presented in Fig. 6, curve A. The peaks at 597, 561 and 548 mμ are identified

with cytochromes *a*, *b*, and *c* respectively (see Estabrook, 1956, 1958). The small shoulder at about 555 mμ is ascribed to cytochrome c_1. In Fig. 6, curve B, the difference spectrum of another pair of muscles is given; here the reference muscle was treated with 2 mM amytal for 85 minutes before freezing. The peak of cytochrome *b* is now stronger relative to cytochrome *c*, while cytochrome c_1, at 555 mμ, is also a little more prominent. Thus it can be concluded that c_1 does occur in muscle tissue, though at a low level, and is slightly reduced in the resting state.

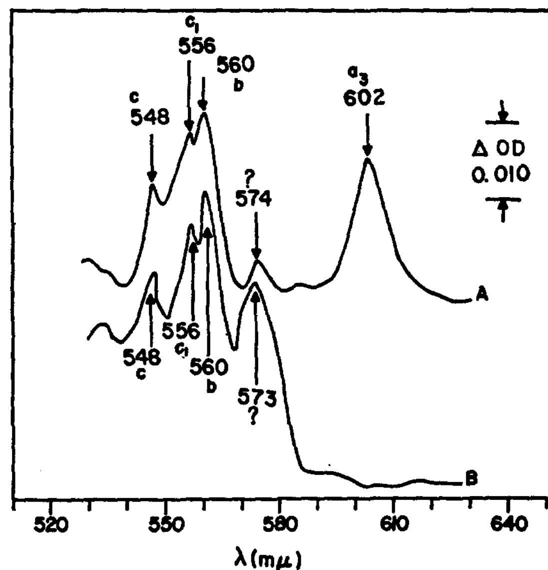


FIGURE 7. Difference spectra of muscles at the temperature of liquid N_2. Curve A, sample 2.5 hours anoxic; reference 2 mM NaCN for approximately 30 minutes. Curve B, sample treated with $Na_2S_2O_4$; reference anoxic for 20 minutes. Two pairs of toad sartorii.

Curve A of Fig. 7 is the difference spectrum of two muscles, one of which was anoxic for 2.5 hours while the other was treated with cyanide. Here more cytochrome *b* has become reduced during anoxia while cytochrome c_1 is also considerably more reduced. The maximum at 602 mμ is attributed to cytochrome a_3. An unknown peak at 574 mμ has developed. Finally, the extra reductions produced by $Na_2S_2O_4$ treatment over those developed during 20 minutes of anoxia are presented in Fig. 7, curve B. It is now obvious that the extra absorption observed at room temperature in this type of spectrum is caused by additional absorption occurring at the maxima of cytochrome *c*, c_1, and especially *b* and by the appearance of a new peak at approximately 573 mμ. It is also of interest that the peak at 591 mμ does not show up dis-

tinctly in any spectrum at this low temperature. The lack of sharpening of the band might indicate that here we are not dealing with a hemoprotein.

III. *Spectra of Intracellular Particles*

Considerable difficulty was encountered in the preparation of good mitochondria from skeletal muscle. Various methods were used, but if respiratory control is taken as the criterion of quality, the results were not satisfactory (Jöbsis, 1963). Some of the results for the toad muscle with the Waring blendor and grinding with sand are given in Table III for the 5000 × *g* fraction. (The spinning speed was purposely kept low in these experiments in the hope that mitochondrial fragments would stay in suspension. The next fraction (13000 × *g*) was small and contained little cytochrome.) It is clear

TABLE III

RELATIVE CONCENTRATIONS OF RESPIRATORY CHAIN COMPONENTS OF TOAD SKELETAL MUSCLE MITOCHONDRIA

Calculated from difference spectra; reference: aerobic + ADP. Concentration of cytochrome *a* = unity, 5000 × *g* fraction, 10 minute spins.

