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OXIDATION-REDUCTION CHANGES OF CYTOCHROMES FOLLOWING STIMULATION OF AMPHIBIAN CARDIAC MUSCLE

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Previous work carried out in this laboratory on aerobic skeletal muscle (Connelly & Chance, 1954; Weber, 1957; Jöbsis & Chance, 1957) demonstrated changes in the components of the electron transport chain in response to contractions. This paper reports similar studies with the cardiac muscle of the toad and the frog. The only previous observations on the heart are those of Arvanitaki & Chalazonitis (1947). The technique used (filters and a single-beam instrument) does not allow the elimination of the non-specific changes, which makes their conclusions not completely definitive.

The cardiac muscle has a high working capacity, that is it can immediately increase its working level and remain there without showing fatigue. Hence it is an ideal material for studying the control mechanisms operating on metabolism. It is known, for instance, that the time course of the oxygen consumption of the heart, studied by polarographic (Ramírez, 1953) or spectrophotometric methods (D. W. Lübbers & J. Ramírez, unpublished) follows the cardiac work without delay when it is modified by changes in the load or in the frequency of contractions.

In the present study the changes of the steady-state oxidation–reduction levels of the cytochromes, pyridine nucleotides, and flavo-protein in the cardiac muscle were followed during and after isometric contractions by means of spectrophotometric techniques as a further step in the understanding of the control of the cardiac respiratory mechanism. The hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate (P) as a consequence of muscular contraction has been postulated (Szent-Györgyi, 1951), but the direct chemical techniques applied to muscle have so far yielded only negative results (Fleckenstein & Janke, 1953; Mommaerts, 1955). On the other hand, the use of spectrophotometric techniques for the

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indirect analysis of the ADP inside the muscle (Chance & Connelly, 1957) has the great advantage of not destroying the muscle structure. One may use the respiratory chain as a qualitative rapid indicator of the intracellular modifications in the ADP concentration, since the ADP is highly specific in its action on the respiratory chain (Chance & Williams, 1956*a*).

For comparison, the sarcosomes, which contain the respiratory enzymes under study, were isolated from the cardiac muscle and the steady-state changes in the respiratory chain components which occur in response to the addition of ADP were recorded (Chance & Baltscheffsky, 1958). The addition of ADP in these experiments is assumed to simulate a muscular contraction. A preliminary account of part of this work has been given previously (Ramírez, 1958).

METHODS

The hearts of the toad (*Bufo marinus*) and the frog (*Rana pipiens*) were used. The animal was prepared by injecting sodium heparin (50 mg/kg) into the dorsal lymphatic sinus. After 15 min the animal was killed by destroying the brain and medulla with a needle, and then perfused with 500 ml. of frog's Ringer (mM: NaCl, 111·4, KCl 2·7, $CaCl_2$ 1·8, Na phosphate buffer 2, pH = 7·1) in order to wash out the blood, which might interfere later in the spectrophotometric measurements. The heart was excised and a piece of ventricular wall was mounted, compressing it gently between two perforated lucite plates (Fig. 1), leaving uncovered a 'disk' of stretched muscle of 5·5 mm diameter. The thickness of the excised tissue, between 0·5 and 1·5 mm, is not completely uniform owing to the spongy internal structure of the ventricle. The preparation was put in an ordinary 1·0 cm cuvette in the spectrophotometer and a continuous jet of oxygenated Ringer's solution was directed on each muscular surface to avoid anoxia during the measurements. Such a preparation does not show spontaneous activity in most of the experiments. To elicit the activity the muscle strip was stimulated electrically by means of condenser discharges. The perfusion liquid and the preparation were maintained at constant temperature throughout the experiment. The difference spectra of the heart strips were obtained in the split-beam spectrophotometer described by Yang & Legallais (1954).

In order to study the kinetics of the oxidation–reduction changes of the different components in response to changes in contractile activity, the double beam spectrophotometer described by Chance (1951) was used. The light was detected by an 'end-on type' photomultiplier (Dumont 6292) located at 3·3 cm from the muscle surface. At this distance the large sensitive surface (15 cm^2) of this tube picks up most of the light scattered by the preparation. The difference in optical absorption at two wave-lengths (less than 20 mμ apart) was recorded as a function of time with a Hathaway high-frequency mirror-galvanometer oscillograph (model S 14-A). An Esterline-Angus recorder was used for monitoring purposes. By this differential technique we cancel out most of the non-specific optical changes due to reflexion, polarization, diffraction, and scattering, which occur during the contractions (Hill, 1953), except in the Soret region of the spectrum where these changes are wave-length-dependent. However, if one waits until the contractions cease, the non-specific changes disappear, and then it is possible to record the modifications of the pigments as in the case of cytochrome a_3 (*vide infra*). With these procedures the spectral changes corresponding to the oxidation–reduction of the respiratory chain components can be studied. If a plate of ground glass is put in front of the preparation in order to diffuse the incident light, one can further eliminate the non-specific optical changes originating during the contractions. The non-specific changes recorded at a single wave-length are five to twenty times bigger than the modifications of the spectra of the respiratory pigments. At the reference wave-length, where practically only the non-specific changes are present, the decrease of the optical transmittance

due to each contraction was used as a convenient measure of the contraction. These changes are bigger than in skeletal muscle, because in the heart the contractions are taking place in a muscle with highly irregular fibre disposition and heterogeneous transmittance. The sarcosomes were prepared from toad's heart according to the method described by Cleland & Slater (1953) and suspended in the magnesium-free medium described by Packer (1957). The oxygen consumption was measured with the platinum oxygen electrode (Chance & Williams, 1955*a*).

