

**EPICARDIAL AUTO-FLUORESCENCE NAD(P)H KINETICS IN THE ISCHEMICALLY PRECONDITIONED LANGERDORFF RAT HEART. EFFECTS OF CAPSAICIN. PART 2.**

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## SUMMARY

The data in Part 1 are preceded by a review of the conventional *ex vivo* model of conditioned ischemic learning. Here in Part 2, the fluorescence kinetics of indistinct pools of reduced pyridine nucleotides and the conventional parameters observed in short, long and preconditioning reperfusion conditions as well as in the common phase of protracted ischemia (30 min) and in the reperfusion phase monitored over 60 min show significant trends in the context of the most recent metabolic observations of heart perfusion after capsaicin pretreatment. The present paper reports the mean kinetic values of the metabolic parameter associated with those from functional sampling, with the emphasis on protracted ischemia following short (15 min) and long (1 h) perfusion with and without short preconditioning ischemic insults in capsaicin-treated and -untreated specimens. We also describe and discuss a number of trends observed in single cases, where the phases of preischemic conditioning, protracted ischemia and reperfusion demonstrated both the protective effect and prevalent damage. The paper examines the significance of this experimental model in the integrated biological and pharmaco-toxicological context.

### 3. RESULTS

In addition to Table 1 and Figures 1-7 in Part 1, Figure 8 shows the percentage normalized t-test-evaluated time courses, means and standard errors of the control and capsaicin-pretreated groups for the NAD(P)H autofluorescence metabolic parameter. The Data Section (5.2) at the end of the general Discussion Section (4) of Part 2 shows the percent values of state 3 (normally oxygenated) vs. state 5 (ischemic, taken as being to 100%) variations of each heart observed, plus the data of Figures 1-7 of all the hearts used. In the Table I, 5.1 Data Section, all the hearts are identified by the dates when each animal was sacrificed, most of the randomized rats having been used to obtain the functional parameters - coronary flow (CF), heart rate (BPM), maximum systolic left ventricular pressure (MSLVP) and end-diastolic left-ventricular pressure (EDLVP) - together with the globally observed metabolic index. The results of the more detailed interactive multi-ANOVA statistical analysis of the same data, after the data presented in Figure 7 (Part 1), will be commented upon here only as single control vs. main treated trends, as in the other figures for the individual parameters, while the results of the specialized analyses on full and interactive data will be presented along with the specific conclusions drawn.

Coronary flow (CF, Figure 1), in the short perfusion (SP) group, presents a substantial drop - around -40%, in both controls and pretreated animals - after persistent index ischemia, similar to that in the long perfusion (LP) control group, which was then improved (to ca. -25%) by pre-treatment. In the preconditioned (PC) group, CF declines gradually during the subsequent short ischemias, whereas after the final protracted ischemia the increase in flow occurs only where pretreatment with the vanilloid is not given, *as if capsaicin exercised an apparent persistent protective effect similar to that resulting from PC, only, however, after prolonged perfusion.* These are immediately evident overall trends, which, nevertheless, have not been described in the known, systematically cited references.

As far as spontaneous heart rate is concerned (BPM, Figure 2), the arrhythmic complexes disturb the study, which cannot, in an oversimplified manner, ignore their presence in terms of the consequences, and the re-occurrence of each of them. The apparently most serious mean profile, however, proves similar in the PC and pretreated groups, particularly in a phase of the final perfusion which lasts a maximum of 10-15 minutes and is already present after exposure to the vanilloid, especially in the LP group. The PC effect, which, in any event, affords dubious protection, would not appear to be similar to that of the vanilloid, unlike that indicated by the CF measurements.

In Figure 3, the trends of the means of the MSLVP percentage values show the typical capsaicin-induced reduction during administration of the substance; this reduction is present at all steps in the PC group and reverts almost to steady 100% levels in the LP kinetics, but reaches values above 100% during final reperfusion in the PC specimens only. Indeed, the final improvement in systolic performance after different lag times (as also shown by the rate pressure product [RPP] - Figure 5 - and the RPP/CF - Figure 6 - trends) was common only in the vanilloid-pretreated groups, and the decrease in systolic efficiency after the first ischemic insult, in both the PC and long index ischemia groups, appears to be mostly associated with the length of perfusion, while PC partial protection may possibly be afforded against the final conditions in which it establishes, triggers and consolidates the early maintenance procedure.

In Figures 4 and 7, the EDLVP time courses and the kinetic trends of ischemic contracture, respectively, are depicted in the 6 experimental conditions (PC, SP, LP: capsaicin-treated vs. controls) and present differences characterized by high levels of statistical probability, associated with more than 4 interacting parameters, as analyzed next. This conclusion which would also appear to be an unprecedented original contribution, in that it is not paralleled in any previous study, in itself warrants focused discussion. Furthermore, the kinetic trend of increasing ischemic contracture in hearts submitted to the ischemic preconditioning (IPC) procedure is quite appreciable, in the repeated PC cycles, mostly earlier and paradoxically rising in the capsaicin-pretreated specimens. Finally, capsaicin administration undoubtedly increases damage at the beginning of final reperfusion in the PC hearts as well as after the typical fading in LP hearts. Nevertheless, as in the case of the LP trend in controls, PC did not appear to afford any protection during final reperfusion, and protection may only be erroneously apparent when compared with the SP samples.

The most evident common features of the two dependent parameters (RPP [Fig. 5] and RPP/CF [Fig. 6]) would not appear to militate against the recovery of better functionality with protracted perfusion and do not appear to be higher at a later stage after PC treatment, especially after capsaicin. The vanilloid effect, however, is confirmed in the PC group at initial resumption of the final perfusion, manifesting the typical greater overshoot compared to the final PC condition, peaking after the first 5-10 minutes at levels only observed after protracted perfusion, planned as a condition of effective control of the ischemic adaptation modality operated; this distinctly singular trend is as surprising as it is unexpected. Figure 8 presents, for the metabolic parameter, the mean kinetic trends of the percentage changes in fluorescent emission, with the SE bars at  $P < 0.05$  every 2 minutes, as for Figures 1-6 in Part

1, Figure 8 is derived from the data and data processing presented in the 5.2 Data Section, whereas Figure 9 presents the overall picture of the 6 trends for the 6 examples of conditions analyzed.

Table 2 presents the kinetic parameter values ( $\tau = 1/k$ ; see Legend, Table 2) of all the samples observed, calculated by approximating the emissions during the oxygenated reperfusion wash-outs after index ischemia to a 1st-order semilog kinetics, then reduced to a monocompartmental model (All the corresponding values of the hearts observed are available at request as indicated in the 5.3 Data Section. The results of the bicompartamental linear and of a more complex model, associated with the analysis of the fluctuation decays, go beyond the scope of the present report).

Lastly, Figure 10 presents the near-infrared spectrometry spectra for a number of the more significant conditions among those systematically characterized by the functional parameters (Part 1) observed in fluorescence.

Figure 8

NAD(P)H autofluorescence global kinetics. Control (♦) vs capsaicin-pretreated (□) time courses of the means and S.E. of the percent values of state 3 (normally oxygenated) vs state 5 (ischemic, taken as 100%). Data from section 5.2 Data Section related XVI-XVIII Tables.

X axis: consecutive measurements at 2 min intervals.  
 Y axis: means and S.E. of the percent values (vertical bars).

