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Genetic disease of mitochondrial function evaluated by NMR and NIR spectroscopy of skeletal tissue

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Abstract

Bioenergetic sufficiency can be quantitatively assayed by nuclear magnetic resonance spectroscopy (MRS) and on a relative basis by tissue optical spectroscopy (NIRS). Nuclear magnetic resonance measures quantitatively the fall of phosphocreatine and the rise of inorganic phosphate necessary to raise mitochondrial adenosine diphosphate and activate ATP synthesis to adequate level to meet metabolic demands. This relationship is readily demonstrated in skeletal muscle where the quality of supply and demand for ATP is observed over a wide range of aerobic exercise. Metabolic and genetic disease of mitochondria is readily detected by the rapid fall of PCR and rise of P_i during mild exercise and has been essential in the diagnosis and therapy of deficiency of cytochrome bc_1 in human skeletal muscle. Insufficiencies of oxygen utilization in relation to oxygen delivery are readily measured optically by the simplest of dual wavelength spectrometers. Instead of deoxygenating hemoglobin during exercise in cases of normal bioenergetic function, a luxury perfusion or hyperoxygenation of skeletal muscles occurs in exercising the energetically deficient skeletal tissue. In this way, a simple screen for metabolic and mitochondrial disease of energy production has been established and demonstrated in a number of clinical cases. Thus, the combination of the absolute evaluations by NMR and the relative indications of light of spectroscopy (NIRS) form essential tools in detection of mitochondrial defects.

Keywords: Mitochondrial function; NMR spectroscopy; NIR spectroscopy; Skeletal tissue

1. Introduction

Two non-invasive methods are under study for the detection and quantitation of functional disability of skeletal muscle mitochondria in the production of ATP from oxygen reduction: optical measurements of oxygen delivery and oxygen utilization on the one hand, and nuclear magnetic resonance spectroscopy of the mitochondrial capability, on the other hand. The methods have direct clinical application as non-invasive methods of tissue study, both applicable in short times ranging, respectively, from 5 min for the optical method to a half an hour for nuclear magnetic resonance.

Fig. 1 illustrates the rationale for the optical method. Here the principle is the relationship between oxygen delivery to a functional tissue ($D(O_2)$) and oxygen utilization by the normal or damaged mitochondria ($V(O_2)$).

NIR spectroscopy of the tissues indicates hemoglobin deoxygenation in the arteriolar, capillary, and venolar bed of the exercising tissue where oxyhemo-globin deoxygenation and delivery to the tissue occurs in amounts regulated by the oxygen demand of the mitochondria.

The needs for energy metabolism of the functional muscle are set by the demands of external work which may be constant or graded and, as shown below, are usually established by exercise of the gastrocnemius in treadmill walking exercise [1]. ATP usage by the myofibrils breaks it down to ADP + P_i which activates the creatine kinase equilibrium to restore ATP levels by expending mitochondrial ATP and returning ADP and P_i to the mitochondria. The mitochondria themselves operate in a feedback loop where the ADP activates mitochondrial electron transport and ATP synthesis to return ATP to the creatine kinase equilibrium, which in turn activates respiration and mitochondrial oxidative phosphorylation.

These two important feedback controls are a part of functional activity in case insufficient oxygen is present to provide the needs of the creatine kinase equilibrium and

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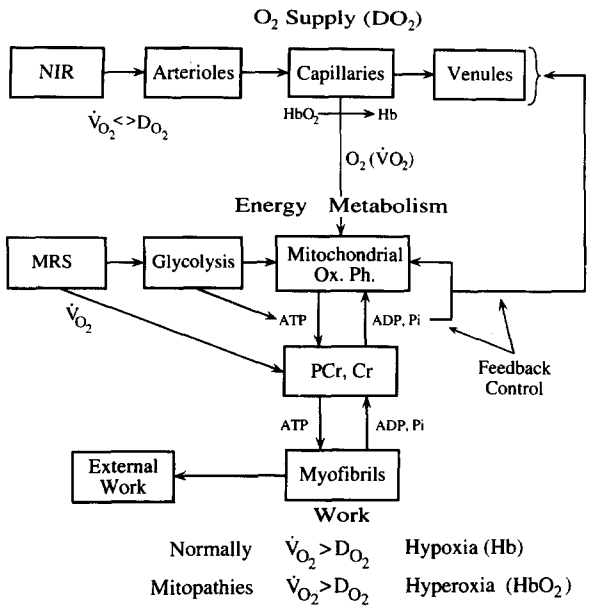

Fig. 1. Rationale of non-invasive studies of mitochondrial dysfunction by measurement of inequalities of tissue O_2 uptake ($V(O_2)$) and tissue O_2 delivery ($D(O_2)$).

