ATP synthesis and proton handling in muscle during short periods of exercise and subsequent recovery

David Bendahan,1 Graham J. Kemp,2 Magali Roussel,1 Yann Le Fur,1 and Patrick J. Cozzone1

1Faculté de Médecine, Centre de Resonance Magnetique Biologique et Medicale, Unité Mixte de Recherche 6612 Centre National de la Recherche Scientifique, Marseille 13005, France; and 2Department of Musculoskeletal Science, University of Liverpool, Liverpool L69 3GA, United Kingdom

Submitted 2 July 2002; accepted in final form 28 January 2003

ALTHOUGH THE NONINVASIVE TECHNIQUE of 31P-magnetic resonance spectroscopy to study proton buffering in finger flexor muscles of eight healthy men (25–45 yr), during brief (18-s) voluntary finger flexion exercise (0.67-Hz contraction at 10% maximum voluntary contraction; 50/50 duty cycle) and 180-s recovery. Phosphocreatine (PCr) concentration fell 19 ± 2% during exercise and then recovered with half time = 0.24 ± 0.01 min. Cell pH rose by 0.058 ± 0.003 units during exercise as a result of H⁻ consumption by PCr splitting, which (assuming no lactate production or H⁺ efflux) implies a plausible non-Pi buffer capacity of 20 ± 3 mmol·l⁻¹ intracellular water⁻¹·pH⁻¹ unit⁻¹. There was thus no evidence of significant glycolgenolysis to lactate during exercise. Analysis of PCr kinetics as a classic linear response suggests that oxidative ATP synthesis reached 48 ± 2% of ATP demand by the end of exercise; the rest was met by PCr splitting. Postexercise pH recovery was faster than predicted, suggesting “excess proton” production, with a peak value of 0.6 ± 0.2 mmol/l intracellular water at 0.45 min of recovery, which might be due to, e.g., proton influx driven by cellular alkalinization, or a small glycolytic contribution to PCr resynthesis in recovery.

bioenergetics; buffer capacity; glycolgenolysis; phosphorus-31 magnetic resonance spectroscopy; skeletal muscle

Despite the costs of measurement in vivo, except indirectly by using 31P-MRS (1). We earlier used an analysis of ischemic exercise (13) to estimate β in human muscle, which was complicated by its pH dependence. Here we set out to minimize such complications by studying the early phase of the rest-exercise and exercise-rest transitions by using 31P-MRS. Because of their bearing on the analysis of ATP turnover, we also consider some quantitative implications of recent proposals that rapid cycles of PCr splitting and resynthesis (3), fueled by anaerobic glycolgenolysis (29), operate during muscle contraction, unobservable by conventional 31P-MRS or biopsy methods. Part of this work has been presented in preliminary form (12).

METHODS

Subjects. The study was conducted on the dominant forearm of eight male volunteers, aged 25 to 45 yr. Subjects were not involved in any arm training and had no physical limitation to exercise. Their written, informed consent was obtained for the study, which was approved by the local ethics committee.

Exercise protocol. During training sessions performed several days before MRS studies, maximum isometric finger flexion force was measured until three reproducible values were sustained for 3 s. 31P-MRS investigations were carried out as previously described (13) by using a Bruker 47/30 Biospec spectrometer interfaced to a 30-cm bore, 4.7-T superconducting magnet. Subjects sat on a chair with the dominant arm, restrained with Velcro straps, resting in the magnet bore at shoulder height (to ensure good venous return). Magnetic field homogeneity was optimized by monitoring the signal from water and lipid protons at 200.14 MHz. Pulsing conditions (1.5-s interpulse delay, 120-μs pulse length) were chosen to optimize the 31P signal obtained with a 50-mm-diameter surface coil (double-tuned for 31P and 1H) positioned over the belly of the flexor digitorum superficialis muscle at the maximum diameter of the forearm. Spectra were time-averaged over 6 s (4 scans). After 10 spectra were recorded at rest (60 s), subjects performed finger flexion at 1.5-s intervals for 18 s (3 spectra), followed by 180-s recovery (30 spectra). This sequence was repeated three times consecutively. Exercise consisted of lifting a weight adjusted to 10% maximum voluntary contraction in a 50/50 duty cycle (i.e.,

1. Morgan. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
0.75-s contraction, 0.75-s relaxation). Contraction-relaxation cycles were gated to magnetic resonance spectra acquisition by using a home-built trigger, with spectra being recorded from the start of contraction. The sliding amplitude and frequency of the weight were recorded by using a displacement transducer connected to a personal computer running ATS software (SYSSMA-FRANCE). Calculated power output was averaged over each 6 s of exercise (i.e., one spectral accumulation).