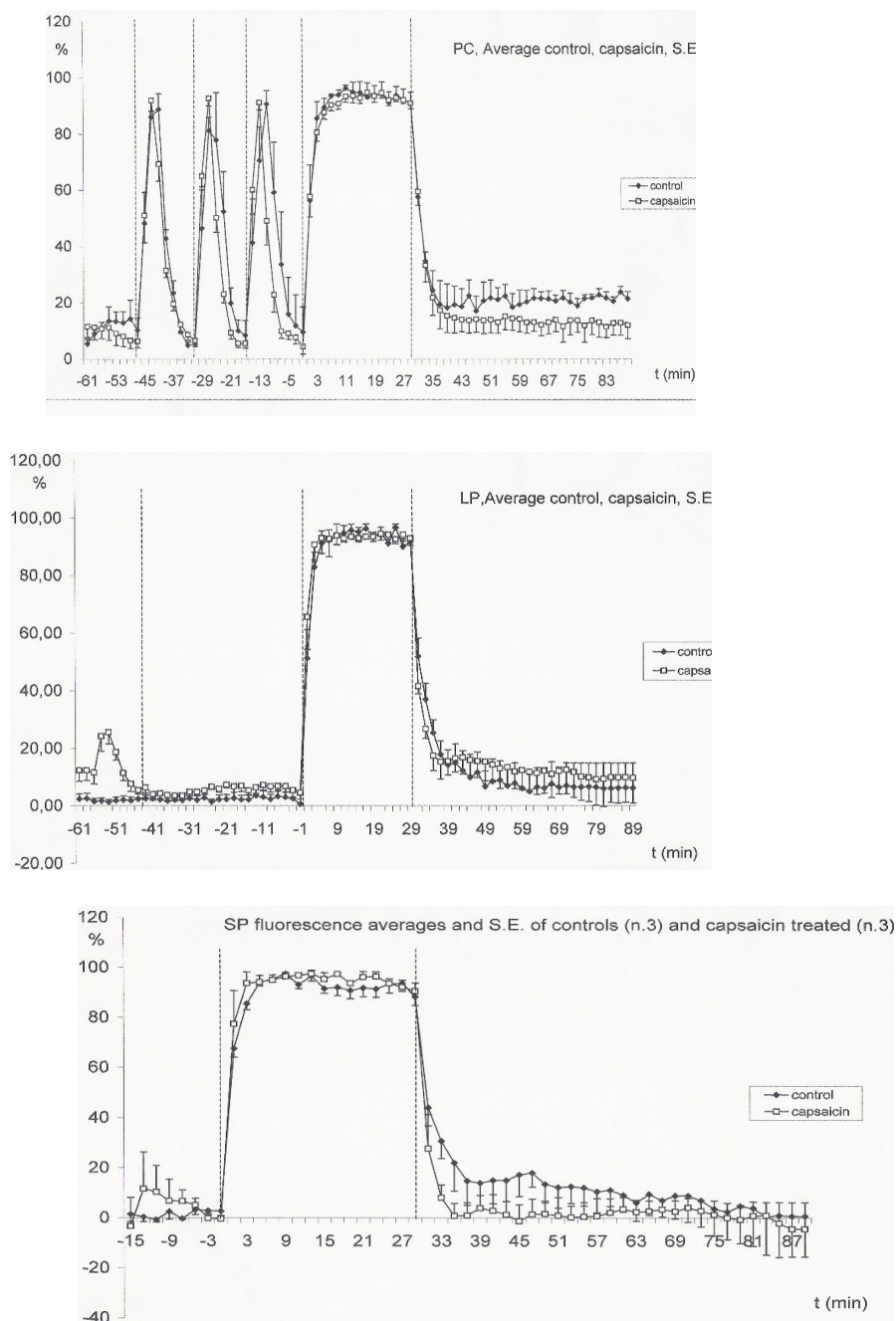


Figure 9

Sample individual specimens presented in Figure 8 as standardized arithmetic means. Reduced generation of pyridine factors in rat heart perfused according to Langendorff after a single 30 min period of ischemia (left) and pretreatment with a final concentration of 10 μmol capsaicin (right) in the conditions described as: a) Preconditioned, b) Long Perfusion, and c) Short Perfusion. From left to right and from top to bottom, hearts identified in the table of 5.1 Data Section in the following order: Nov 25b'98 and Nov 10b'99; Nov 24b'98 and Oct 29a'99; Nov 30a'98 and Oct 26c'98.

In some specimens, in last (oxygenated) reperfusion, after the first most rapid initial decay, fluctuating-decaying behaviours (i.e.: [484]) appear, whose data fitted by the Origin software longly than 30 min decays, did show the best fittings following the  $Y_2$ ,  $Y_3$  and  $Y_4$  functions, characterized by different incoming contributing factors a, b, and c, eventually to be identified (i.e.: [211]):

$$Y_2 = Y_{02} [a_1 / (a_1 - a_2)] [e^{-k_3(x-x_0)} - e^{-k_2(x-x_0)}];$$

$$Y_3 = Y_{03} [b_1 / (b_1 - b_2)] [e^{-k_5(x-x_0)} - e^{-k_4(x-x_0)}];$$

$$Y_4 = Y_{04} [c_1 / (c_1 - c_2)] [e^{-k_7(x-x_0)} - e^{-k_6(x-x_0)}].$$

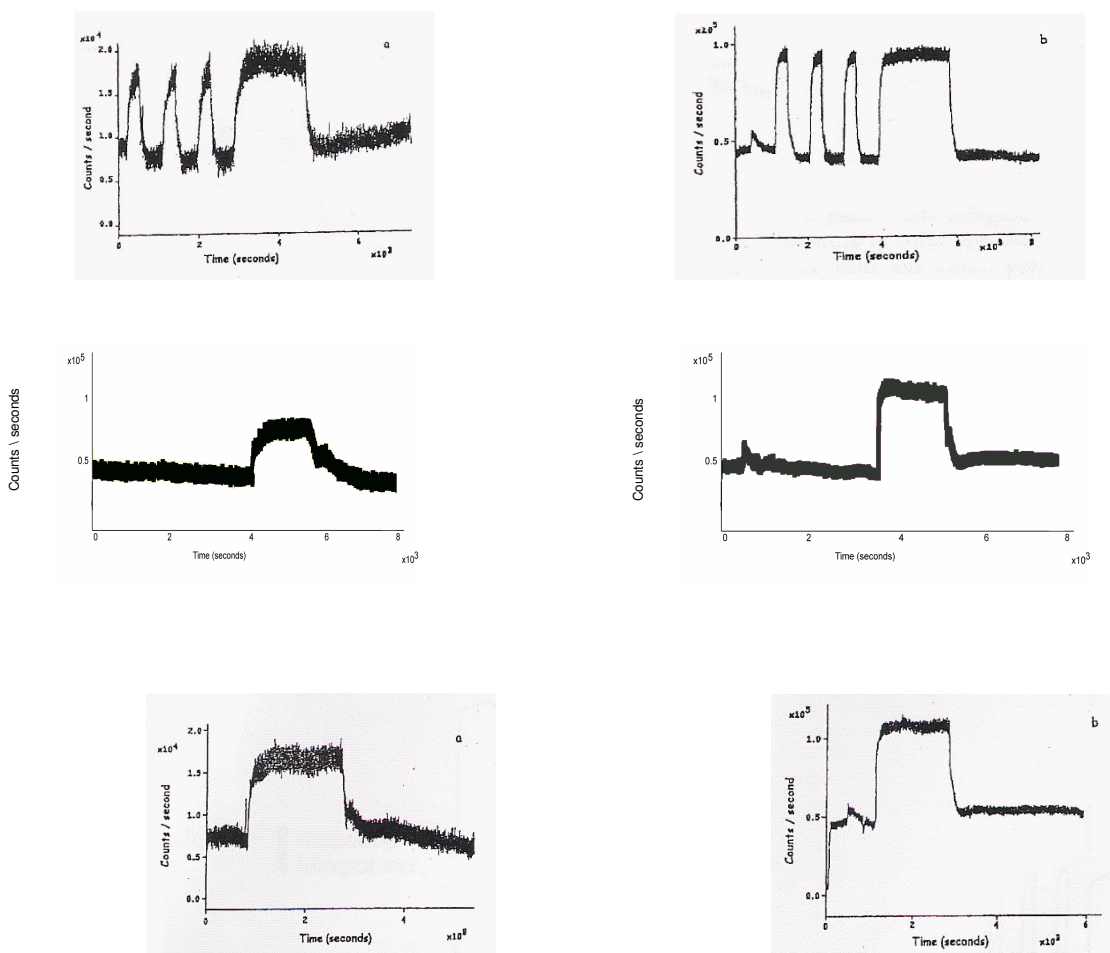


Table 2

Values of  $\tau$ , i.e. of the inverse values of the slopes of the semilog fittings of the original photon-counted fluorescence emission kinetics of the first 6 min intervals of the rapid last reperfusion reoxygenated decays. Original data recalled in section 5.3 for all the hearts observed for fluorescence metabolic parameter have been fitted with the monoexponential linear model:

$$Y_t = Y_{01} e^{-k_1 (x - x_0)},$$

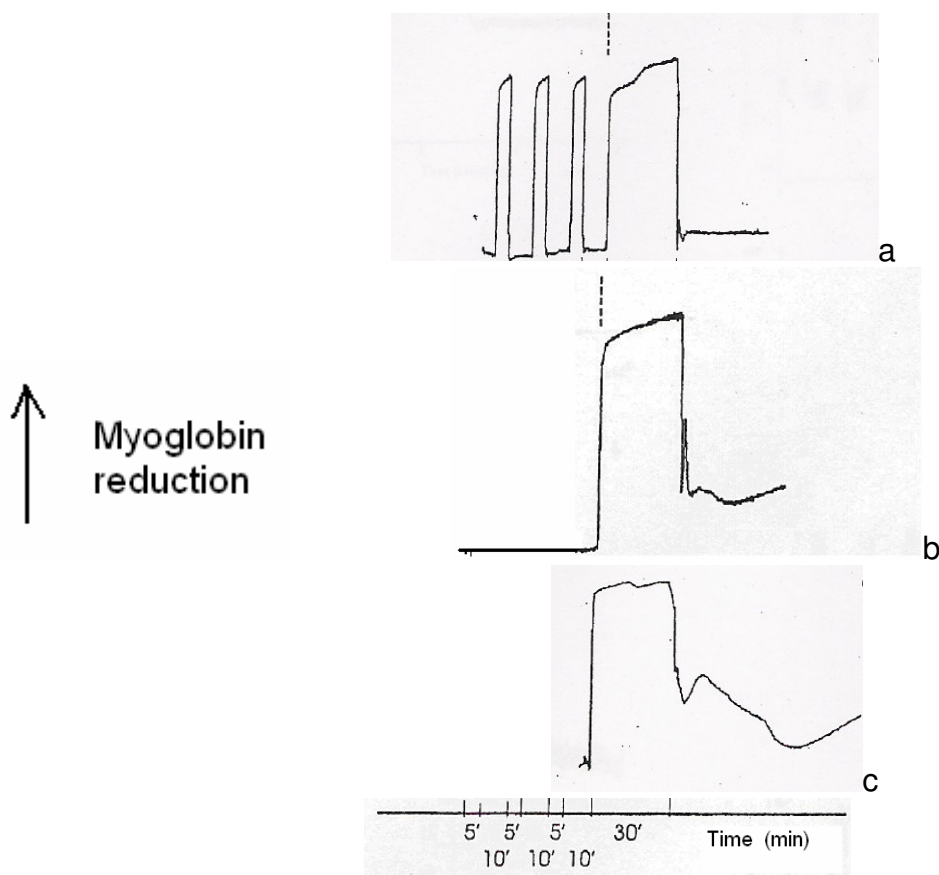
and the  $\tau = 1/k$  values subjected to the t test like the other Table and Figures. Means and S.E. of the 6 groups and P values of the t test (unpaired, two tailed) between control and capsaicin-treated specimens subjected to preconditioning (PC), long perfusion (LP) or short perfusion (SP).

Preconditioned control	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Preconditioned Capsaicin	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Test (t) P	% Difference Average
15. 1 Nov 25b '98	-579,71	0.78	-1892,59	1667,80	833,90	19. 1 Nov 26a '98	-919,12	0.64	-1020,20	704,72	315,16	0.37	60.13
16. 2 Dec 10b '98	-4329,00	0.53				20. 2 Dec 10a '98	-2222,22	0.71					
17. 3 Dec 14a '98	-1166,86	0.66				21. 3 Nov 8a '99	-770,42	0.73					
18. 4 Nov 5b '99 (new)	-1494,77	0.63				22. 4 Nov 10a '99	-952,38	0.69					
						23. 5 Nov 10b '99	-825,76	0.77					
Long perfusion control	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Long perfusion Capsaicin	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Test (t) P	% Difference Average
7. 1 Nov 24b '98	-2232,14	0.77	-1364,41	692,46	346,23	11. 1 Oct 29a '99	-1127,40	0.84	-1115,70	333,78	166,89	0,55	81.77
8. 2 Nov 25a '98	-857,63	0.75				12. 2 Nov 1a '99 (new)	-1529,05	0.60					
9. 3 Nov 27c '98 (new)	-1610,31	0.24				13. 3 Nov 4a '99	-1094,09	0.85					
10. 4.Oct 28a '99 (new)	-757,58	0.90				14. 4 Nov 4b '99	-712,25	0.89					
Short perfusion control	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Short perfusion Capsaicin	Value of $\tau$	R <sup>2</sup>	Average	St. Dev.	S.E.	Test (t) P	% Difference Average
1. 1 Nov 30a '98	-718,39	0.84	-856,15	125,61	72,52	4. 1 Oct 26c '98 (new)	-475,06	0.83	-603,71	277,01	159,93	0,25	70.51
2. 2 Dec 8c '98 (new)	-964,32	0.61				5. 2 Oct 28b '99	-414,42	0.87					
3. 3 Dec 9a '98	-885,74	0.88				6. 3 Oct 29b '99	-921,66	0.73					



Figure 10

Rat hearts perfused as presented in Figures 1-9. Near-infrared measurements of myoglobin redox levels (Cf.: Rossini L, Shaw A, *Near-infrared spectroscopy of Langendorff rat heart exposed to capsaicin pretreatment and preconditioned*. Work in progress) during and after ischemia, preceded in a) by a standard preconditioning protocol. Besides the well-known loss of the cytoplasmic carrier, the preconditioning also induces a reduction in the phase of fluctuating instability associated with the reperfusion injury, which has originally been described for the mitochondrial and cytoplasmic set of pyridine dehydrogenase cofactors.



## 4. DISCUSSION

### 4.1 THE LANGENDORFF MODEL

The Langendorff model [236] continues to be used, so much so that, over the period from 2000 to 2005, 1078 contributions are to be found in PubMed using this keyword (935 when selecting “Langendorff and heart” and 800 when selecting “Langendorff and myocardial”), despite the fact that retrograde perfusion via the aorta presents difficulties with regard to the maintenance of controlled stability. In addition to the references in Part 1 [1-11, 131], we cite those regarding (i) the measurement and/or conditions of the interstitial intra- and extracellular spaces of the preparation [237-240], (ii) the regulation of coronary flow and a number of characteristics of intermittent ischemia, obviously not only *ex vivo* but also *in vivo* [241-247], (iii) the contribution of colloids and the maintenance of parameters of abnormalities or otherwise of energy metabolism regarded as significant, both independent and related to coronary flow and endothelial control of single or repeated ischemia/reoxygenation cycles [248-254], (iv) sometimes with evidence of dichotomies between *ex vivo* and *in vivo* models of young or old animals, species, characteristics, gender [255-257], and different reactive functions, identified as mitochondrial or otherwise [8, 258-260], or of (v) fresh or cultured ventricular myocytes of various and/or the same species [260-264]. Undoubtedly, the control mechanisms of the relationships between edema and hypertrophy in the surviving perfused preparation *in vitro* and *in vivo*, oxygenated differently or otherwise by means of different so-called physiological solutions, and whole or fractionated/diluted blood, need to be better known in order also to be able to better characterize the cardiac adaptation/acquired memory reactivity, while the analytical solutions themselves continue to be a matter of controversy [265-268]. Our research project was focused on limited comparisons of a number of traditional reference parameters and, in addition to the results reported here, on direct observation of a metabolic parameter, at a level of compartmentalized complexity judged to be intermediate [195] in the global preparation perfused by the retrograde (aortal) route, without any recycling of the Krebs-Henseleit buffer, which was selected because it is the one most commonly used. Of the capsaicin effects which have been increasingly analyzed at the same time in detail elsewhere [164-187, 269-274], we certainly had no intention of examining the vascular-endothelial, neuronal and, more properly, myocellular and fibroblastic components, e.g. of spatial dislocations such as the temporal-kinetic interactions of the various systems of renewal of by no mean