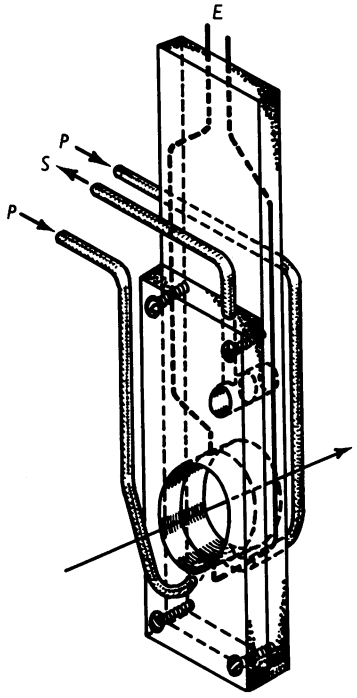

Fig. 1

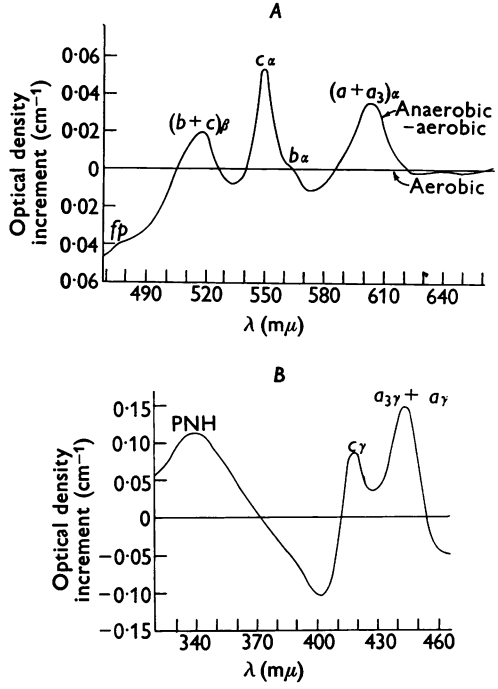

Fig. 2

Fig. 1. Chamber for cardiac muscle. The muscle (not shown) is put between the two perforated lucite plates. These plates are held together with four screws. *E* indicates the stimulating electrodes, *P* the tubes carrying oxygenated Ringer's solution to each muscle surface, and *S* the suction output for Ringer's solution. The whole chamber is put in a 1·0 cm cuvette. The large arrow shows the direction of the spectrophotometer light beam.

Fig. 2. Difference spectrum of oxygenated minus anoxic toad's ventricle. The base line represents the 2 strips oxygenated. *A*. α and β bands of the cytochromes and flavoproteins (fp). *B*. Soret (γ) band of the cytochromes and reduced pyridine nucleotides (PNH). For explanation see text.

RESULTS

Resting state absorption spectra

The difference spectrum of the toad heart strips recorded with the split-beam spectrophotometer is shown in Fig. 2 as the difference between the aerobic and the anaerobic states. Two strips prepared so as to be as similar as possible

were gassed with oxygen or nitrogen and the difference spectrum was recorded. The base line corresponds to both muscles being in the aerobic steady state. In the anaerobic-minus-aerobic spectrum, the peaks and troughs corresponding to the difference between the reduced and oxidized pigments of the respiratory chain are apparent. The peak at 605 mμ ($a+a_3$) α corresponds mainly to the α peak of cytochrome a with a small contribution of cytochrome a_3 (Chance, 1957). The shoulder around 564 mμ ($b\alpha$) corresponds to the α band of cytochrome b. At 550 mμ ($c\alpha$), the α band of cytochromes $c+c_1$, is seen. The β bands of cytochromes $c+b$ appear at 518 mμ. A trough at 465 mμ corresponds to flavoprotein (fp) and is followed by the γ band of cytochrome a_3 at 445 mμ. The γ band of cytochrome b is masked by the γ bands of the other cytochromes. The γ band of cytochrome c is apparent at 419 mμ, and the peak of the reduced pyridine nucleotides (PNH) is found at 340 mμ. It is evident that the toad's heart does not contain a measurable amount of myoglobin.

Figure 3*A* shows the difference spectra of the frog's heart recorded similarly. In this case there is no clear indication of the reduced cytochrome peaks, as in the toad. Instead, peaks are evident at 600, 557 and 513 mμ, and two troughs at 557 and 537 mμ. This is rather characteristic of the haemoglobin or myoglobin differential spectra but the position and size of the peaks is different. This spectrum can be resolved into two main components, the spectrum of the respiratory-chain components and the spectrum of an intracellular myoglobin-like pigment. The spectrum of the respiratory chain was evidenced by reducing the respiratory pigments of one muscle by adding 1–2 10^{-3} M-NaCN and bubbling oxygen to both strips. Under these circumstances, the intracellular myoglobin-like pigment is oxygenated in both muscles and in the differential trace its spectrum is cancelled out. Only the characteristic peaks of the respiratory chain, similar to those of the toad's heart spectrum, then appear (Fig. 3*B*). The intracellular pigment was recorded without the interference of the peaks of the respiratory pigments, by adding NaCN to both muscles and passing nitrogen to one strip and oxygen to the other. By this procedure the respiratory chain is reduced in both muscles and its spectrum disappears in the differential trace, leaving only the spectrum corresponding to the reduced-minus-oxygenated forms of the intracellular pigment (Fig. 3*C*). A similar trace is obtained also by subtracting the spectrum 3*B* from the spectrum 3*A*. This trace is very similar to the spectrum of the oxygenated-minus-reduced frog's haemoglobin in the erythrocytes. The positions of the peaks and troughs are practically identical (Table 1). This intracellular 'myoglobin-like' pigment is concentrated preferentially in the upper left part of the heart, giving a pink colour to it. The physiological role of this compound is like that of myoglobin in other muscular tissues (D. W. Lübbers & J. Ramírez, unpublished). Since the absorption bands of this pigment are so large in some preparations that they mask the peaks of the