Preparation	Reduction by	Concentration relative to cyt *a*				
		a_3	$c + c_1$	b	fp	DPNH
Waring blendor high speed, 10 sec.	Succinate + CN^-		0.33	0.17	0.69	0
Waring blendor low speed, 30 sec.	Succinate + CN^-		0.44	0.22	1.1	0
Grinding with sand	Succinate + CN^-		0.83	0.40	1.7	0
Grinding with sand	Anoxia (succinate)	1.0	0.97	0.46	2.3	0
Grinding with sand	$Na_2S_2O_4$	1.1	1.2	0.63	2.7	

that blending produces more damage than grinding as witnessed by the lower cytochrome *c* values after the use of the blendor. The cytochrome distribution pattern of the particles prepared by the grinding technique is quite comparable to those of the intact toad muscle, with the exception of the DPNH values. Some increased absorption would usually develop in the 320 to 375 mμ region, but no true DPNH peak could be identified. It can, therefore, be assumed that even these mitochondria have sustained sufficient damage to allow DPN to leak out but not enough to cause much depletion of other respiratory chain members. One thinks immediately of possible swelling damage, which might produce the observed lack of respiratory control.

The occurrence of extra absorption bands observed in intact muscle upon treatment with hydrosulfite or by long anoxia was also noticed in these preparations. A difference spectrum of long *vs.* short anoxia is shown in Fig. 8. The peaks are located at 591, 560–562, and 436 mμ. They agree well with those

observed in the muscles. The peak at approximately 614 to 615 mμ was observed only occasionally. In the mitochondrial preparations (5000 × *g* fraction) prepared by grinding with sand, the ratio of the cytochrome *c* absorption (550 to 540 mμ) in short anoxia or in the presence of CN^- to the 561 to 575 mμ absorption in long anoxia or in the presence of $Na_2S_2O_4$ was 2.2 (average of 5 experiments). This ratio was 2.5 (average of 3 experiments) in the 13000 × *g* fraction. For the 591 to 575 mμ peak the ratios were much higher: 5.3 and 11.6 respectively for the same two fractions. A difference exists, therefore, between the distribution of the two components. The 561 mμ peak has approximately the same relative strength in the particulate fractions as in the whole muscle, while a considerable amount of the 591 mμ component appears to be lost during the preparative procedure; in the intact muscle its ratio was found to be 2.2.

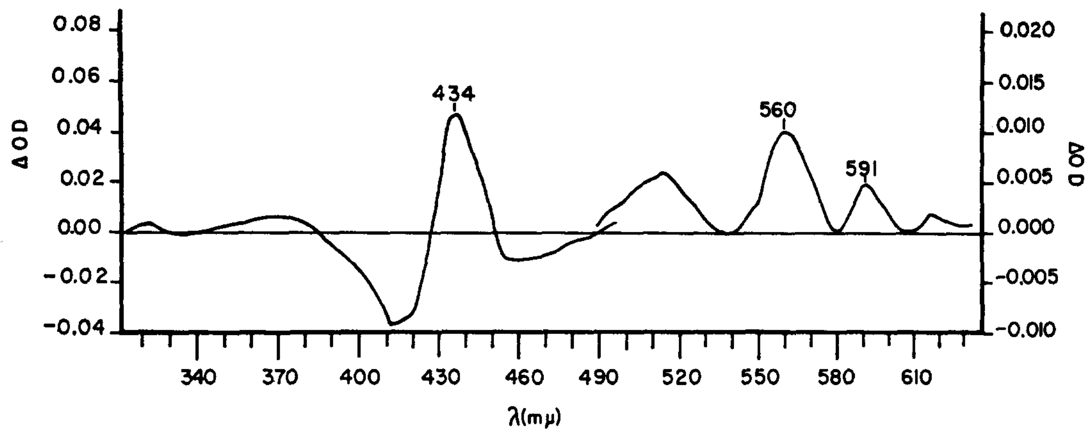


FIGURE 8. Difference spectrum of isolated mitochondria. Sample anoxic for 95 minutes; reference anoxic for 5 minutes. Mitochondria prepared from toad skeletal muscle by grinding with sand procedure.