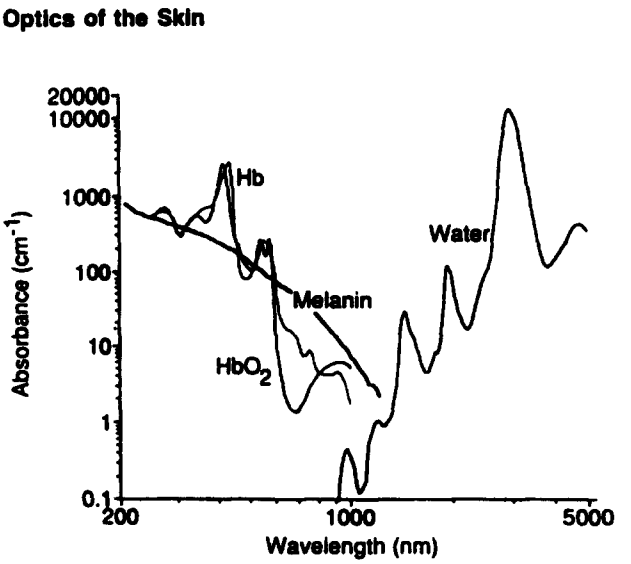

Fig. 2. Absorption spectra of major skin chromophores. The values shown are absorption coefficients for pure water, human blood at 11 g/100 ml hemoglobin concentration (oxygenated and deoxygenated), and DOPA-melanin, which has a 300–1200 nm spectrum that is very similar to pigmented epidermis, at 15 mg/100 ml concentration in water. The DOPA-melanin concentration shown is approximately equivalent to heavily pigmented (dark brown) human epidermis [4].

myofibrils in turn. The first is glycolysis which provides ATP, if ADP rises above its K_m and mitochondrial activity is no longer adequate to maintain ATP. Furthermore, as oxygen utilization rises to the limit of oxygen delivery, signaling to the microvascular systems, occurs through the rising level of ADP, AMP and adenosine, triggering vasomotor response in the arteriolar system to increase flow, blood vessel volume and oxygen delivery.

This homeostatic system can be viewed from the standpoint of the degree of oxygenation of hemoglobin. The steady state oxygenation level of hemoglobin is dependent upon the relative magnitudes of $D(O_2)$ and $V(O_2)$. Under physiological conditions, $V(O_2)$ is greater than $D(O_2)$ and exercise induces a mild hypoxia leading to hemoglobin deoxygenation to a large extent. On the other hand, in mitochondrial disease, incapability of the mitochondria to utilize oxygen to form ATP is expressed by the condition of low $V(O_2)$, massive but futile signaling for increased O_2 and a hyperoxia where the HbO_2 levels can be near saturation. In summary, the rationale for the optical method is to observe HbO_2 to be low in normal tissue, and to be high in bioenergetically inefficient tissue, both under conditions of exercise.

2. Methodology

The near infrared portion of the optical spectrum was shown first to be useful for transmissions through tissues by Matthes and Gross [2] and was exploited by Jobsis for propagation of photons through the neonate head [3]. The rationale for the use of this portion of the spectrum is illustrated by the window displayed in Fig. 2 which indicates that not only is Hb absorption smaller in this region, but that of melanin decreases and that of water has not increased sufficiently with increasing wavelengths in the region 700–800 nm to cause difficulties [4].

Furthermore, Fig. 3 shows that the dual wavelength technology for balancing increases of absorption at one wavelength due to Hb deoxygenation (760 nm) against decreases of absorption at a second wavelength (850 nm) due to Hb oxygenation, gives a balanced response employed in dual wavelength spectrophotometers since 1951 [5]. At the same time, measurement of the sum of the absorbency at the two wavelengths will measure the total hemoglobin or blood concentration by making small ad-

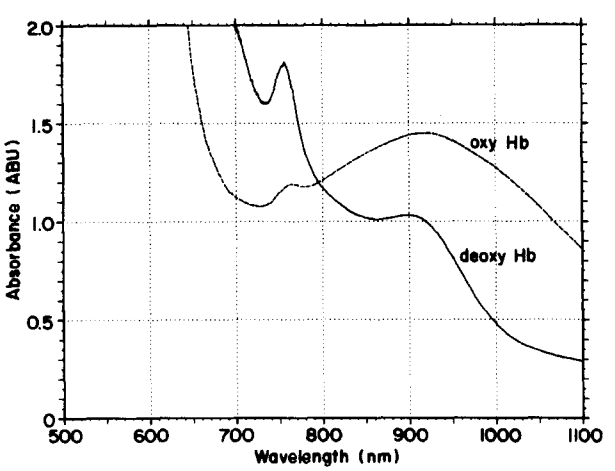

Fig. 3. Absorption spectrum of oxy and deoxy hemoglobin at 700 to 1100 nm region.