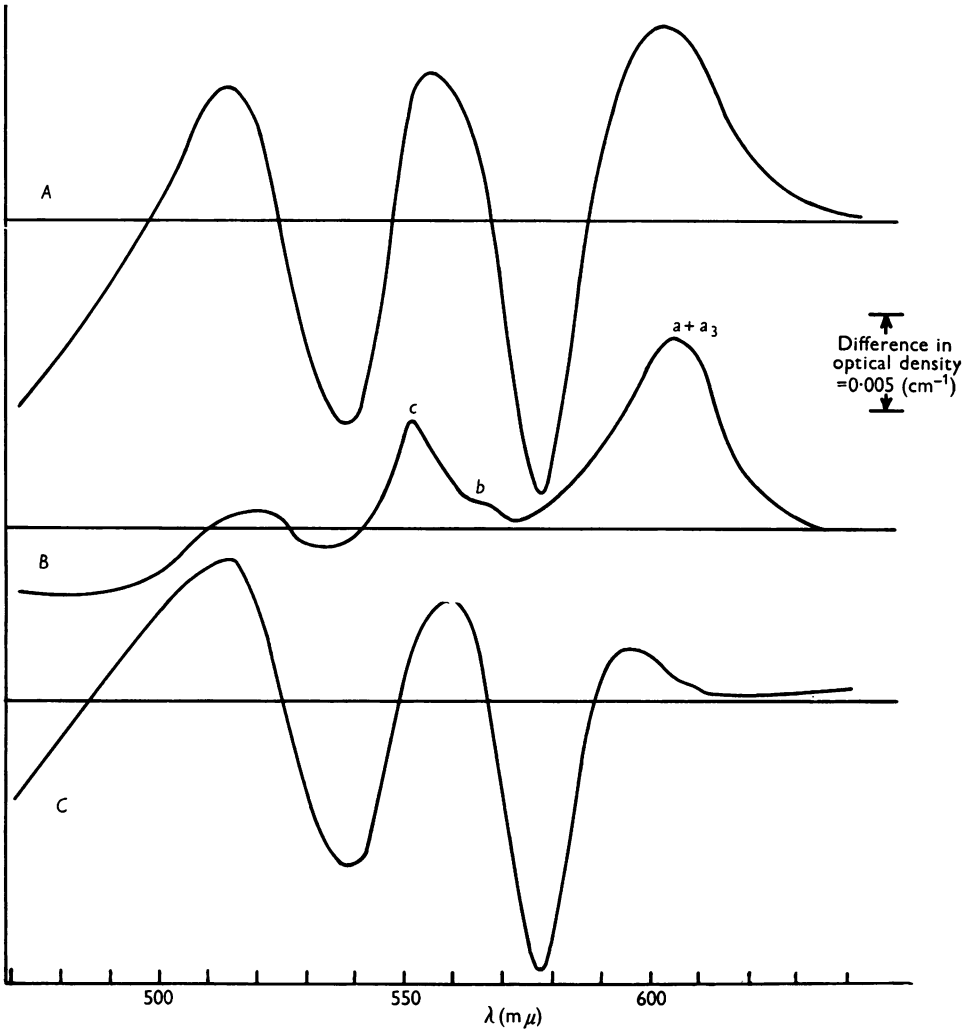

Fig. 3. Difference spectra of frog's heart strips. *A*, oxygenated minus anoxic muscle; base line, both strips in presence of oxygen. *B*, respiratory pigments reduced in cyanide-treated muscle, oxygen in both preparations. *C*, intracellular nitrogen reduced 'myoglobin-like' pigment minus the oxygenated pigment; respiratory chain reduced in both preparations with cyanide.

TABLE 1. Absorption bands in a difference spectrum (oxygenated minus reduced) of haemoglobin in frog's erythrocytes and intramuscular haemoglobin-like pigment

	Wave-length (mμ)				
	Peaks			Troughs	
Haemoglobin	597	558	512	577·5	537
Pigment	597	559	513	576–577	538

cytochromes in the reduced state, as is seen in the Fig. 3*A*, we prefer the toad's heart, which does not contain the pigment, to the frog's heart. Also, the concentration of cytochromes is about three to five times greater in the toad's heart.

By the application of the calculations of Chance (1957) to the spectroscopic data on the strips of the toad's heart it was found that the difference in concentration between the aerobic and anaerobic states of each cytochrome was equivalent to 20–45 μmoles/kg wet wt. depending on the preparation. This compares with about 10–20 μmoles/kg wet wt. in the toad's sartorius muscle (F. F. Jöbsis, personal communication) and 2–3 μmoles/kg wet wt. in the frog's sartorius muscle (Weber, 1957). The relative differential concentra-

TABLE 2. Relative differential concentrations of the respiratory pigments (cytochrome *a* = 1) in toad and in rat muscle

	Toad			Rat*
	Intact heart	Heart sarcosomes		
Pigment	muscle†	0·32 M sucrose	0·086 M sucrose	Heart sarcosomes
Cytochrome *a*	1	1	1	1
Cytochrome a_3	0·8	0·8	1·9	1·1
Cytochrome *b*	0·4	0·3	0·61	1·0
Cytochrome $c+c_1$	1·1	0·6	0·86	1·3
Flavoprotein	1·3	1·5	1·1	1·1
Pyridine nucleotides	5·8	—	—	4·3

* Chance & Baltscheffsky (1958). † Average of 6 muscles.

tions of the respiratory components, taking the concentration of cytochrome *a* as unity, are shown in Table 2. These ratios of the components are similar to those in toad's heart sarcosomes and also to the ratios in rat heart sarcosomes described by Chance & Baltscheffsky (1958). The main differences are that the cytochrome *b* is in higher concentration in the rat heart sarcosomes than in either the whole heart or heart sarcosomes of the toad. The cytochrome *c* is in a greater concentration in the heart. In toad's heart sarcosomes the ratio $a_3:a$ in 0·32 M sucrose is less than the ratio in hypotonic medium (0·086 M sucrose), which is in agreement with a similar observation in liver mitochondria (Chance & Williams, 1955*b*).

The spectral curve of the respiratory pigments in the toad heart sarcosomes is similar to that of intact muscle and is practically identical with the spectrum of rat heart sarcosomes (Chance & Baltscheffsky, 1958).

The oxidation–reduction level of cytochromes a_3, *a*, *c* and *b* in the resting aerobic steady state is about 92–98% of complete oxidation, as was shown by 2–8% further oxidation when the perfusion liquid contained either 1000–2000 μM Na amytal or 200 μM 3-hydroxy-1-heptylquinoline-*N*-oxime (HOQNO). These substances inhibit the electron flow to the cytochromes, stopping the respiration. It should be noted that whereas cytochrome a_3 is

95 % oxidized in the intact muscle it is more than 99 % oxidized in the isolated sarcosomes.