negligible diffusive and interactive localized microvolumes of myocardial and/or endothelial nitrogen and oxygen radicals [84, 188, 205, 275-283], which are all problems that go beyond the necessarily limited bounds of the possible aspects focused upon in the present study. We are well aware of the cytoprotective mechanism of the heat shock protein 70 system [115-118, 284-289], also in local cardiac warming *in vivo* [290], which has necessitated appropriate checks above all in the perfused organ observed within the superconductive magnet in the no-reflow phases of global ischemia. The duration of the thermal shock necessary for the transformation of the organ for study *in vitro*, not evaluated by Langendorff [236], does not, however, condition the isolated preparation [291]; nevertheless, the importance and significance of the initial alterations of cell volume and of the metabolic parameters evaluated, also during repetition of the stresses/ischemic shocks/reperfusions, which are currently in the course of in-depth analysis [245, 292-293], will also be further examined here. Those relating to anterograde vs. retrograde perfusion [294], which are tackled in the various comparisons implemented with the working preparations [295-307] and prove equally elusive in terms of conclusiveness, present abundant scope for the development of studies with the same original model, where, however, differences that prove substantial do not remain unresolved, e.g. where the down-regulation of ventricular contraction in the initial ischemic phase is dependent above all on flow, and not so much on the pressure of coronary perfusion [308], which was maintained constant [80 mm Hg] in our assays. The problems of temporal stability of the preparations, which we recall here once again, obviously mandate the use of a control group, and not only normal vs. treated animals (including pharmacologically treated animals), but when undertaking the study of possible differences, where preconditioning cycles of more or less protracted duration may or may not be used, which may have been decisive proof - and this is not always encountered in the references [cfr. 123] - in the drawing of apparently contradictory conclusions, in addition to species-specific diversity factors, e.g. specific to the rat and rabbit, both *in vivo* and *in vitro* [cfr.123 vs 263, cited; see also here below].

#### **4.2 STUNNING, HIBERNATION AND PRECONDITIONING IN THE GLOBALLY ISCHEMIC NO-FLOW LANGENDORFF MODEL**

Ischemic preconditioning, the process of adaptation to the low-energy state due to oxygen deprivation [309], was evaluated in the first place *in vivo* as protection afforded in terms of attenuating the structural damage, reducing the infarcted area, preventing arrhythmias and improving functional recovery, which, however, was not judged to be the most evident marker [310] and was regarded as

overlapping the initial and permanent ischemic phenomena and the later phenomena resulting from reoxygenation reperfusion. Stunning, described by Heyndricks *et al.* in 1975, is a term introduced by Braunwald and Kloner in 1982, denoting temporary postischemic systolic and diastolic dysfunction of the myocardium evident when coronary flow is apparently normal (or supranormal), and is different from hibernation, a condition described in 1989 by Rahimtoola as a reduction in contractile activity associated with a tolerable reduction of blood flow [311-313], whereas the two phenomena have been judged to be insufficient to cause the protective effect of preconditioning [314-320], not attenuated even in the Langendorff preparation [12]. In this model, too, in reperfusion, the overall beneficial effect of IPC may be reduced or even opposed to one or more components of the reflow process that may prove detrimental and act to reduce the extent of recovery as well as contributing independently to the functional recovery slowed by the superimposed adaptation and mediated by factors of triggering, mediation/modulation (mediator phase) and subsequent maintenance, otherwise not involved in the initial kinetics, and co-determinants of late, protracted effects (effector steps), and multiple interactions, which, however, may also be common to both stunning and hibernation [314-320]. We would recall here the introductory remarks of this paper, regarded as a useful premise to the examination of the functional results presented in Part 1 (Figures 1-6) recorded in the three control groups, which possibly cannot be dissociated from having excluded the preselection of acceptance limits and/or excluding the preparations examined in the present study. More to the point, it is relevant here to recall the identification of differently isolated, opposing (e.g. different roles of iso-PKs [321-324]) or alternative mechanisms judged to be detrimental [325-327], which, in those cases where there exists a motivated doubt as to the unique validity – or otherwise! – of the conclusions of currently successful experimental and behavioral trends, have to date given rise to claims as fruitless as they are alarming [328].

As representatives and co-founders of the WHO participating centres, (cfr. 5<sup>th</sup> Meeting of the Representatives of National Centres participating in the WHO International Drug Monitoring Scheme, Portonovo di Ancona, 4-5 October 1982) we have advocated the need for the institution and joint development of forms of standardization and regulation of clinical studies along with those of an experimental nature, in the belief that these may constitute by no means negligible, mandatory precedents (see [329]). Nevertheless, even in the most accredited literature support is expressed for results which are only apparently significant, obtained in frankly dishomogeneous non-randomizable populations, that perpetuate the damage done by “illusory statistics” [330], which is an ongoing theme that can be extended to the acceptance of spurious, essentially untenable and impossible correlations,

which are merely an expression of the contaminations of current scientific knowledge. In this regard, it is now perfectly clear that we can no longer continue to apply Ockham's razor in favour of solutions that are only apparently simpler, but actually devastating.

Accepting a physiological definition of ischemia, suggested by the increased interest in cardiovascular research [331], which today could be extended to research into trauma and stress [115-118, 284-289], we concede that fundamentally related dynamics [279, 332-352] may initiate with closure of the slow membrane channels, cellular-syncytial entry of  $\text{Ca}^{2+}$  and disinhibition or unmasking of ATP-dependent  $\text{K}^+$  exit channels, described in the seminal paper by Noma, published in 1983 [353] - co-participating sensors in maintaining metabolic homeostasis [354]. At least two recent reports deal appropriately and in depth with the analysis of the relevant updated information [150 and 355, of reference 431], also emphasizing the often decisive and by no means negligible themes of the contested specificity of *pharmacological preconditioning* [cfr. 109-111, 113, 118, 120, 122, 126, 131, 137-138, 140-141, 143-144, 201-203, 205-207, 247, 357, 360-363]. Both in reperfusion and in the processes of ischemic preconditioning, the  $\text{K}_{\text{ATP}}$  channels, if ever they were the principal agents, would still appear to be contested if superficial, sarcoplasmic or mitochondrial, whereas, in both young and old rats, even a very brief calorie restriction improves the ischemia tolerance independently of their being open or not [260], and this, moreover, is not associated with PC due to  $\text{Ca}^{2+}$  [124]. The opening of mitochondrial channels with substantial conductance of  $\text{Ca}^{2+}$ , activated by  $\text{K}^+$  ( $\text{BK}_{\text{Ca}}$ ), is cardioprotective [67, 356], and their modulation (in carotid glomus cells) occurs via heme-oxygenase-2 (constitutive HO-2) [358], apparently as a result of the CO generated [359], hence the known interference in cardiac metabolic regulatory pathways, as in the case of NO, whereas deprivation not of oxygen (hypoxia and ischemia), but, for example, of glucose, the prototype of "*metabolic PC*", increases the resistance (in cardiac fibroblasts) resulting from ischemia by means of up-regulation of the expression of heme-oxygenase-1 (HO-1) and cyclo-oxygenase-2 (COX-2) isoactivity, both of which inducible [205, 364], the latter being identified by synergistic co-interaction with the equally inducible NO-synthase-2 [365]. For HO-2 and COX-2, the induction occurs via p38-mitogen-activated protein kinase (p38-MAPK) - and protein kinase C (PKC) - reported as having a gradual potentially bifrontal, not to say, detrimental effect [326]. Therefore, as things stand at present, it cannot be conclusively claimed that every reaction process with a metabolic deficiency, including ischemia and glucose deprivation, as conditions of stress and even heat shock, can only occur and express itself with protection mechanisms, albeit apparently prevalent. Obviously, the hormetic hypothesis [366], amongst others, may also be applicable here.