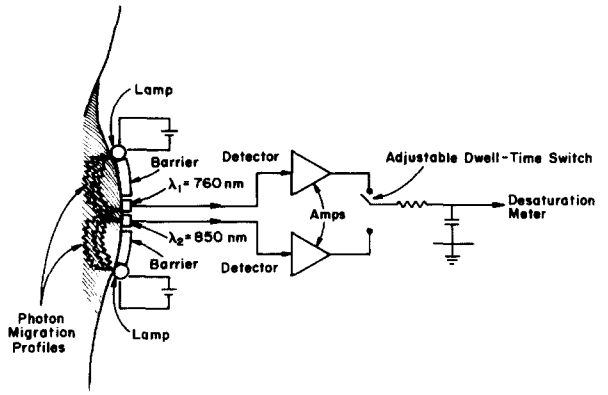

Fig. 4. Illustration of photon migration patterns of the DC tissue hemoglobinometer using 760 and 850 nm wavelength of the detectors and white light for the illumination. The mean penetration of the optical photon migration patterns is approximately half the distance between the light sources and the detectors.

justments in the relative contributions to ensure freedom from crosstalk between the deoxygenation signals and the blood concentration signals.

Thus, a simple differential spectrophotometer will operate satisfactorily as shown in Fig. 4 where dual light sources (only for averaging the signals from a large volume of muscle tissue) are separated from the pair of detectors at 760 and 850 nm as determined by evaporated film, optical filters, and silicon diode detectors. The output is connected to a differential circuit for measuring the difference of absorbances and (not shown) the sum of absorptions at the two wavelengths.

Fig. 5 illustrates the entry of photons by a diffusion process into the tissue in two banana shaped patterns characteristic of the light source detector pattern employed here, the depth of penetration is half the distance between light source and detector. Thus, for a separation of 4 cm, a penetration on the average of 2 cm is obtained. The

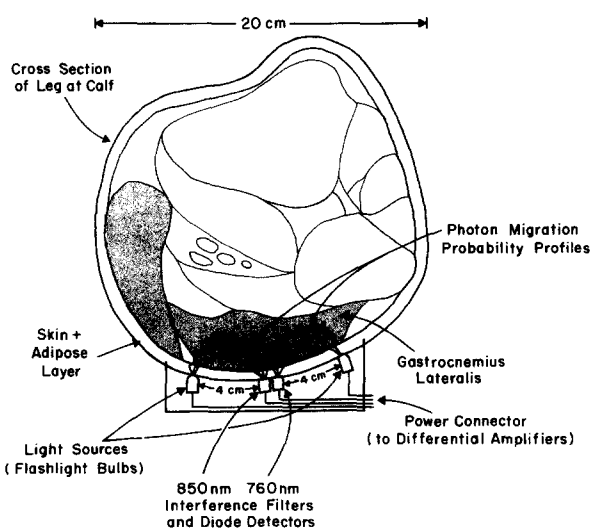

Fig. 5. Illustrating the relationship of the optical pattern with the portions of the medial and lateral gastrocnemius.

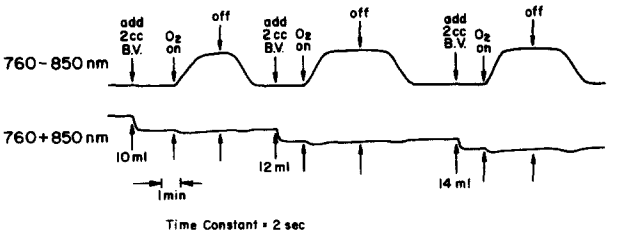

Fig. 6. Illustrating the response of the difference channel to hemoglobin oxygenation to the exclusion of blood concentration increases (top trace) and the response of the sum channel to increases of blood concentration to the exclusion of changes of hemoglobin deoxygenation.

characteristics of the migration pattern are evaluated by solutions of the diffusion equation obtained by finite element methods or, conveniently, by Monte Carlo simulation as carried out especially by my colleagues Feng and Zheng [6].

3. Tests of the efficacy

Tests of the efficacy of the device in measuring hemoglobin deoxygenation and blood concentration changes without crosstalk interference are obtained by a 7–10 cm diameter circular vessel filled with a scattering material simulating that of muscle (0.5–1% intralipid), hemoglobin in the amount characteristically observed in skeletal tissue by the optical method around 100 μM, and finally yeast cells to simulate tissue respiration. Thus, Fig. 6 shows, from left to right, serial additions of blood causing downward jogs of the blood volume trace, serial oxygenations of deoxyhemoglobin showing upward cyclic responses since respiration removes the added oxygen after a minute or so in three additions of oxygen and three additions of blood. It is noted that negligible impact of blood addition to the oxygen trace and of oxygen addition to the blood volume trace is observed. Thus, the function of an optical device in measuring hemoglobin oxygenation, on the one hand, and blood concentration change, on the other, is distinctive.