Changes of the respiratory components as a consequence of muscular activity. When the resting muscular strip is electrically stimulated at a constant frequency, the oxidation–reduction levels of the respiratory chain components are modified and a new steady state is reached. This state is established very rapidly; the half maximum change is reached in 2–4 contractions, which is after about 4–8 sec ($t\frac{1}{2}$ ON). Moreover, it is the same for the different components in the respiratory chain. The number of twitches is the same when different preparations are used and is independent of temperature and stimulating frequencies.

After stimulation the respiratory chain components immediately start returning to their previous oxidation–reduction level; the half time for this return ($t\frac{1}{2}$ OFF) is quite variable in different preparations. It is about 4–6 sec for cytochromes *b*, *c*, *a* and flavoprotein, but is usually 2–3 times longer in the case of cytochrome a_3 and pyridine nucleotide (see Table 3). These fast changes in the oxidation–reduction level of the respiratory chain components agree with the rapid changes in oxygen consumption (Ramírez, 1953).

The typical changes of the steady state of cytochrome *b* during the muscular activity in the frog's heart are shown in the upper trace of Fig. 4*A*. Although this heart contains a 'myoglobin-like' pigment, its interference with the cytochrome absorption changes can be eliminated by perfusing the muscle with oxygen-saturated Ringer's solution. Such a procedure always maintains the 'myoglobin-like' pigment in a fully oxidized state. The lower trace indicates the change in transmittance recorded at only one wave-length, 540 mμ, and indicates the contractions. During the muscular activity the cytochrome *b* becomes oxidized. The total change is very small. In this preparation it is 0·0017 optical density units, which represents an oxidation of 2 % of the cytochrome *b*. A similar oxidation of cytochrome *b* in skeletal muscle, produced by contractions, has been described by Weber (1957), and by Jöbsis & Chance (1957).

During isolated contractions it is possible in some preparations to see changes in the cytochromes (Fig. 4*B*). These tracings show spontaneous contractions every 10 sec, and the corresponding changes in cytochrome *b*. There are very early changes in the oxidation–reduction steady state of cytochrome *b* during the contraction and a fast reduction during the resting period. Note that in this preparation the non-specific optical changes originating during the contraction are practically absent in the differential trace.

The total change reached during the contractions is a function of the stimulating frequency (Fig. 4*C*). A similar curve is obtained with the other components of the respiratory chain. At the highest frequency that the heart can contract (in these circumstances about once per second), there is still not

a saturation in the cytochrome change. The limiting factor is the refractory period of the heart. The total change at the maximum is 2–6% of the total cytochrome concentration (Table 3). In skeletal muscle the changes are about 5–10%.

When muscle strips 1 mm thick are subject to stimulation at about 1 c/s at 15° C, anoxia occurs in the centre of the muscle owing to the large increase in oxygen consumption. In this case the oxygen is used up by the outer muscle fibres, leaving those in the centre without oxygen. Spectroscopically this is shown by a pronounced cytochrome reduction superimposed on the steady-

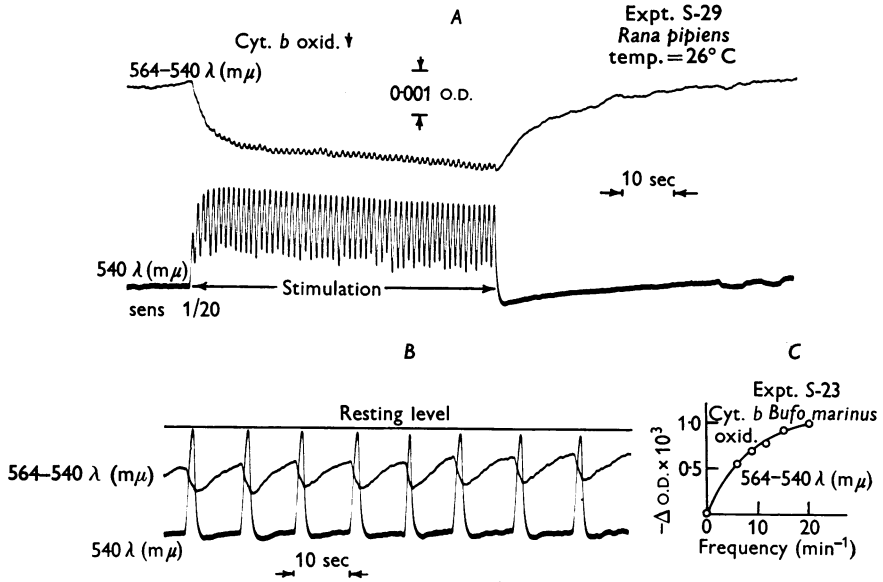

Fig. 4. Changes of cytochrome *b* produced by heart contractions in the frog. *A*, stimulation at 1 per sec: upper trace, cytochrome change recorded as the difference of absorption at 564–540 mμ; lower trace, contractions recorded by the changes of transmittance at 540 mμ. *B*, isolated contractions in the same preparation. *C*, cytochrome *b* oxidation in optical density units versus stimulation frequency in toad heart muscle.

state cytochrome changes due to the muscular contractions. This reduction disappears very slowly after the activity when the oxygen diffuses back again. To obviate this difficulty, stimulation frequencies of 0·5 c/s or less were used.

The behaviour of the other components is similar to that of cytochrome *b*. Fig. 5*A* illustrates the reduction of cytochrome *a* during the activity, recorded at 605–620 mμ. The cytochrome *c* in most preparations becomes more reduced during the contractions but in some experiments its oxidation–reduction level did not change. The flavoprotein and pyridine nucleotide become more oxidized during activity (Fig. 5*B*). The characteristics of the cytochrome changes are about the same at temperatures between 7 and 27° C.