DISCUSSION

A perusal of Table II brings out some interesting differences in the cytochrome concentrations in various muscles. Comparison of the cytochrome *a* contents of the sartorii of the toad, the frog, and the bullfrog shows quite different levels to be present in these closely related species. It would be interesting to follow up these results with a comparative study among various species. Until that time it cannot be decided whether the variation is caused by differences in climate (tropical *vs.* temperate) or in behavior (active hunting *vs.* a sedentary wait and see attitude), or whether these differences are spurious.

The variation in the levels of the cytochromes in different muscles of the

same animal is also great enough to be intriguing. The quickly contracting and slowly relaxing retractor capitis collique, used by the turtle to draw back its extended neck and keep it retracted, has less than one-tenth the concentration of the coraco-hyoideus, a muscle with a more usual myogram. In this connection it is also of interest that the cytochrome content of intact toad heart muscle is 2 to 4 times that of the sartorius of the same animal (Ramirez, 1959). Of the three muscles the heart has, of course, the most constant and frequent alternation of contraction and relaxation followed by the sartorius and the coraco-hyoideus and, finally, by the neck muscle. In view of the turtle's ability to keep its head withdrawn for long periods against a considerable opposing force, it may mean that considerably more energy turnover is involved in a succession of contraction and relaxation cycles than in the maintenance of the contracted state. It could also be, however, that the lower level is related to an interference with blood flow during the contraction. The tissue would then depend on glycolysis for a continued energy supply. Some correlative studies between the respiratory and glycolytic systems and the physiological function of various muscles might be enlightening.

The relative concentrations of the components of the chain form a pattern without much variation among the various muscles. This pattern also resembles closely that of mammalian ascites tumor cells (Chance and Hess, 1959) and with certain, accountable differences that of extracted mitochondria (Chance and Williams, 1955).

The fact that DPNH levels differ between most isolated mitochondria and the intact muscle may well have its cause in the impossibility of oxidizing all of it in the reference muscle. A true difference with mammalian liver mitochondria is, however, not excluded: the DPNH content of rat heart mitochondria is lower than that of liver mitochondria (Chance and Baltscheffsky, 1958). The former have a DPNH to cytochrome *a* ratio of about 4.3 to 1 when reduced by a DPN-linked substrate but 10 to 1 when assayed with succinate, while the latter preparation shows ratios of 20 to 40 to 1 with DPN-linked substrates. However, the liver mitochondria contain considerable quantities of TPNH, which is indistinguishable from DPNH at 340 mμ, and, thus, would tend to lower the difference. Intact ascites cells also show low pyridine nucleotide levels: 6 to 8 times cytochrome *a* in intact cells, 10 times in their isolated mitochondria. This difference has been ascribed to a failure to produce a completely oxidized respiratory chain in the whole cell because it is impossible to deplete the intracellular substrates completely (Chance and Hess, 1959).

The cytochrome *b* and flavoprotein concentrations of the intact muscles are also somewhat low in CN^- or anoxic *vs.* oxygenated difference spectra, as compared with such spectra from mammalian liver or heart mitochondria. The differences are, however, not as great as among the DPNH levels. Rela-

tively low titers of these 2 components are also found in intact ascites cells (Chance and Hess, 1959) and toad heart muscle (Ramirez, 1959). The discrepancies in cytochrome *b* levels should partially be ascribed to the occurrence of a considerable reduction of cytochrome *b* in the resting steady state of the muscle. Nevertheless, the level assayed by the use of amytal still shows a lower content in the muscles. There is, however, no guarantee that this component is really completely oxidized in the presence of amytal. Complete oxidation would seem especially doubtful if considerable levels of succinate or other substrates dehydrogenated by flavoprotein enzymes (such as α-glycerophosphate) are present in the cell.