4. Response of normal gastrocnemius to treadmill exercise

The application of the small optical pickup and a small differential amplifier to the human leg is indicated, where the device is held in place by Velcro elastic straps, and the optical unit is positioned over the lateral portion of the gastrocnemius (Fig. 7), which is appropriately activated in the power stroke of walking exercise, usually on the typical treadmill operated for the study of mitochondrial disease at low speed, i.e., a few miles an hour [1]. Typical responses to exercise are shown in Fig. 8 where upper deflection of the differential trace indicates deoxygenation

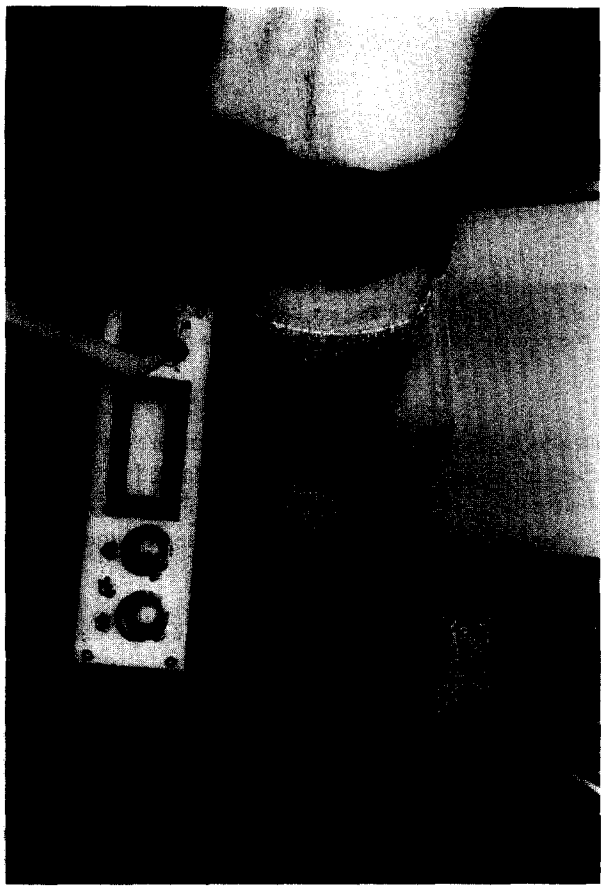

Fig. 7. Illustrating the attachment of the optical probe to the gastrocnemius in the adult human subject together with differential amplifier; the amplifier providing the difference and the sum of absorbances at the two wavelengths.

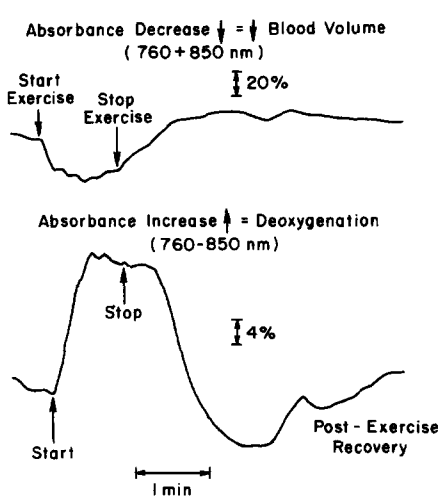

Fig. 8. Illustrating the optical responses of the sum and difference traces to treadmill exercise of the left gastrocnemius of a normal adult, approx. 70 years old. The upper deflection of trace B indicates deoxygenation of hemoglobin (and myoglobin) during exercise with delayed recovery following cessation of exercise. The blood concentration changes show the diminution of blood concentration upon the start of exercise and the refilling of the limb thereafter.

of the tissue hemoglobin occurring promptly on start of exercise corresponding to an absorbance change of 0.09 units. The deoxygenation starts promptly on the initiation of walking exercise and is maintained over the interval of exercise. On cessation of exercise, recovery of oxygenation occurs with a time characteristic of the activity of mitochondrial energy metabolism. Correspondingly, blood concentration is decreased on initiating extra blood flow through the limb and is recovered on cessation of exercise as stasis of blood flow allows blood to accumulate in the limb. In this case, the normal response shows the $V(O_2)$ is greater than $D(O_2)$ and extensive deoxygenation occurs. More severe deoxygenation and a slower recovery occur in the case of diseased limb with impaired $D(O_2)$ and in which there is normal $V(O_2)$, and $V(O_2) \gg D(O_2)$.

5. Responses to mitochondrial dysfunction

Fig. 9 illustrates the paradoxical response of the limb in which oxygen uptake is compromised by deletion of cytochrome oxidase in the majority of the tissue mitochondria. Initiation of treadmill exercise activates a small $V(O_2)$ in the gastrocnemius muscle and a massive breakdown of ATP as indicated by signaling for increased oxygen delivery to the tissue incompetent oxygen demand, i.e., $D(O_2)$ is greater than $V(O_2)$ (see Fig. 1). The oxygenation of hemoglobin occurs promptly upon initiating exercise as shown by the downward deflection of the trace. The oxygenation remains at a plateau value over the duration of the exercise and promptly, on cessation of exercise, a similar paradoxical deoxygenation occurs with a return to a level of slightly more deoxygenation than the rest value. Thus, a qualitative difference between the response of the limb containing normal mitochondria and dysfunctional mitochondria is shown by the comparison of Fig. 8.