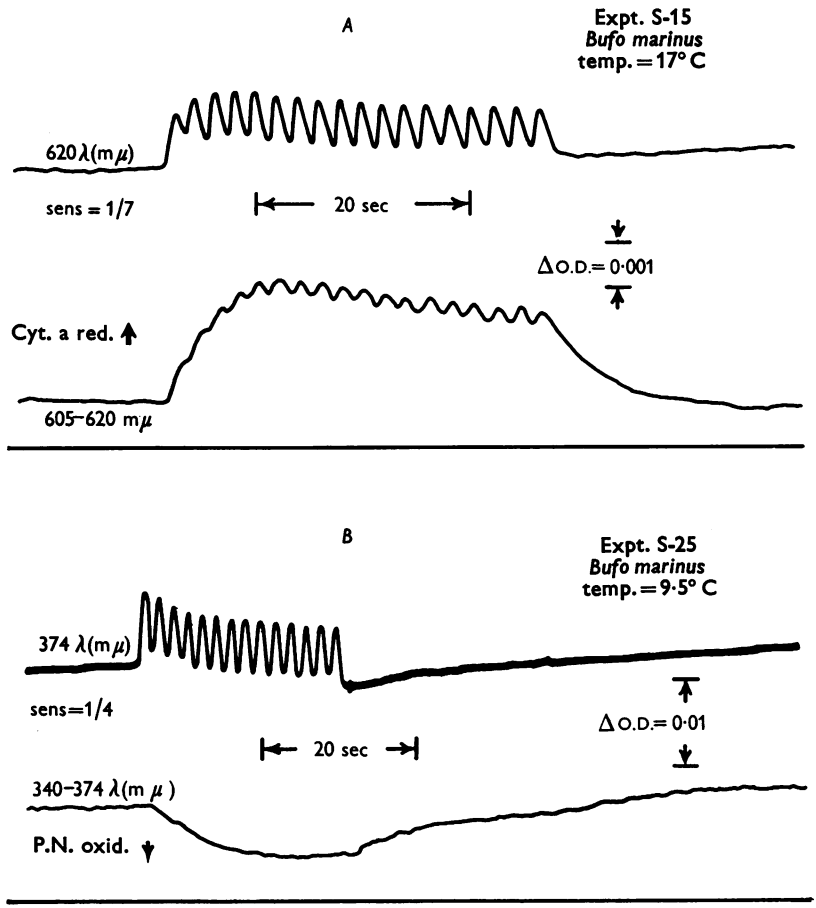

Fig. 5. Changes of cytochrome *a* (*A*) and pyridine nucleotides (*B*) as a consequence of cardiac contractions. The lower curves show the change in the respiratory pigment recorded as a difference of absorption at two wave-lengths; upper traces, changes in transmittance at one wave-length that indicates the mechanical activity. Calibration in optical density units.

TABLE 3. Typical experiment showing the changes occurring in the respiratory pigments as a consequence of cardiac activity*

Pigment	Change in optical density†	Change (μmoles/dm³)	Total concn. (μmoles/dm³)	Change in total concn. (%)	$t_{\frac{1}{2}}$ ON‡ sec	$t_{\frac{1}{2}}$ OFF sec
Cytochrome a_3	−0·0028	−0·51	19·3	−2·7	4	11
Cytochrome *a*	+0·0013	+0·83	24·4	+3·4	3–4	3·5
Cytochrome $c+c_1$	+0·00054	+0·30	26·9	+1·2	4§	6·9§
Cytochrome *b*	−0·0011	−0·57	9·8	−5·8	4	7·5
Flavoprotein	−0·00080	−0·77	38·5	−2·0	5	7·5
Pyridine nucleotides	−0·0052	−9·2	154·0	−6·0	5–6	12

* Stimulating frequency, 0·5/sec; temperature 20·8° C; strip thickness, 0·095 cm.
† +reduction; −oxidation.
‡ $t_{\frac{1}{2}}$ ON, etc., see text.
§ Subject to some uncertainty because of the small change in optical density.

The oxidation of pyridine nucleotide during the contractions in skeletal muscle has been reported by Connelly & Chance (1954). The changes in the various components are summarized in Table 3, and Fig. 8.

Action spectra

As a further step in the identification of the respiratory chain components participating in these reactions, similar observations were repeated at various wave-lengths in a double-beam spectrophotometer, maintaining the reference wave-length constant throughout the measurements. The optical density changes reached at the activity steady state are then plotted against the

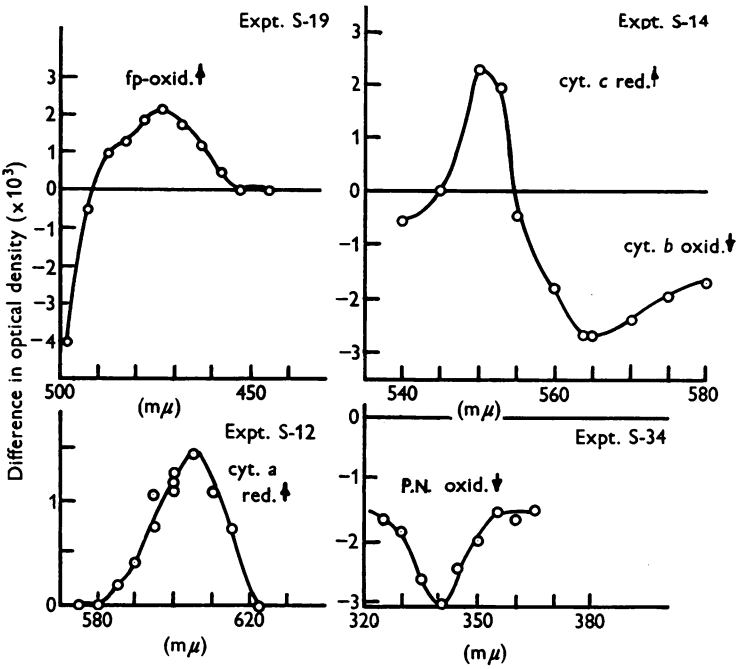

Fig. 6. Maximum change in optical density at different wave-lengths produced by the contraction in the heart (action spectra). Upper curves: left, demonstrates an oxidation of flavoproteins; right, shows a reduction of cytochrome *c* and an oxidation of cytochrome *b* during the activity. Lower curves: left, reduction of cytochrome *a*; right, oxidation of pyridine nucleotides.

wave-length and an 'action spectrum' is obtained indicating at what wave-lengths the maxima and minima occur (Fig. 6). During the contractions a maximum absorption change occurs around 470 mμ, indicating an oxidation of flavoprotein. There is a maximum at 550 mμ and a minimum at 564–565 mμ. The latter is not very sharp, possibly because the non-specific changes in the absorption are relatively larger in that region. These changes indicate a reduction of cytochrome *c* and an oxidation of cytochrome *b*, respectively. The

oxidation of cytochrome *b* has also been observed in the Soret region (*vide infra*). There is also a reduction of cytochrome *a* with a maximum at 605 mμ and an oxidation of the pyridine nucleotides with a minimum at 340 mμ. Since all these maxima and minima correspond to the absorption peaks of the components in question, these spectra provide a good verification of the participation of the members of the respiratory chain during the contractions.