In the first place, one could emphasize, as emerges from the functional parameters investigated, the extensive variability of the results obtained, which in individual samples may represent alternative trends, as opposed to the mean values accepted as significant [cfr.: the complete data tables of 5.1. DATA Section, and Figures 1-6 in Part 1.). Here we can only reiterate what we have said above with regard to the misleading criterion dating back to Ockham, when the premises - and the relevant references - tend to bear out the by no means negligible consistency of prevalent complexities.

#### 4.3 INDEX ISCHEMIA CONTRACTURE PARAMETERS

In the various control situations (without PC), the contracture of protracted global ischemia, which cannot be observed in perfusions (Langendorff rat heart) with glucose-enriched Tyrode solution [367], is accelerated in the same preparation in Krebs-Henseleit buffer and glycogen depletion due to glucose omission, administration of pyruvate, 2-deoxyglucose, acetate, adrenaline or glucagon [96, 368-371]. In rat ventricular skinned fibers, where the increasing stiffness may reach the condition of rigor corresponding to the “stone heart”, particularly implicated are the relatively greater decrease in phosphocreatine and the local increase in  $Mg^{2+}$ -ADP [372], with reversal of Pasteur’s effect - inhibition of ATP-induced glycolysis - with subsequent paradoxical acceleration of the glycolytic flow itself due to the endogenous accumulation of other metabolites and also possibly depletion/leakage, extracellular accumulation and initial loss of those which certainly participate in the damage in the course of reoxygenation/reperfusion [cfr.: pyridinic coenzymes [373], oxypurines and nucleotides [374], lactate dehydrogenase [71], carbohydrates and sum of purines - hypoxia due to flow reduction - [79], neuropeptides - not excluded by the capsaicin/vanilloid effect - [269], creatine kinase [375], and dystrophin [376]. In the Langendorff IPC rat model, doubling of the time of reduction of myocardial oxygen consumption ( $MVO_2$ ) on demand has been interpreted as the most efficient adaptive mechanism of preservation during sustained protracted index ischemia, followed thereafter by improvement in postischemic cardiac functions [345]. The reduction of latency and paradoxical exacerbation of the index ischemia contracture, reported in 1992 [37] and associated with glycogen depletion [377], was analyzed in the same blood-perfused rat heart model [18-23]. A delay, but no exacerbation, was found in pacing at 2 Hertz [50]; rapid pacing and intermittent ventricular fibrillation can induce PC protection during ischemia and reperfusion without ischemia in the PC period and some exacerbation of ischemic contracture [23, 69].

In adaptation and in the occasional, by no means negligible maladaptation of IPC, and of hypoxic PC, e.g. due to perfusion flow reduction [79], the tissue loss of multiple factors has never been and could never be fully assessed, particularly during the cycles of standardized PC protocols, where, moreover, the first brief global ischemic insult may be enough to trigger the protection [373], which tends to diminish after the fourth [245]. The availability of glucose, also via the control of transport, as in glycogenolysis, therefore probably entails a reduction of glycolytic activity, while the adaptation to ischemia without PC is better with greater tolerance if the glycolytic flow is high, associated with greater glycogen reserves [377-380]. Of interest, also in ischemia, is activation of the heterotrimeric AMPK kinase complex by protein-threonin-kinase (LKB1), identified in the liver as the target of the action of serum-glucose lowering biguanides such as metformin [381], where the ATP generated by creatine phosphokinase intervenes, modulating its activation in different ways according to the dynamics [382, 384]. As is known, the creatine-kinase system also intervenes in the regulation of functions of the cytoskeleton [383], not to mention functions of local coordination postulated for glycogen [380], whereas IPC, which involves the sequestration of beta-adrenergic receptors [385-386], demands and/or favors the translocation of iso-protein-kinase PKC [387], hence the distinct mechanisms in acute or protracted ischemia [214], and also the induction of transient glucose privation upgrades the expression of HO-1 and COX-2 [364]. Worthy of a brief mention here are a number of traditional mechanisms of diastolic relaxation preceding hypoxic-ischemic contracture [388] and that related to reoxygenation reperfusion [389]. The torsion deformation of the ventricle - twist -, which is inversely proportional to the end-diastolic volume occurring during relaxation and filling and is determined by and related to the titin isoform ratios (the larger/more extendable vs. the smaller/less extendable ones in passive stiffness) may contribute to the depressed contractile performance and increased stiffness to the point of ischemic contracture [390], a fact which may lead to imprudent, ongoing, misleading accepted conclusions. Another factor examined sectorially, with updates of greater holistic globalization [391], is the acidosis associated with the damage postulated by some authors as a result of compartmentalized or diffusive concentrations of lactate, independent or otherwise of IPC. A review of the literature presents us here with findings which are not always coherent, where, in the isovolumic Langendorff rat heart model, IPC attenuates the acidosis (and postischemic dysfunction) [37], pyruvate elevates the threshold of the same [71], and acetate, as a substitute for glucose and in glucagon-induced glycogen depletion, lowers lactate without reducing the damage [96].

The lowering of the internal pH is not thought to induce damage attributable to lactate [74, 392-393], as a result of the inhibition of glycolysis due to the increase in the redox NADH/NAD<sup>+</sup> ratio, of Na<sup>+</sup>/H<sup>+</sup> exchange, judged to be of greater significance for critical mitochondrial damage [61-62], and, lastly, possibly of Ca<sup>2+</sup> antiport with an increase in intracellular Na<sup>+</sup> (cfr.: chick cardiac cells [394]). In contrast, in the analogous murine model, the expression and translocation of activated PKC-ε proved protective, and not the ischemic tissue acidification, which is not necessary for IPC [38] as in the above-mentioned contractures during the first and subsequent ischemic insults. Nevertheless, in simulated hypoxia, the increase in NADH proves simultaneous, in the first 8 seconds, with the reduction in isotropic activity. Lowering of the pH and an increase in the lactate/pyruvate ratio follow at 15-30 seconds [395]. In contrast, in the isolated rabbit heart, the onset of tissue acidosis precedes any decline in mechanical functions [396].

Coming now to the functional parameter patterns of the 6 mean trends for each series of controls/treated animals in each of the three conditions examined (PC, LP and SP), Figure 6 of Part 1 shows the kinetics, which are different in the various conditions, but do not correspond to the cited published reports. Our functions are not attributable to less than 4 parameters, including not individually separable interactions of isolated factors, hitherto regarded as decisive. The kinetic variants of the metabolic parameter in the rapid transient initial phases and following the conclusion of the ischemia warrant appropriate discussion; precise knowledge of these is mainly conditioned by the modalities of realignment of the unified data to correspond to the standard repetition frequencies of the measurements, scaled down, for the purposes of the multiANOVA analysis, to intervals of every two minutes, which is an aspect we will comment upon in section 4.4 here below. The trends, however, which at first sight seem more stable in the central phases of index ischemia, prove, on the basis of comparison of the stationary states “3” vs “5” seminally defined by Chance *et al.* [397-400], the distinct, undeniable decoupling of the kinetic data presented to date, focusing, as they do, on the trends of the only resulting parameter describing ischemic contractures over a standardized sustained phase of 30 minutes. The central ischemic phases of all specimens, in fact, correspond to redox levels of global pools of pyridine-nucleotide co-factors, which are practically undifferentiated and stable around the maximum common levels reached and prove to be dissociated from those of the sigmoid functions, increasing up to the peak values with subsequent fadings to the point of conclusion of the global ischemia; these fadings are typical and differentiated for the 6 experimental groups studied.