A second feature of the limb containing incompetent mitochondria is shown by Fig. 10 where cuff ischemia was employed to identify the nearly completely deoxygenated state of the limb at rest. The cuff is applied during the walking exercise where hemoglobin is highly oxygenated. Cuff application causes a prompt deoxygenation to a level only slightly in excess of the resting state prior to exercise. Release of the cuff allows a transient oxygenation which subsides to the initial resting level.

Similar application of the cuff to the resting normal limb would show a highly significant deoxygenation, much greater than that of the diseased limb. It is apparent that the oxygen demand of the diseased limb is sufficiently low that the set point for hemoglobin deoxygenation is largely deoxygenated, yet a suitably low concentration of oxyhemoglobin adequate to supply the respiratory deficient mitochondria is available. Thus, two paradoxical optical signals are available for the detection of mitochondrial dysfunction, a set point for oxygenation that is highly deoxy-

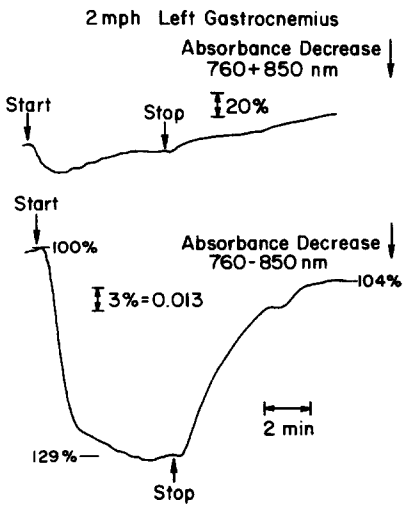

Fig. 9. Illustrating the paradoxical response of the limb of a cytochrome oxidase deficient patient through treadmill exercise. The same apparatus configuration as in Fig. 8 is used, however, the response of the oxygenation trace is now in the direction of increased oxygenation during exercise and decreased oxygenation during recovery. The blood concentration changes are similar, i.e., a decrease of blood concentration on initiating exercise, however, the recovery is considerably prolonged, over that of the normal control.

genated at rest and a prompt and large oxygenation initiated on starting exercise. Both of these responses are the converse of that observed in the normal muscle containing functional mitochondria.

This method has been used in preliminary studies of the detection of mitochondrial disease; cytochrome oxidase disease as already shown, in McArdle's disease, PFK

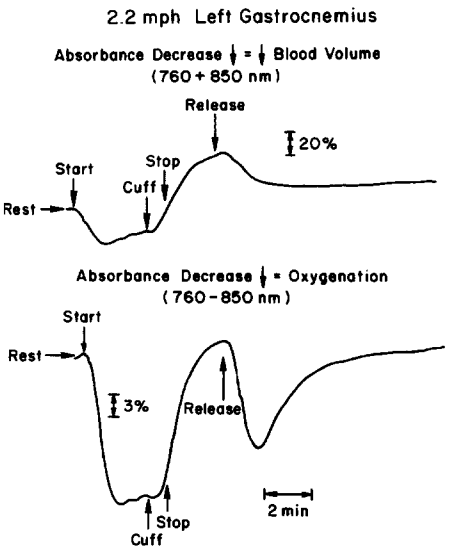

Fig. 10. Illustrating the effect of cuff ischemia on the oxygenation of the limb of the cytochrome oxidase deficient subject. It should be noted that the application of the cuff at the time of cessation of exercise creates a deoxygenation only slightly in excess of that of the resting state and release of the cuff causes a momentary oxygenation which subsides to the resting level. Thus, cytochrome oxidase deficient limbs have a resting deoxygenation almost equal to that of cuff ischemia.

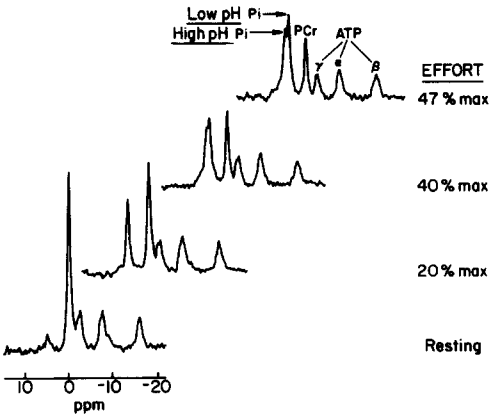

Fig. 11. Illustrating the high PCr/P_i ratio of the resting limb and the near equality of PCr and P_i in the exercised limb.

deficiency, all of which show the response characteristic of $V(O_2)$ less than $D(O_2)$. A disease minimally affecting mitochondrial capability for ATP synthesis, carnitine phosphoryltransferase, gives a normal response of $V(O_2)$ greater than $D(O_2)$ as do normal controls.

Obviously the census of subjects is inadequate for a thorough going statistical study and cross referrals from various laboratories and clinics are highly desirable.