The changes in cytochrome a_3 followed at 445 mμ in muscle deserve special consideration. The action spectrum calculated from the steady-state values during the contractions does not show any distinct trough but only an irregular

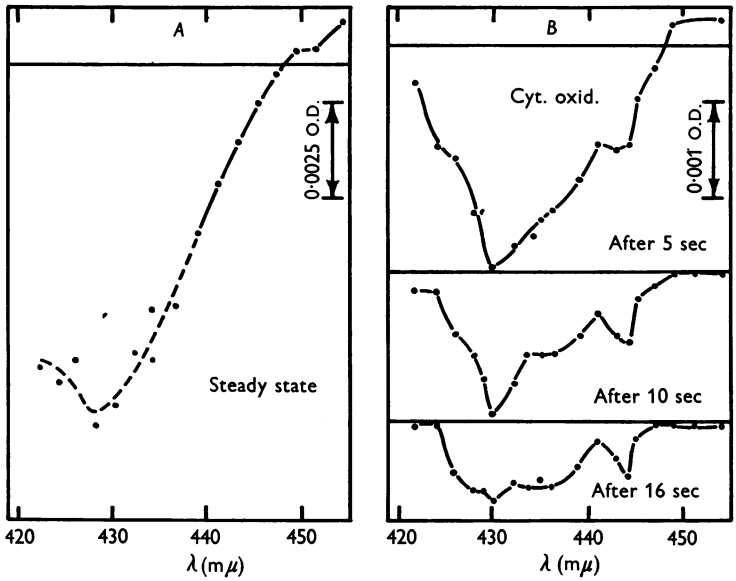

Fig. 7. Decrease in optical density at different wave-lengths in the Soret region as a consequence of muscular contractions in toad's heart. Temp. 25° C, stimulation frequency 0·5/sec, reference wave-length 455 mμ. *A*, maximum changes during the activity steady-state. *B*, changes persistent 5, 10 and 16 sec after the stimulation period; the peak at 444 mμ indicates an oxidation of cytochrome a_3 and the peak at 430 mμ an oxidation of cytochrome *b*.

curve (Fig. 7*A*). If 'action spectra' are calculated from the oxidation–reduction changes at later times after the stimulation, a distinct trough around 444 mμ appears. In Fig. 7*B* this trough is already apparent at 5 sec after stimulation and still remains after 16 sec. The wave-length at which the maximum optical density appears after stimulation in several experiments is tabulated in Table 4. It is between 444 and 445 mμ, and on this basis the absorption band is identified with cytochrome a_3. The extent of oxidation of cytochrome a_3 during the muscular activity is less than the total oxidation obtained by the action of an inhibitor like amytal at concentrations which stop respiration. The lack of a distinct trough during the contractions

is due to a great change in the light-scattering properties of the tissue at the Soret band, where changes of cytochrome a_3 have to be measured. Another factor that tends to mask the trough is the oxidation of cytochrome *b*, which has a maximum absorption at 430 mμ close to the peak of cytochrome a_3. That the return to the resting steady state of cytochrome *b* after the activity is faster than the return of cytochrome a_3 is readily observable from Fig. 7*B*. The appearance of the oxidized trough at 445 mμ in the 'action spectrum' can be seen even at the steady-state level during contractions if $\sim$ 10 μM antimycin *A* (5·4 mg/l.) or 20 μM-HOQNO is present (Ramírez, 1958). These inhibitors decrease the extent of cytochrome *b* oxidation during activity by partially blocking the chain between cytochrome *b* and *c* (Chance, 1956).

TABLE 4. Maximum change in optical density of cytochrome a_3 in the Soret region after the stimulation of the heart

Expt.	Animal	Inhibitor	Wavelength of maximum change (mμ)	Time after stimulation (sec)	Change in optical density units*
S-22	Toad	—	445	2	−0·001
S-23	Toad	—	445	12·5	−0·0003
S-25	Toad	—	445	2·5	−0·0012
S-27	Toad	—	445·5	2·5	−0·0002
S-39	Toad	—	444	6·5	−0·0008
S-52	Frog	—	445	2	−0·0005
S-25	Toad	*A.A*†	445	2·5	−0·006
S-27	Toad	*A.A*	444·5	2·5	−0·002
S-30	Toad	HOQNO‡ 20 μM	445	3	−0·0005
S-21	Toad	NaN_3 100 μM	444	0	+0·0006

* + reduction, − oxidation. † 10 μM antimycin *A*. ‡ 3-hydroxy-1-heptyl-quinoline-N-oxime.

In the presence of 100 μM-NaN_3, the response of cytochrome a_3 during activity is in the direction of reduction rather than oxidation, and in the 'action spectrum' a peak at 445 mμ appears indicating a reduction of cytochrome a_3. This reduction in the presence of azide was shown previously in skeletal muscle by Weber (1957) and also in mitochondria from liver (Chance & Williams, 1956*b*).