Calculation of pH and metabolite concentrations. As described elsewhere (13, 23), MRS signals were processed by using NMR1 software (New Methods Research) by 15-Hz line broadening, baseline correction, Lorentzian peak-fitting, and correction for differential magnetic saturation. Absolute concentrations were calculated assuming [ATP] = 8.2 mmol/l intracellular water (brackets denote concentration). Free [ADP] was calculated from the creatine kinase equilibrium, intracellular water (brackets denote concentration). Free lactate and that lactate production is negligible, so that H⁺/H11001 is relevant to the stoichiometric calculations, which are described below. To test the possibility that this change during the rest-exercise and exercise-rest transitions, we monitored the chemical shift of the central resonance of β-ATP (relative to PCr at −2.45 parts/million).

Kinetic analysis. To obtain the half time of PCr recovery, we fitted the time-dependent [PCr] changes during the exercise-rest transition to a monoexponential equation (24) and assumed that the PCr resynthesis rate approximates that of oxidative ATP synthesis at the end of exercise (10). The same approach is used to estimate the initial rate of PCr depletion in exercise (a measure of total ATP turnover) (9). Due to uncertainty about steady-state [PCr] in such short exercise, a free fit is unreliable, and we constrained the half time to be the same as in recovery, in accordance with a standard model of aerobic exercise (24). In both cases, we corrected for the bias involved in sampling an exponential time course with finite time resolution (30). Because we will argue that glycolytic ATP production is negligible during exercise, we estimated oxidative ATP synthesis rates as the difference between the instantaneous and initial rates of PCr depletion (24).

Proton balance calculations. The Lohmann reaction (PCr splitting to maintain constant [ATP], despite mismatch between ATP supply and demand) can be written as ΣPCr → ΣP, + creatine + γH⁺, where the coefficient γ (negative, representing net H⁺ consumption) is the difference between the net charges on Pi and PCr (13), given empirically by a cubic function of pH (4, 19). Conversely, in the reverse Lohmann reaction (net PCr resynthesis during recovery), ΣP, + creatine + γH⁺ → ΣPCr, representing net H⁺ generation. During exercise, the net H⁺ load that results in pH change is the difference between glycolytic H⁺ production (= change in lactate) and H⁺ consumption by PCr splitting. We will argue that lactate production is negligible, so that H⁺ load = −f(yd[PCr], which is negative and results in alkalization. During recovery from acidifying exercise, pH returns to basal, despite net H⁺ generation by PCr resynthesis, because of net H⁺ (acid) efflux from the cell by various means (18). Here, where muscle alkalizes during exercise, we will argue that H⁺ efflux during recovery is negligible, so that H⁺ load = −f(yd[PCr], which is positive and results in acidification (8). In general, the total β(β2) is estimated by β2 = −d[H⁺ load]/dpH. If there is no lactate production in exercise, this becomes β2 = γβ/[PCr]/6pH. A component of this is due to P, (13), which is subtracted from β2 to yield the true β.

It was suggested (14), based on an analysis of cellular H⁺ balance (21), that oxidative ATP synthesis is a net producer of a small amount of H⁺, generated by carboxylic acid formed from newly generated CO₂. Taking account of the carbon-ATP stoichiometry of glucose oxidation (21) and the physiological negative log of dissociation constant of carboxylic acid (6.1) (28), the net amount of H⁺ (m) generated per mole of oxidatively generated ATP is m = 0.18/[1 + 10(6.1−pH)] (15). If this is taken into account, then, in exercise, the revised (negative) net H⁺ load resulting from PCr splitting is H⁺ load = mψQ dt − f(yd[PCr], where Q is ATP synthesis rate and is estimated as above, and t is time. Similarly, in recovery, the (positive) H⁺ load resulting from PCr resynthesis is H⁺ load = f(m − γ[PCr]). This correction tends to make the H⁺ load more positive and increases estimated β. However, we argue later that only a small fraction of this potential H⁺ load remains in the cell during recovery.