6. NMR study of $V(O_2)$ as a measure of mitochondrial dysfunction

Referring to Fig. 1, we can see that MRS can measure the deficiency of $V(O_2)$. The deficiency must be measured on an absolute scale and is not referred to $D(O_2)$ as in the case of the optical hemoglobin study.

The rationale for the measurement of $V(O_2)$ by Nuclear Magnetic Resonance is illustrated in Fig. 11 and is also explained in detail in the accompanying presentation by my colleague G.K. Radda [7]. Fig. 1 illustrates the role of creatine kinase in equilibrating the energy demand of the myofibrils and the energy production of the mitochondria. Thus, the level of PCr and P_i, both readily detected by NMR, gives the balance of energy production and energy need for accelerated oxidative metabolism of the mitochondria. For example, in Fig. 11 [8], the resting state of normal mitochondria shows a very high PCr, P_i whilst the exercise limb diminishes PCr and raises P_i to the point of approximate equality.

A measure of $V(O_2)$ is obtainable from the maximum value of P_i/PCr as a function of work load as shown in the example of Fig. 12 [9]. This chart illustrates the hyperbolic relationship between work, external work (see Fig. 1) and the displacement of the creatine kinase equilibrium by ATP breakdown in the myofibrils as balanced against ATP synthesis in the mitochondria. As pointed out by Dr. Radda, this imbalance is represented by a control signal or error signal for the adjustment of ATP synthesis by the

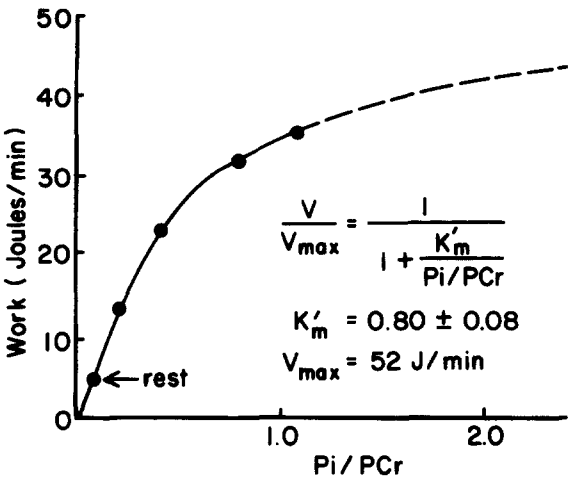

Fig. 12. Illustration the hyperbaric relationship between external work in Joules per min and the rise of P_i/PCr tending towards a plateau value at the maximum value of $V(O_2)$. ^{31}P-NMR graded exercise protocol, right arm test [9].

mitochondria exactly to balance ATP depletion by the myofibrils. The ADP level adjusts the rate of ATP synthesis so that perfect balance is obtained over the range of the hyperbolic relationship, the balance being nearly perfect in the linear portion of the curve and increasingly imperfect as the plateau of the relationship is reached. It is not possible to operate the muscle at the maximum rate of mitochondrial ATP synthesis because the gain of control loop is inadequate to maintain homeostasis. Instead, extrapolation to $V(O_2)_{max}$ is obtained with the usual type of reciprocal plots, and a typical Hanes plot for three normal subjects is shown in Fig. 13. This plot affords, as the intercept on the x-axis, the K_m for ADP which is assumed to be the same for all subjects because it is an intrinsic

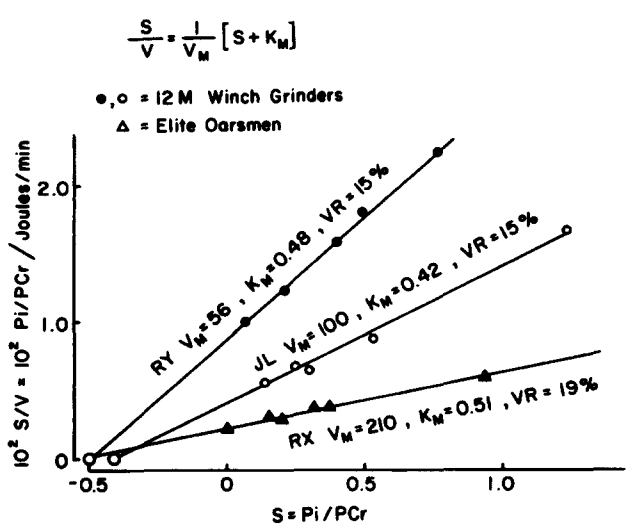

Fig. 13. Application of Hanes plot analysis of arm exercise of three subjects, two athletes engaged in Yacht racing and one elite oarsman. It is noteworthy that the zero intercept of these two occurs at the approximate K_m of mitochondria for ADP in terms P_i/PCr, approx. 0.5 whilst the inverse slopes give their maximal value [9].