Response of the toad's heart sarcosomes to the addition of ADP

Since the behaviour of the toad heart sarcosomes is similar in detail to that of rat heart sarcosomes described by Chance & Baltscheffsky (1958), it will be described only briefly. The sarcosome suspension in an oxygenated Mg^{2+}-free medium (M: sucrose 0·32, Na ethylene diamine tetraacetate 0·001, KCl 0·01, phosphate buffer 0·016, pH 7·4), in the presence of 1–3 mM-Na α-ketoglutarate or Na succinate as substrate, presents a slow respiration. Upon addition of 30–300 μM-ADP the oxygen consumption increases until the ADP is phosphorylated (respiratory control). During this increase cyclic changes of the

respiratory components occur; namely pyridine nucleotides, flavoprotein and cytochrome *b* become oxidized while cytochromes *c* and *a* become reduced. Thus we see that the direction of the changes in the components are the same as those observed in the intact muscle during the contractions. Cytochrome a_3, however, does not change or shows a very small reduction (less than 0·3% of the total concentration) in the sarcosomes, although it shows over a 2% oxidation in the intact muscle. High P:O ratios around 3·7 were obtained with the sarcosome preparations when α-ketoglutarate was used as substrate.

When creatine alone or in the presence of ATP was added to the sarcosome preparation, no changes in the spectra of the cytochromes were observed.

DISCUSSION

Changes in respiratory-chain components and ATP hydrolysis

The similarity of the changes of the respiratory-chain components in heart muscle upon activity and in the sarcosomes, on addition of ADP, strongly suggests the view that ADP is released during the muscular contractions, However, our present data do not allow us to make quantitative comparisons of the energy released by ATP hydrolysis and the total energy expenditure, since the work performed by the heart and the changes in heat production were not measured.

In our conditions the accurate time relationship between contraction and cytochrome changes is very difficult to establish because of the impossibility of completely eliminating the interference of the non-specific optical changes originating during the activity. Nevertheless, we can say that the cytochrome change begins during the contraction phase of the heart twitch.

Comparison with skeletal muscle

The fast changes in the steady-state level of the cytochromes when muscular activity starts and stops (Table 3) indicate a very rapid mechanism of control and interaction between the contractile systems and the energy-producing mechanism of the respiratory chain. This is in contrast to skeletal muscle, where three times as many twitches are needed to reach half the maximum oxidation–reduction change of the respiratory pigments (Chance & Connelly, 1957) and the cytochrome changes persist several minutes after a short tetanus. In fact, the resting steady state in sartorius muscle is reached in some cases after 20–30 min (Weber, 1957; F. F. Jöbsis, personal communication).

There are several factors which might be responsible for the difference in behaviour between cardiac and skeletal muscle.

(1) There are a large number of sarcosomes contained in heart muscle (Weinstein, 1954; Harman, 1956; Sjöstrand, Andersson-Cedergren & Dewey,

1958). The sarcosome size is small, *ca.* 0·8 μ, and they exist along practically every fibril. This morphological arrangement greatly decreases the diffusion time between the fibrils and sarcosomes, thus increasing the synchronization of the sarcosome response with the fibril activity. In the frog's sartorius muscle, however, the sarcosomes are bigger, 10×1 μ, and are rather widely spaced in the muscle, having several fibrils between them. This difference in the number of sarcosomes between skeletal and cardiac muscle is in agreement with the fact that the equivalent concentration of cytochromes is greater in the heart.

(2) There is a low concentration of creatine phosphate (CP) in the cardiac muscle as compared with the five to ten times higher concentration found in skeletal muscle (Clark, Eggleton, Eggleton, Gaddie & Stewart, 1938). Furthermore, in the cardiac muscle the ATP–creatine transphosphorylase activity is six times lower (Oliver, 1955). Hence the competition of the respiratory chain and the ATP–creatine transphosphorylase in the rephosphorylation of ADP is probably not so important in the heart and is in favour of the respiratory chain. In the skeletal muscle, when ADP is produced as a consequence of muscular activity, the ATP–creatine transphosphorylase system could rephosphorylate ADP to ATP and produce creatine (C). Upon cessation of the activity, when the ADP production stops its concentration decreases owing to the action of the respiratory chain. Then the equilibrium of the reaction

$$CP + ADP \rightleftarrows ATP + C$$

catalysed by the ATP–creatine transphosphorylase, is reversed, that is displaced to the left, producing CP and ADP. This ADP interacts with the respiratory chain, where it is finally rephosphorylated. As a consequence of this interaction between the transphosphorylase system and the respiratory chain in the skeletal muscle, the cytochrome changes and the oxygen consumption are prolonged in time.

Moreover, there is some evidence that besides creatine phosphate, the phosphate compounds of carnosine and anserine (Severin & Meshkova, 1953; Meshkova & Zajieva, 1953; Nagradova, 1956) can also rephosphorylate the ADP to ATP in the skeletal muscle whereas the cardiac muscle is devoid of the carnosine–anserine fraction (Schmidt & Cubiles, 1955).

(3) The effect of variations in temperature on the cytochrome changes during the activity in cardiac and skeletal muscle is also different. In the heart muscle cytochrome kinetics between 7 and 27° C are relatively constant. In frog skeletal muscle the amplitude of the cytochrome change decreases with increasing temperature and almost reaches zero at 25° C. (B. Chance, personal communication). As with cardiac muscle, the cytochrome changes in isolated sarcosomes are about the same at different temperatures (Chance & Sacktor, 1958). These data may mean that in skeletal muscle extra-sarcosomal ATP

phosphorylating systems which are temperature-dependent are present, but in cardiac muscle these systems are not active.

All these observations indicate that in the cardiac muscle the main source of resynthesis of ATP is the respiratory chain, and therefore the coupling between the contractile mechanism and the cytochrome system is 'tight'. In the skeletal muscle other systems (creatine, carnosine, anserine) can suddenly produce a high amount of ATP, and in the end the respiratory, or glycolytic, mechanisms 'recharge' these systems and the cytochrome chain in these circumstances works over a considerable period of time to fulfil the instantaneous high energy requirements of the muscle.