To compare recovery and exercise, we calculate, for all intervals of recovery, the amount of H⁺ generated by PCr resynthesis. Comparing this with the amount of H⁺ buffered, assuming the β established during exercise, we will find an excess H⁺ load (i.e., required to account for the pH being more acid than expected), whose cumulative expression is excess H⁺ load = f(βδpH + γ[PCr]). We also make this point by calculating the predicted pH change from the H⁺ load from PCr resynthesis and the β established in exercise: fδpH = f(γ[PCr]/β).

Statistical analysis. Values are reported as means ± SE and are compared by two-tailed t-tests, paired and unpaired as appropriate. Curves are fitted by least squares.

RESULTS

Kinetics of pH and PCr. The time courses of [PCr] and cytosolic pH are shown in Fig. 1. A and C. As the three exercise-recovery cycles gave statistically indistinguishable results (see right side), these were combined. As expected, [PCr] fell during exercise and rose more slowly during recovery (Fig. 1A). In aerobic exercise, PCr changes in both exercise and recovery are expected to obey the same exponential kinetics (24), from which PCr recovery half time = 0.24 ± 0.02 min (= rate constant 3.0 ± 0.2 min⁻¹). Whereas it is impossible to assess whether the three exercise data points obey similar kinetics, the fit is acceptable as a first approximation (Fig. 1A) and gives a total ATP turnover rate of 43 ± 5 mmol·l−1 intracellular water⁻¹·min⁻¹. [This is close to that given by the method in Ref. 6, which is based on estimating what the PCr change would be if oxidative ATP synthesis (assumed to have the same ADP dependence in exercise and recovery) were absent.] The initial rate of postexercise PCr resynthesis, a measure of end-exercise oxidative ATP synthesis rate (10), is 21 ± 2 mmol·l⁻¹ intracellular water⁻¹·min⁻¹, which is, therefore, 48 ± 2% of the total ATP demand.

Because of the creatine kinase equilibrium, calculated [ADP] changes opposite to [PCr], rising from 6 ± 1 to 20 ± 2 μmol/l intracellular water during exercise and then recovering with a half time of 0.19 ± 0.01 min (not shown). Another contribution to this change is pH (solid symbols in Fig. 1C), which rose during exercise as a result of H⁺ consumption consequent on PCr
significantly different from observed pH (proton handling is simply the reverse of that in exercise (see text).)

predicted pH change in recovery, based on the assumption that

METHODS.

rst 90 s of 180-s recovery.

loads. Time course of changes are shown during 18-s exercise and the

Fig. 1. Time course of pH, metabolite concentrations, and proton

losses. Time course of changes are shown during 18-s exercise and the

and equal (triangles in Fig. 1E). Comparison of the pH change and this (negative) H+ load in exercise gives

DISCUSSION

β. The aim of this work was to obtain an estimate of

pH increased during exercise as a result of H+ consumption, consequent on PCr splitting, and decreased again during recovery as a result of H+ generation accompanying PCr resynthesis (Fig. 1, A and C). On the simplest analysis, the relationship between the PCr fall and pH rise during this brief period of exercise is consistent with a purely oxidative (i.e., nonglycolytic) response. This resembles the response to brief

significant power was designed to be constant, but showed a small but significant (P = 0.03 by paired t-test) increase during exercise (Fig. 1D).

Analysis of proton balance. Figure 1E shows the time course of the “proton loads” resulting from these changes. The main actual component is due to the Lohman reaction, consuming H+ during exercise when PCr is falling and generating H+ during recovery when PCr is resynthesized. These two components are opposite and equal (triangles in Fig. 1E). Comparison of the pH change and this (negative) H+ load in exercise gives

β = 20 ± 3 mmol·l intracellular water−1·pH unit−1 (see Fig. 2). Using this value, we have calculated the expected pH change in recovery resulting from PCr resynthesis (squares in Fig. 1C). Unexpectedly, the predicted rate of reacidification is significantly slower than observed, suggesting that extra H+ (open triangles in Fig. 1E) are being added in recovery by H+ influx and/or glycolytic ATP production.