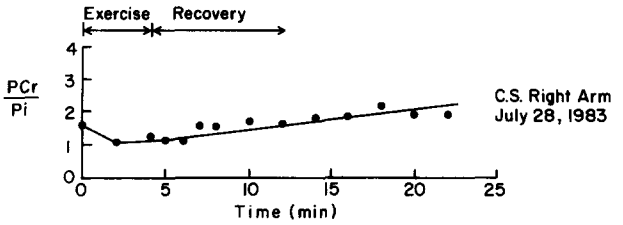

Fig. 14. Illustrating a subject exhibiting cytochrome bc_1 deficiency who has initially a low PCr/P_i (1.8) and exercise causes very little diminution PCr/P_i. Finally the recovery time is of the order of 15 min for the cytochrome oxidase deficient right arm [12].

property of the mitochondria. The V_{max}, however, differs depending upon the amount of mitochondria which is maximal for the endurance performer (lowest trace), less for the 'weight lifter', and least for the football player. The method is sensitive to the amount of functioning mitochondria that are mobilized in the particular exercise regime. There are few methods of verifying the V_{max} of the mitochondria that are equally accurate, appropriately localized and non-invasive.

Correlations with the NIR method would show minimal deoxygenation in the slow twitch endurance performer, and maximal deoxygenation in the fast twitch 'weight lifter' muscle [9].

7. Application to bioenergetic dysfunction

Drs. Buist, Kennaway and Capaldi [10,11] obtained biopsy material, enzymatic analysis, and DNA studies on a 17-year-old lady who was unable to walk and showed severe fatigue symptoms. She was referred to the Johnson Foundation for NMR examination to verify the biopsy findings [12]. On presentation, she was a wheel chair case able to do wrist flexor in-magnet studies against a calibrated weight set at the minimum value. The remarkable feature of her NMR examination shown in Figs. 14 and 15 was the low PCr/P_i value which was initially 1.8 and, on wrist flexor exercise fell to 1.0. Equally remarkable was the fact that her recovery time was very prolonged, the halftime of over 10 min, whilst usual recovery times are as short as half a minute, for normal subjects. Thus, immedi-

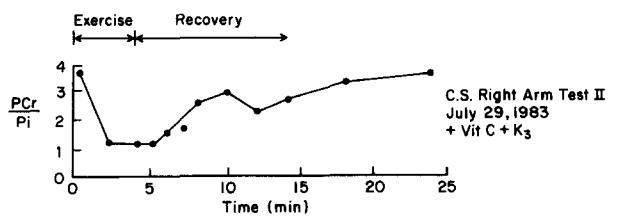

Fig. 15. The effect of vitamin K_3 + C supplement to the right arm test (carried out on the same day as the control study). It is seen that the resting value of PCr/P_i is nearly 4 and a fall to the same level as in the previous study during exercise, but with recovery time of approx. 5 min [12].

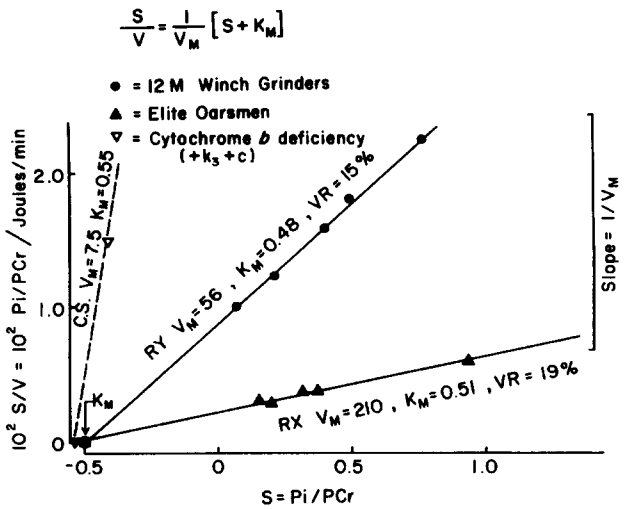

Fig. 16. Hanes plot of the exercise performance of the mitochondria deficient subject following supplementation with K_3 and C to have a V_{max} of 7.5 as compared with normal subjects in the range of 50–200 [9].

ately the diagnosis of a severe bioenergetic deficiency was verified to the satisfaction of those supervising the tests on July 28, 1983. The same day it was decided that the biochemical bypass of the cytochrome bc_1 site afforded by vitamin K_3 with ascorbate as a reductant to ensure that K_3 was in the reduced state was prescribed by the attending physicians. Thereafter, the subject was retested. The PCr/P_i value was now as high as 4 and was depleted rapidly with wrist flexor exercise to the previous value of 1.0, from which there was a bypasic recovery, the larger portion of which occurred in less than one minute, and the later portion in several minutes. Thus, an immediate effect of the 'biochemical bypass' was observable.