Crossover points

When the condition exists between two adjacent components of the respiratory chain such that upon increase of the respiratory rate there is an oxidation of the component on the substrate side and a reduction of the component on the oxygen side, then the point between these two components is

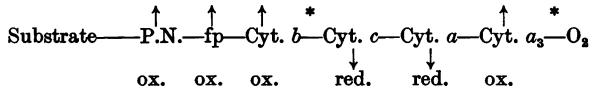

Fig. 8. The respiratory chain. The upward arrows indicate an oxidation, the downward arrows a reduction of the components during the heart activity. The stars signify the crossover points.

called a 'crossover point' (Chance & Williams, 1956*b*). In the intact heart two crossover points in the respiratory chain are found when the electron flow increases upon mechanical activity (Fig. 8). One point is between cytochromes *b* and *c* and the other between cytochrome a_3 and oxygen. In the sarcosomes, upon addition of ADP only one crossover point was identified and that was between cytochromes *b* and *c*. According to the crossover theorem of Chance & Williams (1956*b*), these points indicate sites of phosphorylation, although it is possible for a site of phosphorylation to be located at a place where no crossover appears.

In heart muscle NaN_3 at low concentrations changes the response of cytochrome a_3 during the muscular activity from an oxidation to a reduction which makes the crossover point between cytochrome a_3 and oxygen disappear. A possible explanation of this effect is that the NaN_3 prevents the phosphorylation between cytochrome a_3 and oxygen, since it is known that 10^{-4}M azide decreases the phosphorylation efficiency, that is, the P:O ratio in heart sarcosomes with α-ketoglutarate as substrate (Slater, 1955). However, Chance & Williams (1956*b*) found no decrease in P:O ratio in liver mitochondria oxidizing succinate in the presence of $2{\cdot}76 \times 10^{-4}$M-$NaN_3$ and Loomis & Lipmann (1949) found a decrease in P:2Fe $(CN)_6^{4-}$ ratio only with 1×10^{-3}M azide, in kidney homogenates. If the phosphorylation between cytochrome a_3 and oxygen exists,

then intermediates involved in the reaction are apparently labile since in sarcosomes isolated from toad's heart, in a procedure which gives respiratory control and the usual values of the P:O ratio, no crossover point between cytochrome a_3 and oxygen is found. In addition, it has not been possible to obtain a consistent demonstration of the oxidation of cytochrome a_3 in other types of muscle (toad sartorius, frog sartorius and turtle neck muscle, F. F. Jöbsis, personal communication.) The cardiac muscle could therefore have a uniquely high P:O ratio (i.e. around 4·7 with a substrate like α-ketoglutarate) if the additional phosphorylation site of the cytochrome a_3 level is taken into consideration, since in the isolated sarcosomes the P:O ratio is approximately 3·7. Work is in progress to confirm this point in a more direct way.

The spectroscopically observed oxidation of cytochrome a_3 is not contradictory to the fact that an increase in respiration rate is accompanied by an increase in the concentration of reduced cytochrome a_3 (see Appendix).

SUMMARY

1. The components of the respiratory chain in the intact cardiac muscle of the toad and of the frog have been identified by means of spectrophotometric techniques. The relative concentrations are similar to those in toad heart sarcosomes. The concentrations are 2–3 times higher than in the toad's sartorius muscle.

2. The oxidation–reduction level of the cytochromes in the resting state, including cytochrome a_3, is 92–98% of oxidation.

3. During mechanical activity the oxidation–reduction steady-state levels of the respiratory chain components change very rapidly; the time for reaching half the maximum change is attained during 2–4 contractions. The maximum change is nevertheless very small, about 2–6% of the total concentration. These characteristics are approximately the same for the different components.

4. 'Action spectra' of the heart muscle taken during the contractions show optical absorption changes having maxima and minima corresponding to the peaks of the respiratory-chain components. By this procedure it was shown that pyridine nucleotides, flavoprotein, and cytochromes *b* and a_3 are oxidized during activity and cytochromes *c* and *a* are reduced. In some preparations the cytochrome *c* does not change.

5. The changes of the respiratory-chain components in the sarcosomes on addition of ADP are the same as in contracting muscle. This is in favour of the idea that ADP is released during muscular activity.

6. The finding that cytochrome a_3 does not change on addition of ADP to sarcosomes suggests that there is an additional phosphorylation site in the intact heart muscle, which has not been demonstrated in intact skeletal muscle or in isolated mitochondria.

7. The fast changes during activity of the respiratory components in

cardiac muscle, as compared with skeletal muscle, are interpreted in terms of higher concentration of cytochromes, greater number and smaller size of sarcosomes and lower concentration of creatine phosphate, ATP–creatine transphosphorylase and similar ATP regenerating systems.

8. It is concluded that in the heart the respiratory chain is the main source of ATP resynthesis.

9. The observed oxidation of cytochrome a_3 is compatible with the idea that in order to increase the oxygen consumption the concentration of reduced cytochrome a_3 must increase.

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APPENDIX

The unusual oxidation of cytochrome a_3 during heart-muscle activity can be shown to be consistent with the fundamental equation of oxygen consumption in a cytochrome oxidase system (Chance, Williams, Holmes & Higgins, 1955).

$$-\frac{d[O_2]}{dt} = k_1[a_3''][O_2].$$

The equation states that the oxygen consumption is proportional to the concentration of reduced cytochrome a_3, $[a_3'']$, and to the oxygen concentration $[O_2]$. Since oxygen concentration is constant in our experiments, the cytochrome a_3'' concentration must be increased during the activity in order to explain the increase in oxygen consumption. At first glance this might seem to be in contradiction to the observed cytochrome a_3 oxidation. However,

if a crossover point between cytochrome a_3 and oxygen does imply that there is a phosphorylation at this level, we may postulate the existence of a compound $a_3'' \sim I$, the inhibited form of a_3'', that stores energy in the resting state. It is assumed that spectroscopically there is no difference between a_3'' and $a_3'' \sim I$ (Chance & Williams, 1956*a*), and $a_3'' \sim I$ does not combine with oxygen. During the muscular activity $a_3'' \sim I$ breaks down, owing to the increase in ADP concentration, and therefore the concentration of free a_3'' increases; in consequence the rate of oxygen consumption is raised, as is required by the above equation. Part of the free a_3'' is oxidized, as the spectrophotometric data indicate, but the ratio in these circumstances

$$\frac{\text{free } [a_3''] \text{ (activity)}}{\text{free } [a_3''] \text{ (rest)}} > 1,$$

and the respiratory increase is proportional to the newly released a_3''.