Figure 2 shows the relationships between pH, PCr, and the resulting H+ loads in a time-independent way (these are similar to plots for ischemic exercise in Ref. 13). Figure 2A plots the fall in [PCr] against the (negative) fall in pH in exercise (solid circles). PCr decrease is linear with pH increase from rest to the end of exercise; the exercise data points track a line representing the theoretical line for pure PCr splitting, (i.e., no H+ efflux or glycolytic or oxidative H+ production) given β = 20 mmol·l intracellular water−1·pH unit−1 (see above). Figure 2 also shows lines of increasing [ADP] (a function of pH and [PCr]); the experimental point moves in the direction of increasing [ADP] during exercise and returns during recovery (open circles, dashed line). The difference in the “outward” and “return” trajectories can be seen. Figure 2B pursues this by plotting the H+ load resulting from PCr changes (a function of PCr and pH) against the pH change (13). Also shown are lines of constant β: the data are consistent with β = 20 mmol·l intracellular water−1·pH unit−1 (as shown also in Fig. 2A).

The mean ± SE chemical shift of β-ATP was −18.59 ± 0.02 in the 10 spectra recorded at rest, −18.58 ± 0.02 in the 3 exercise spectra, and −18.59 ± 0.01 in the first 10 spectra in recovery. These are essentially identical, ruling out any change in [Mg2+].
contractions in dogfish white muscle (8), and the response to exercise in McArdle’s disease, in which lactate is not produced (16).

After acidifying exercise, pH recovers, despite net H⁺ generation by PCr resynthesis (16), because of acid efflux, due partly to the Na⁺/H⁺ antiporter and to lactate-H⁺ cotransport (18). In the absence of acid pH change or lactate accumulation (as here), there is no driving force for H⁺ efflux, and so the return of pH from alkaline to basal is, to a first approximation, the consequence of PCr resynthesis H⁺ generation alone, as in the dogfish muscle study (8). However, the relationship of pH to PCr (Fig. 2A) and, more importantly, to the PCr consumption of H⁺ (Fig. 2B) is different in recovery and in exercise.

Given that the absolute value of γ proportionately influences the estimate of β, one could argue that the discrepancy between observed and predicted pH in recovery (Fig. 1C) could be due to a calculation artifact associated with errors in the Lohmann coefficient γ. To explain this discrepancy, we would have to postulate that some factor could alter the γ coefficient between exercise and recovery, so that there is, in truth, no “extra proton load” (as postulated in Fig. 1E). There are no obvious candidate causes of such a change. No appreciable change in [Mg²⁺] was likely with such small changes in pH and with no change in [ATP], and our data confirm that no such change occurred. Also, it should be kept in mind that [Mg²⁺] has a very small effect on the Lohmann coefficient, as previously reported (19). The required change in γ, which could explain such a discrepancy, is actually very large: for the first two recovery data points, a γ value of 0.49 ± 0.08, i.e., at least twice as large as the value of 0.20 ± 0.004 derived from the published analysis that we use (19), would be required to eliminate the discrepancy between observed and “predicted” pH changes (Fig. 1C). This hypothetical value is, for example, larger than that of 0.40, which occurs in the complete absence of K⁺ (19) (and, in any case, intracellular K⁺ would tend not to fall but to rise after the contractions, if anything, as Na⁺ entering during the action potential is pumped out in exchange for K⁺). It is very unlikely that the stoichiometry of the Lohmann reaction could be this different at essentially the same [PCr] and [Pi] (and a very similar pH (all very little different from resting values) during exercise and recovery.

So far we have ignored the argument (15, 21) that oxidative ATP synthesis involves a small amount of net H⁺ synthesis (see METHODS). The effect of assuming that this occurs at the start of recovery is to make the estimated β much closer to that at the start of exercise, as one might wish. However, if we assume that this process operates in later data points (albeit at a decreasing rate as PCr synthesis slows down), we find that the predicted acidification is significantly faster than observed, leading to an overshoot of pH (not shown). This point can be seen more directly: total H⁺ consumption due to PCr splitting during exercise is almost exactly balanced by total H⁺ generation due to PCr resynthesis during recovery. However, the hypothetical oxidative H⁺ production reduces H⁺ consumption during exercise but increases H⁺ generation during recovery, throwing these out of balance. Given the size of the H⁺ load potentially available for oxidation, only a small fraction of this is necessary to reconcile
exercise and recovery observations (circles in Fig. 1E). It may be, therefore, that the muscle is more or less an open system to CO$_2$ (28) in exercise, but not fully so during recovery. This cannot, at present, be tested.