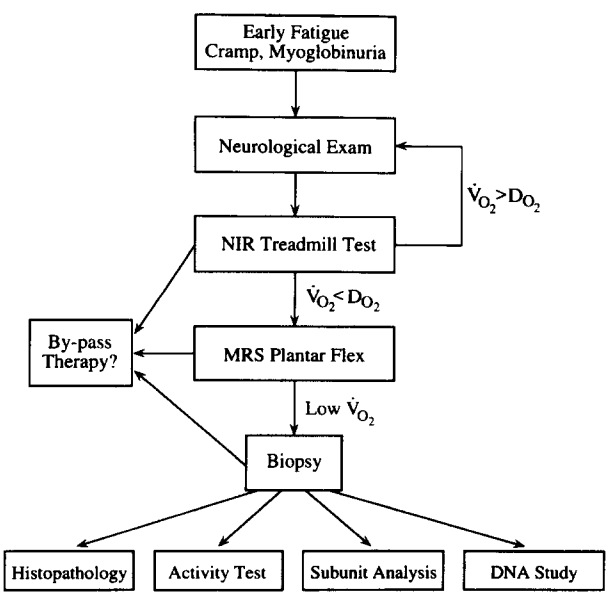

Fig. 17. Illustrating the rationale of the clinical study of patients with suspected bioenergetic deficiencies at the Neurological Clinic HUP.

In order to quantify the $V(O_2)$ available from the $K_3 + C$ supplemented limb, the Hanes plot is presented in Fig. 16 and the very sharp slope of the inverse plot testifies that in spite of encouraging effect of the bypass, the $V(O_2)_{max}$ was still a small fraction of full functional controls shown to range from 56 to 210. In a subsequent session, it was verified that the therapy was still operative, and indeed a most important dose response curve was obtainable. In the ensuing 11 years, the subject has led a life of restricted activity, but normal in many respects, walking ability is significant as is modest stair climbing ability. Further tests are contemplated to re-determine the dose curve and to examine this subject for the first time with NIR.

8. Discussion

The NMR method represents a gold standard of quantitative determination of the functional activity of mitochondria whose ATP production is stimulated by ADP to meet the needs of muscle function in the bioenergetic deficient limb. In the case of bioenergetic deficiency initially observed of cytochrome bc_1 deletion, there was no hyperbolic relationship to be obtained without therapy, PCr/P_i was low and lowered only slightly with minimal exercise. Thus, on a scale of Hanes plot above, it is estimated that the $V(O_2)$ was of the order of 1, a few percent of the minimum normal response and a fraction of a percent of the maximum normal response. Thus, the dynamic range of the NMR method is excellent for quantifying small residual ADP stimulated ATP production in the human limb, and should be applied wherever possible in the initial phases of the work-up of a mitochondrial/metabolic disease.

The NIRS method is of similar high sensitivity but gives a measure of stimulated respiratory, relative to the stimulation of oxygen delivery, and indeed is highly sensitive, perhaps more so to the function of severely dysfunctional limbs, and is of course convenient, safe, and economical to employ.

8.1. Clinical approach

On the basis of several years of collaborative study with Dr. William Bank of Neurology, we have developed a rationale for study which may be useful to others (Fig. 17) [13].

The patient population obtained from the Neurologic Clinic and from numerous referrals often complain of chronic fatigue, early fatigue, cramps following exercise and frequently myoglobinuria. Following a neurological examination, the subjects are transferred to the Johnson Foundation in a wheel chair in order to ensure that they are in a 'resting state' prior to the NIR examination. In that examination, they remain seated while the probe is applied

to the leg, and rise to a standing position 30 s before the treadmill is operated and walk at the slowest value, 2.2 miles per h on the treadmill. Exercise at that level is acceptable but often the speed is doubled in case the response is ambiguous. Thereafter, the patient is immediately reseated and allowed to recover. Several examinations can be made providing the exercise is for a short interval, i.e., 2 or 3 min.

The results are directly interpreted as to whether the oxygen uptake exceeds the oxygen delivery. In this case, the patient is returned to the Neurologic Clinic for further examination for neurological defects which give the symptoms of fatigue but may not involve a defect of energy metabolism.

In those in which the oxygen uptake is less than oxygen delivery during exercise, where possible, an MRS examination is prescribed and a determination of the V_{max} on an absolute scale can be obtained for the particular limb under study. In this case, plantar flexion may often be used and calibration for normals for plantar flexion is employed. If a low $V(O_2)$ of the NIR test is verified by MRS, the biopsy is prescribed and histopathology, enzymatic activity tests, subunit analysis, and DNA study are carried out to determine the site of the lesion. If this study identifies a cytochrome bc_1 deletion then therapy with K_3/ascorbate seems appropriate.

Other therapies may also be considered and post therapy examination particularly by MRS and NIRS as well are necessary to evaluate the effectiveness of any putative or real therapeutic procedures. In view of the increasing population of clinical cases involving mitochondrial and metabolic dysfunction, this procedure is recommended since the NIRS test is simple, rapid and effective and provides solid justification for the more expensive and lengthy and the subsequent biopsy procedure and tissue biochemistry study.

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