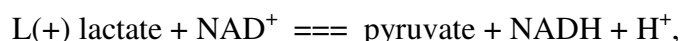


The percentage data relating to the NAD(P)H/NAD(P)<sup>+</sup> profiles of the individual specimens are given in 5.1. Data Section, while the 6 kinetic trends of the mean values in Figure 7 correspond, for the metabolic parameter, to those of Figure 8. The figures corresponding to the original fluorescence tracings, in the common 30-minute ischemia section, do not reveal the double kinetics described in the amphibian heart, interpreted as an initial prevalence of the reductions of the cytoplasmic pools, dissociated from the mitochondrial compartmentalized ones [217], but they do enable us to recognize the differentiated oscillatory phases during reperfusion, discussed in the following paragraphs. Cardiac proteomics, which has already been initiated [85], has not yet enabled investigators to produce a conclusive topological definition with complete mapping of the connections between the metabolic catalytic units possibly classified as receptorial, or their possible multiple interactions, i.e. the structural connectivity of the various architectures, characterized, for example, by the “small-world networks” of intermediate degrees between traditional and random ones [401-404], the local clusterings of the high frequencies of contacts and the mean length of the synchronicity entrainment paths, according to the laws of scaleless power, indicating the possible presence of systems in a state of auto-organized growth [405] and denoting that the configuration of the network will influence its non-linear dynamics in the future. In addition to the changeable, increasingly complicated image of the mythical “Kafkaesque mixture” of evidence present, the dimensions of the results of the apparent present-day temporary conclusions, which tend to be merely contingency-based or opportunistically reductionistic, will just go on multiplying to the point where they cease to have any meaning at all. Not without learning that even the most recent homeomorphic acquisitions already evince interactions between separate functional submodules of diversely differentiated cells, which can express themselves by combining distinct topologies [406], establishing increasingly theoretical forms of modelling that can hardly fail to resolve the by no means irrelevant existing confusion, contradictions and omissions - including the singular factual citations currently discernible - and which satisfactorily clarify, for example, also the temporal anticipations of ischemic and postischemic contractures.

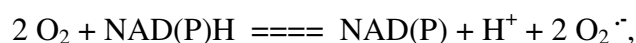
#### *4.4 IPC CYCLES AND POST-INDEX ISCHEMIA REPERFUSION, CONTRACTURES AND RECOVERY*

It has been claimed that the loss of pyridine enzymes, reported in canine ischemia, contributes to determining the transition from the reversible to the irreversible phase of ischemic damage [373]. Even more than the loss of concentrations judged to be free of substrates, used for estimating redox

compartmental quotients - and phosphorylation quotients, as will be discussed here below, - of dehydrogenases, this depletion leakage is an indicator of the heterogeneity of  $H^+$  diffusion, that is to say, of the dynamic spatio-temporal characteristics of the local intracellular pH as well as the redox regulatory gradients currently undergoing new forms of modelling [195, 391, 407]. In particular, the flows corresponding to the stoichiometry of lactate dehydrogenase:



must be associated, for example, with those of NAD(P)H oxidase:



which is known to play a role in cardiovascular biology [408], where it is by no means irrelevant that, in the first place, for the homologue *NOH-1*, there exists a codification for alternative splicing of the  $H^+$  channel prototype [409]. First and foremost, in the early variations of the mechanical functions, and associated alterations of energy metabolism proper to short IPC cycles, a number of instances of which were mentioned towards the end of the previous section, and also in the protracted reperfusion of the index ischemia, up until the recovery, if any, monitored over the last 60 minutes of the individual kinetic time courses, it is not without importance that, in the oxidation of NAD(P)H, the superoxide free radical is generated, which participates in the oxygen-nitrogen exchange network, referred to both in the protection mechanisms [152] and, above all, in damage of mitochondrial origin, and both in the short triggered ischemias of PC [148] and in the acute phase of the reperfusions [88, 99, 146, 268, 275-283, 361]. It should be noted, moreover, that in our IPC model, the reduction in cardiac oxygen consumption ( $MVO_2$ ), which is an expression of the above-mentioned “down-regulation of the cellular energy demand” and is supposed to reduce energy expenditure, may stabilize itself as from the very first short ischemia cycle, even in the presence of KCl (15 mM), which impedes contraction and is not indispensable for the conditioned process, which, on the other hand, is impeded in this experimental condition by NO, which was assumed back in 1988 to be due to blocking of the mitochondrial respiratory chain [345]. In contrast, the steady redox level of the NAD(P)H/NAD(P)<sup>+</sup> pool, reached in our early PC ischemias is maintained substantially constant in all the subsequent measurements, as can be seen in Figure 8 for the averaged tracings and in Fig. 9 (original tracings) and with the same myoglobin kinetics observed in the identical model in Figure 10 [211]. In actual fact, in the first 30 seconds of the final reperfusion, there is known to be a strong burst of radical production (see Fig. 1, page 21, of ref [297]), and in this initial period we almost constantly found severe sudden decreases in contractile function, which, however, do not appear in the blood-perfused model (cfr.: Fig 5, p 39, of

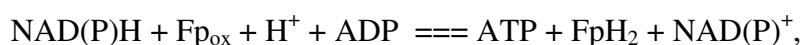
ref. [297]); these are followed by a hyperactivity increase as early as the first minute of reoxygenation, which continues as a dysfunction measured at the subsequent 2-minute observation intervals. Rapid trigger phenomena can obviously give rise to subsequent kinetic trends, whose essential characteristics cannot be recognized with insufficiently continuous standard measures at inappropriate intervals of longer duration. The author of this paper has already expressed himself clearly on this point [297, pp. 37-38] - albeit in vain, for many of those who followed - stating that The “heterogeneity of individual transients and irregularities in the amplitude, phases and localization of the transients might not be revealed by time-averaged measurements of any intra- or extracellular effector of redox radicals,  $Ca^{2+}$ , messengers, etc.”, which, on the contrary, require spectral analyses in the frequency domain [197-198, 410-412], avoiding the falsifications implicit in the application of the apparent simplifications of the current Ockham’s razor criterion, where internal events shorter than the repetitive measurement interval adopted are expressed as arithmetical or geometrical means and/or are commonly subjected to transformations involving non-time-independent variabilities. In any event, Figures 1 (CF), 3 (MSLVP), 5 (RPP) and 6 (RPP/CF) in Part 1 show conclusively, for the PC groups, the drop in percentage values of these functional parameters as early as the onset of the initial stress of the first brief ischemic insult, and the functional deficit emerges all the more clearly at the end of the common final perfusion phase after the full duration of 60 minutes, typical of the early first window of protection. In the control groups, as in those pretreated with the vanilloid, the metabolic fluorescence index which rises rapidly, as an apparently monotonic function not analyzed here, reaching stable levels in the three PC cycles, corresponding to those expressed equally in the central trend, represents a new, initial element differentiating between the functional responses and the metabolic index; this dissociation implies an acquired permanent memory uncoupling right from the very first conditioning cycle. Consequently, the early kinetic triggering and possibly interacting metabolic profiles may be reconsidered here as markers of the potential energy availability and expenditure, not associated with the coincidental mechanical features, extending even as far as the windows of any late recovery [68, 77, 104-106, 148, 152, 161, 212, 243, 245, 261-262, 289, 308-309, 311, 413-414]. We do not intend here to develop the discussion any further in relation to the possible regulation of interacting redox and phosphorylative potentials with locally compartmentalized networks, which will be the subject of our next very relevant  $^{31}P$  NMR observations [271]. Nevertheless, the currently relevant references are to be found in [295-306, 332-352] as well as in [413-434]. Further information on the possible multi-scaled spatial and temporal compartments associated with the genetic and epigenetic

interactions/regulations found is available in studies such as those organized analytically [435] and these topics are overviewed in [434] or described in [427-437]. The hypercontracture, which extends, in the Langendorff rat heart model, to the early failures after sustained ischemic stress, pending confirmation as to whether the peptide co-deprivation described [269] and/or any specific vanilloid/capsaicin pretreatment free-radical-induced activation and desensitization [164-187, 269-274], counteracted as in cultured adult cardiomyocytes by particulate guanylate cyclase stimulators, such as ANP or urodilatin [438], may be worth reporting here, inasmuch as dysfunction, contracture and rigor [262] during reperfusion have been attributed to conditions interpreted as no-reflow conditions [20-21, 52, 68].