On this basis, analysis of the changes in exercise yields an estimate of $\beta = 20 \pm 2$ mmol·l$^{-1}$·pH unit$^{-1}$. This is close to estimates derived from ischemic exercise using the same assumption of zero lactate production in very early exercise (13) and consistent with estimates derived from inferred lactate accumulation later in ischemic exercise (13) (note that these experiments used the same subjects as the present experiments, apart from one substitution, and the same coil and exercise rig). This can be seen by comparing Fig. 2B with the similar Fig. 4, E and F, in Ref. 13, where data points in ischemic exercise move at first into the lower left quadrant (as in the present experiments), representing pure PCr consumption of H$^+$, and then move far into the upper right quadrant as lactate production outweighs H$^+$ consumption by PCr splitting (13).

As we have discussed in detail elsewhere (13), comparison of $\beta$ with published values is complicated by technical factors. The present estimate and our earlier estimate in ischemic exercise (13) are similar to other MRS-based estimates in forearm muscle (4) and calf muscle (25, 31). The muscle best studied by needle biopsy is quadriceps, but this seems to have a higher $\beta$ than forearm or calf muscle, $\sim -40$ mmol·l$^{-1}$·pH unit$^{-1}$ (see references in Ref. 13). A recent attempt to predict $\beta$ from chemical composition suggests $\sim -50$ mmol·l$^{-1}$·pH unit$^{-1}$ (26), but other versions of this calculation differ considerably, mainly because of the difference in the assumed contribution by protein-bound histidine residues (see references in Ref. 13).

**Glycogenolysis during aerobic exercise?** If the assumption of zero lactate production in exercise is wrong, then we have overestimated true $\beta$; however, any smaller estimate is difficult to reconcile with published biopsy and in vitro data, which are discussed in detail elsewhere (13). It is unlikely that there is any appreciable lactate generation in this exercise, given the match between the implied $\beta$ and estimates obtained in ischemic exercise, not just before lactate production appears to start, but also later on where lactate changes can be calculated indirectly (13).

This is pertinent to a current debate. A study of rat muscle using a gated $^{31}$P-MRS technique found rapid decrease and recovery of [PCr] on a $\sim$10-ms timescale during twitch contractions (3). It has recently been proposed on kinetic grounds that the energy for this rapid within-twitch PCr resynthesis comes from glycogenolysis, which must be balanced on any but very short timescales by glycogen synthesis, forming a "glycogen shunt" (29). Predictions based on this model depend on detailed hypotheses about timing. Whereas such PCr depletion-repletion cycles within contraction, below the time resolution of the present experiments, would have, in themselves, no acid-base consequences (H$^+$ consumption during net PCr hydrolysis being balanced by H$^+$ production during net PCr hydrolysis), the result of a rapid fall in PCr followed by resynthesis funded by glycogenolysis to lactate (29) would be a net lactate and H$^+$ load of $\frac{3}{2} \approx 0.67$ per PCr "turned over." This is a slight overestimate if, as has been postulated, oxidation of some of this lactate had met the ATP requirement for glycogen resynthesis (29), but we calculate that this full shunt still generates 0.625 lactate per PCr turned over, and although this might in the end leave the cell (29), its intracellular concentration must rise first. In the whole exercise period, the net [PCr] change of $7.0 \pm 0.8$ mmol/l intracellular water consumes $1.5 \pm 0.2$ mmol/l intracellular water of H$^+$ via the Lohman reaction (see Fig. 1E). The fact that the pH increased shows that there was net H$^+$ consumption. Thus H$^+$ production by glycogen shunting cannot have exceeded 1.5 mmol/l intracellular water, corresponding to PCr "turnover" of $1.5/0.625 = 2.4$ mmol/l intracellular water, or $\sim 30\%$ of the net PCr depletion observed during exercise. However, if shunting even approached this limit, the implied $\beta$ would be very much lower than has ever been reported, inconsistent with in vitro data (13). We conclude that glycogen shunting in this form, if it occurs, cannot be quantitatively significant, unless there are unknown routes for lactate-H$^+$ efflux, which can operate, despite negligible lactate accumulation and intracellular alkalosis.