A slightly discordant note is introduced by the analysis of the cytochrome patterns of isolated, skeletal muscle mitochondria (Table III). Pyridine nucleotides are completely missing here, and cytochrome *b* is somewhat lower than in mammalian liver mitochondria. Too much importance should not as yet be attached to these figures, as it is more than likely that the present particles were seriously damaged, as witnessed by a lack of respiratory control. This liability to damage during isolation procedures may well be due to the extreme size of the mitochondria of anuran skeletal muscle. Electron micrographs, kindly sent to us by Dr. Birks and Dr. A. F. Huxley of Cambridge University, show the mitochondria of frog muscle to range up to 10 μ in length with a width of 1 or 2 μ. The isolation of such large particles in intact form presents special problems, especially in a tissue with the toughness of skeletal muscle. Granting that some damage has occurred during isolation, it seems likely that the lack of pyridine nucleotides is caused by a leakage of these compounds from the mitochondria. The rather low cytochrome *b* figures are not as easily explained and must perhaps be taken as a true variation from the usual pattern of the respiratory chain in mammalian mitochondria. This agrees also with the lower levels of cytochrome *b* found in the intact muscles.

A fact of more than passing interest does emerge from these attempts at the isolation of the mitochondria. In the better preparations (grinding with sand procedure) the cytochrome *a* to cytochrome *c* ratios are almost identical with those in the intact muscle. This observation shows that there is no appreciable amount of cytochrome *c* present in the cytoplasm; all of it appears to be contained in the mitochondria. The occurrence of cytochrome *c* as a shuttle system between cytoplasmic dehydrogenases and mitochondrial oxidases, can, therefore, be ruled out, unless the assumption be made that all of this hypothetical cytochrome *c* is maintained in the reduced form: a state of affairs which would be difficult to reconcile with the expected characteristics of such a shuttle system. Chance and Hess (1959) arrived at very much the same conclusion for the ascites cells.

It was found that the toad sartorius contains some extra pigments which

are not affected by cyanide but become reduced upon long anoxia or treatment with hydrosulfite. In difference spectra these peaks are located at approximately 591, 561, and 434 mμ. The occurrence of a hemoglobin- or myoglobin-like compound was excluded. From low temperature spectra (liquid N_2) it was possible to ascribe the rather broad band at room temperature with a maximum at about 560 to 562 mμ to increases in the cytochrome *b*, c_1, and *c* peaks and perhaps to the occurrence of a new band at 573 to 574 mμ at −160°C. The band at 591 mμ did not show appreciable sharpening at this low temperature.

The location of these absorption maxima prevents us from identifying any of them with those of mammalian cytochrome b_5, which has maxima at 556 and 427 mμ. The possibility remains, however, that the peaks at approximately 561 and 434 mμ are due to a pigment functionally similar to cytochrome b_5. This would then be the only qualitative, spectroscopic difference between the mammalian and toad skeletal muscle cytochrome complements.

The mitochondrial fraction from skeletal muscle also gives rise to these peaks upon $Na_2S_2O_4$ treatment. It does not follow that the compounds responsible for these peaks are necessarily located in the mitochondria. They might also occur in other particles coming down at the same centrifugal force as the mitochondria. It would be an unlikely coincidence, however, if these two types of particles had the same size distribution, as should be concluded from the constancy of the ratio between cytochrome *c* absorption and the extra 561 mμ peak in the whole muscle and in the two fractions. It is, therefore, justifiable to conclude tentatively that the unidentified reducible compound absorbing at about 561 mμ is associated with the mitochondria.

The peak at 591 mμ, common to muscle and the mitochondrial fraction is, according to the same type of reasoning, probably not caused by a compound contained in the mitochondria. Its intensity relative to cytochrome *c* is too variable. The possibility does exist, of course, that this compound washes out partially during the preparatory procedure or that it is a sarcoplasmic constituent partially and variably bound to the particles during preparation. The peak at 614 mμ, occasionally found in the mitochondrial fraction but not yet observed in the whole muscle, may be an artifact of the preparatory procedure. But it could also be that the compound giving rise to it is always in the reduced form in the intact tissue and became oxidized in certain of the mitochondrial preparations. The whole question of occurrence, type, and function of the compounds responsible for these extra absorption peaks developed upon treatment with hydrosulfite or during long, continued anoxia might well be a fruitful subject for future research.

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