#### **4.5 REAPPRAISAL OF AUTOFLUORESCENCE ASSOCIATIONS AND MEANINGS**

Our initial application of physio-pharmacological, non-invasive *in-vivo* read-out techniques [192-193, 228, 235, 329, 439-441] and global spectrometric optical approaches, including the (near-)infrared methodologies, afforded an opportunity to directly evaluate the extent of matrix water volumes. Together with nuclear resonance, they yield integrated indices of the spatial and temporal heterogeneity of the probands, and are well known in autofluorescence studies of reduced pyridine co-factors and their important effects on cardiac and coronary activity [225-227, 232-233, 442]. Possible adequate resolutions, even of a metabolic and functional nature, by means of associated procedures of reconstruction of (microimages) [443-446] were not adopted here because this was not one of the objectives of the present analysis. The observation of autofluorescence of homogeneous and cellular tissues thus permits partial resolution of the microdomains involved in circulatory functions, and the quantitative identification of the origin and of traditional mitochondrial, cytosolic and microsomal-sarcoplasmic involvement proves difficult [447] owing to the free and/or bound levels of both the pyridine (reduced) and flavin (oxidized) co-factors. The initial observations of fluorescence in the Langendorff rat heart model did not yield results in terms of level which were substantially different for the complexes of either type of factor [216], while, on the other hand, the tracings were out-of-phase in the spontaneous pulsations of the homogeneous amphibian heart [194, 217]. Interpreted as fluctuations of subcompartments in obviously unstable temporal equilibrium in the most variable conditions of stress, that is to say, the balancing of reciprocal oscillations on the same side of the basic/general stoichiometric equation indicated at the time [194].

Today we would say “lumped”, this being an example of a model of common pools, without any circumscribed intracellular spatial structure or, at best, only an apparent one:



which may remind one of the intercellular cyclic ones of the confirmed astrocyte-neuron lactate shuttle hypothesis [448], which are recognized solely for the milieus of pyridine factors. The flavin out-of-phase states and fluctuations, described by the Marban group [95, 126-127, 129], both mitochondrial [449] and cellular [450], commented upon as being conclusively dubious (cfr.: section 3.4, pp. 23-24 [355]), have not been further analyzed. However, confining our attention to the emissions of reduced pyridine-nucleotides, delays and specular anticipations (cfr.: paradoxical ischemia), time intervals and consequent periodicity synchronization phase shifts have been attributed to different protracted periods of accumulation and aggregation permanence in protide heterodimers (PER and TIM, in *Drosophila melanogaster* [451]), the temporal kinetic profiles of which were initially only studied singly. Nevertheless, it has already been proved that there exists (in *Saccharomyces cerevisiae* [452]) a functionally structured compartmentalized organization, not only spatial but also temporal, ultradian, of clusters of expression of genetic cell processes, of redox metabolic and mitochondrial aggregate respiration translation, which is also associated with NADH, thus confirming the complex dynamics, and the seminal synchronized periodicity phenomena observed more than 40 years ago also in extracts of cardiac cells [453], by the researchers of the group coordinated by Britton Chance [303, 410-412, 454]. It has been ascertained - and reported [423, 429] - that, in the Langendorff model, it is not the products of ATP catabolism, resulting from imposed incremental heart rate steps (in the electrically stimulated perfused rabbit organ with the sino-atrial node destroyed with glucosated Tyrode solution at 28°C), that activate mitochondrial respiration by simple diffusion, while, in the isovolumic model of contractile function assessed as rate pressure product (RPP), the creatine-phosphate energy shuttle is effectively bypassed [425]; mitochondrial ATPase, during IPC, is not involved and functionally compartmentalized glycolytic activity [224, 455] is *activated* or *inhibited* [94]. In the hepatic mitochondria, devoid of any iso-creatine kinase, the increase in NAD(P)H fluorescence proves to be related, in a declared linear manner, to the increase in O<sub>2</sub> consumption [456], and redox dynamics of enhanced cellular oxidation have been associated with IPC [68], with greater postischemic recovery as a result of activation of pyruvate dehydrogenase [457].

The subject of evaluations of response time, defined as the first statistical moment of the system's impulse response function [458-459], analogous to the mean transit time of pharmacotoxicokinetic

parametrizations, is controversial, where the primary parameters of the model, from which the above-mentioned derivative values result [460], are not defined. Continuing to concentrate here only on the emission of pyridine co-factors (in addition of those already mentioned and discussed (e.g. [222, 231]), see also refs. [461-462]). The complex interpretation of contradictory results already reported is further complicated by the findings, for example, of an increase in aortal pressure (Langendorff rat heart; substrate  $\beta$ -hydroxybutyrate) together with a *decrease* in the NADH/NAD<sup>+</sup> ratio, prevalently mitochondrial with an unchanged lactate/pyruvate ratio; an increase in work due to increased frequency together with an *increase* in NAD(P)H (Langendorff rat heart [464]), a *decrease* in NADH/NAD<sup>+</sup> (Langendorff rat heart, Tyrode; substrate: glucose, pyruvate [465]), or *no variation* (isolated working rabbit heart, glucose  $\pm$  pyruvate [299]). In the same glucose preparation, a *decrease* was observed in the (G-3-P/DHAP)-cytosolic NADH/NAD<sup>+</sup> ratio, but an *increase* in the mitochondrial ratio on pyruvate addition and an *increase* in some cyto- and mito- ratios if lactate is added [300]. Once again it is necessary to refer here to our overview [195], where the substrates mentioned definitely do not present kinetics of homologous redox derivative values, but homogeneous assumptions. Nevertheless, *no changes* in the NADH/NAD<sup>+</sup> ratio with an increase in the frequency of stimulation in rat and guinea-pig cultured myocytes have been depicted more recently [466]. We are operating, then, in the context of now knowing in repeated detail a number of particular microscopic-molecular features (e.g. [467-471]) and intermediate, interacting and overlapping regulation, modulation and control structures in “small world networks” (cfr.: [195, 402-403, 405]), certainly not in any analytically complete manner, but rather as multiform, intermingled factors conditioned by dynamically and temporally compartmentalized spatial microarrays, which have long been addressed theoretically [472], but ignored in practice. Descriptions of channelling flows in substrates - including the pyridine coenzymes [473] - and metabolic networks [474], also in preconditioned and metastable adaptations, of flows in loops reductive of diffusion, segregation and transport, possibly evaluated in isolation (cfr.: [391, 407]), in restrictive conditions which are still not fully known and which, in view of their limited stability, still undermine the ponderable predictivity of satisfactory general integrated conclusions. The integrated cardiovascular modellings, which, despite being in their infancy, are already the subject of highly coordinated and rapid development [476], do not permit conclusions to be drawn, having been declared elusive and ongoing, in a situation where “the sensitivity of the networks to variations in the descriptive biochemical parameters has been modelled quantitatively and shows adaptation properties as a consequence of the network’s connectivity, not requiring fine tuning of some parameters” [477].

Yet, for example, “the formation of disulphide bonds from thiol groups of two cystein residues generates two protons and two electrons, which need to be accepted by an oxidant to yield the covalent structure of the polypeptide chain. So, the oxidative protein folding is coupled to the electron transport chain” [469] and “14 Å or less spacing of redox centres provides highly robust engineering for electron transport with high directional specificity over greater distances by multi-step tunnelling, which slows roughly linearly with distance along the chain, instead of the sharply exponential slowing in all other directions” [470]. Parameter sensitivity as a theoretical criterion for evaluating and comparing the performance of biochemical systems was presented in the ‘seventies [478], and the organization of some biochemical systems from microspace to macroscopic structures was analyzed in the ‘eighties, but, remarkably (p. 8 in [472]), it was envisaged at that time that “if isolated, each of the various levels retains its own autonomous function and structure, although only the coupling of these functions at any level through transport and diffusion yields the physiological properties, which are lost on destruction, and biochemical analysis might yield non-sense information”.