**Glycogenolysis in recovery?** It is generally assumed that lactate is not produced in recovery, although strictly what has been shown is that, apart from a small transient component after 60-s calf muscle exercise in a recent report (7), PCr recovery requires oxygen (27) (as lactate production would not). If PCr recovery were funded by glycolysis, synthesis of one PCr would produce (as lactate production would not). If PCr recovery were funded by glycolysis, synthesis of one PCr would produce (as lactate production would not).

$\gamma$ $\approx 0.21$ and acidify the cell by $0.04$ units; compare this with the H$^+$ load resulting from PCr resynthesis alone (29, 30) and the H$^+$ load resulting from PCr synthesis and funded by oxidative ATP synthesis with "fully retained" H$^+$ (m $\gamma$ $\approx 0.35$). In fact, the required "extra" H$^+$ load peaks at $0.2$ min and then gradually declines to zero (Fig. 1D). It is impossible to say whether this represents the temporary retention of 60% of the H$^+$ from oxidation (21), a small (9%) glycolytic contribution to PCr resynthesis (7) (although this was apparently absent during exercise, see above), or even H$^+$ influx, perhaps in response to cellular alkalization (which is opposite to what has to postulate on the glycogen shunt hypothesis, see above).

**PCr recovery kinetics.** Although it is often useful to treat the PCr recovery rate constant (or, inversely, the half time) as a system property (24), it characterizes a dynamic response that does depend, to some extent, on initial conditions. The effect of pH is well recognized (2, 32), but it must also be assumed to depend somewhat on initial [PCr]. Thus the appropriate literature com-

---

1 This is $0.625 = (1/3)(2 - (2/16))$, where 3 is the glycogenolytic ATP yield from 1 glucosyl unit, and the first 2 is the corresponding lactate yield; 16 is the ATP yield from oxidation of 1 lactate, and the second 2 is the ATP required to add 1 glucosyl unit to glycogen (based on Ref. 29).
parison for the PCr half time seen here (0.24 ± 0.02 min) is with studies showing, let us say, <30% PCr depletion and <0.2 pH change: among these we find PCr half times of 0.15–0.3 min in voluntary dynamic quadriiceps exercise (22), ~0.3 min in two studies of isometric calf muscle exercise (11, 20) and one of calf muscle stimulation (5), and in voluntary dynamic finger flexion (17). Even without the known complication of pH change, it probably cannot be assumed that linear extrapolation of PCr resynthesis rate to complete PCr depletion gives a valid estimate of maximal mitochondrial ATP synthesis rate in the present experiments (32), in view of the degree of extrapolation implied from the minimal PCr perturbation.

Possible limitations of this work. The sample volume of the coil (~30 ml) might include a component of nonexercising muscle. The absence of split Pi peaks argues against this, although, in the absence of large pH changes, this would be difficult to detect. This would lead to underestimation of changes and rates of changes, but would not affect the ratios used to estimate buffer capacities and predict pH changes, for example. All MRS data are the weighted mean of changes in different fibers and thus fiber types, so possible effects of changing patterns of recruitment cannot be excluded. This could be avoided in further studies with the use of electrical stimulation.

Significance and implications. Why does this matter? First, that these experiments can be plausibly interpreted as a purely oxidative (nonglycolytic) exercise response, consistent with a β of 20 ± 2 mmol·1−1·pH unit−1, lends support to the buffer-based approach to estimating glycolytic ATP synthesis by 31P-MRS (see the introduction). However, that pH recovery is faster than expected implies H+ generation in excess of that produced by PCr splitting, either by H+ influx due to cellular alkalization, or by a small glycolytic contribution, or by some other mechanisms not identified so far. Second, uncertainties about glycogen shunting (29) counsel caution in calculating ATP turnover in exercise on this timescale, quite apart from other possible complications, such as varying recruitment patterns and the series elastic component. It is difficult to rule out possible real changes in contractile efficiency, in the absence of firm evidence that glycogen shunting is either negligible, as suggested here, or constant in rate and stoichiometry (29).

We acknowledge the support of Centre National de la Recherche Scientifique, Association Francaise Contre les Myopathies, Programme Hospitalier de Recherche Clinique, and Association pour le Developpement de la Recherche Medicale.

REFERENCES