In particular, it is the auto-organized fluctuating structures that demand more detailed in-depth attention, even when these structures are out of phase but synchronizable [479], and regarded as basic, yet rendered elusive in those cases where they rest upon all too inadequate experimental design analyses - e.g. in repetitive, statistical analyses [480] of the discontinuity of exaggerated lag times, that are unjustified and engender irremediably disinformative confusion of such a nature as to mean that investments of increasingly limited resources are wasted, not to say counterproductive. Research projects strongly backed in the implementation phase are already classifiable as doomed to rapid obsolescence, deserving only of consideration as a sort of denied inertia, where “data models” and the related simulations, instead of the necessary modelling hypotheses that are the prerequisite of any prediction supported by experimental observations, have constituted mere surrogates with all their rationalizable shortcomings, as encountered in the field of preconditioning due to exercise, hypoxic and ischemic stress. The example indicated of anticipation of “paradoxical ischemic contractures” not analytically related to any properly designed, accredited modelling, is pathognomonic; the accredited modelling approach is the one originally presented only here, with analysis of fluctuation trends in reoxygenation phases and prolonged wash-outs (cfr.: Figure 9), which offer no possibility of integration, unless through the most systematic chemical analyses, including phospho- and nitroproteomic analyses [85, 188, 481-483] as well as functional analyses extended to the frequently mentioned spatio-temporal domains, without excluding known approaches [484].

The advances in bioinformatic data integration, functionally integrated systems science and multi-scale, structurally integrated bioengineering modelling [475-476] offer mandatory opportunities for development, not unlike the type advocated in reiterated proposals [187], which are the subject of current verifications regarding the spin-offs of the experimental biological and clinical-therapeutic pharmacotoxicological rationale for the use of cannabinoids and vanilloids (cfr.: [187, 485]).

#### *4.6 CONCLUSIONS*

When optimal individual ideation has been achieved and after conducting the resulting initial trials, eventually to be presented as an abstract if the predictions are subsequently supported by the experimental observations, the globalization of science today requires, as soon as possible, the on-line distribution and publishing of the accepted biological monitoring, standardized according to the broadest, upgraded consensus justifying the most complete availability of the detailed protocols conceived and on the basis of the fullest possible data, in order to produce results that can be re-evaluated in any reconstructive (statistical) analysis and that potentially do not need to be repeated. This is not the case with the current research in the area of the most extensively performed studies, including those using the Langendorff rodent models. We have attempted to obtain our data in the most widely accepted Langendorff rat heart specimens for ischemic PC at one of the best, officially accredited animal facilities in the world, and have registered a substantial animal variability, which, after proper randomization, afforded no chance of rejecting those observed patterns that may have presented opposite features. The aligned 2-minute trends, obtained by submitting the data for controls vs. capsaicin-pretreated animals to bivariate analysis of percentage normalized (arithmetical) means, show both protection and detrimental features in the long perfused vs. preconditioned control hearts. The individual results of parameters, such as ischemic contracture, and the reduced pyridine-nucleotide enzyme pool testify to the integration of at least 6 interacting, possibly independent, analyzed factors. The discussion can neither be extended nor summarized any further at this point. A full multivariate study of the entire data set obtained and listed below – next Section, such as only on-line publishing can permit -, and the specific point-by-point conclusions will be presented therein, in a following Part 3. We agree, of course, with those who maintain that this fertile area of investigation is rapidly advancing [204-205, 328, 486-488], as both previous and new, singular results [489-499] make a constant, continuous, global reappraisal of the whole area absolutely mandatory.



5. DATA SECTION

5.1 TABLE OF ALL RATS (HEARTS) USED, Part 1 and 2: 41 for the 6 Functional Parameters A), plus 6 added for B), the 1<sup>st</sup> Metabolic Parameter only

A) Functional Parameters:  
(1-CF; 2-BPM/Hz; 3-LVMSP;  
4-LVEDP; more 5-RPP, and  
6-RPP/CF)

B) 1<sup>st</sup> Metabolic Parameter  
(1-NAD(P)H global fluorescence)

I - Short Perfusion Controls

1	1 Nov 30a '98	1.	1 Nov 30a '98
2	2 Dec 7a '98		
		2.	2 Dec 8c '98 (new)
3	3 Dec 9a '98	3.	3 Dec 9a '98
4	4 July 21a '99		
5	5 July 21b '99		
6	6 July 27a '99		
7	7 July 27b '99		

II - Short Perfusion Capsaicin

		4.	1 Oct 26c '98 (new)
8	1 Nov 30b '98		
9	2 Nov 7b '98		
10	3 Dec 8b '98		
11	4 Oct 28a '99		
12	5 Oct 28b '99	5.	2 Oct 28b '99
13	6 Oct 29b '99	6.	3 Oct 29b '99

III - Long perfusion Controls

14	1 Nov 24a '98		
15	2 Nov 24b '98	7.	1 Nov 24b '98
16	3 Nov 25a '98	8.	2 Nov 25a '98
17	4 Nov 27a '98		
18	5 Nov 27b '98		
		9.	3 Nov 27c '98 (new)
19	6 July 19a '98		
20	7 July 20b '98		
21	8 July 28a '98		
22	9 July 28b '98		
		10.	4 Oct 28a '99 (new)

IV - Long perfusion Capsaicin

23	1 Nov 26b '98		
24	2 Dec 1a '98		
25	3 Dec 8a '98		
26	4 Oct 29a '99	11.	1 Oct 29a '99
		12.	2 Nov 1a '99 (new)
27	5 Nov 4a '99	13.	3 Nov 4a '99
28	6 Nov 4b '99	14.	4 Nov 4b '99

V - Preconditioned Controls

29	1 Nov 23a '98		
30	2 Nov 25b '98	15.	1 Nov 25b '98
31	3 Dec 10b '98	16.	2 Dec 10b '98
32	4 Dec 14a '98	17.	3 Dec 14a '98
33	5 July 22a '99		
34	6 July 22b '99		
35	7 July 27c '99		
		18.	4 Nov 5b '99 (new)

VI - Preconditioned Capsaicin

36	1 Nov 26a '98	19.	1 Nov 26a '98
37	2 Dec 9b '98		
38	3 Dec 10a '98	20.	2 Dec 10a '98
39	4 Nov 8a '99	21.	3 Nov 8a '99
40	5 Nov 10a '99	22.	4 Nov 10a '99
41	6 Nov 10b '99	23.	5 Nov 10b '99









Table V (Figures 5, p 152 and 6, p 153, Part 1)

**SHORT PERFUSION: data for calculation of RPP and RPP/CF from the averages only (without any statistical evaluation).**

RPP = RATE PRESSURE PRODUCTS OF % AVERAGES: RPP = (MSP-EDP)xHz %. At right: RPP/ CF % VALUES.

	Means		Means Caps.		Means		Means Caps.		Means		Means Caps.		RPP		RPP / CF					
	CORONARY FLOW (CF)				FREQUENCY BEATING				MAXIMUM SYSTOLIC PRESS				END DIASTOLIC PRESS				Controls		Treated	
-15	100,0	100,0	100,0	100,0	100,0	99,8	7,3	7,9	-15	9274,6	9190,2	-15	92,7	91,9						
-13	100,0	98,1	100,0	100,0	100,0	93,9	7,3	8,7	-13	9274,6	8523,7	-13	92,7	86,9						
-11	100,0	99,2	100,0	102,9	100,0	94,3	7,0	9,8	-11	9303,1	8691,2	-11	93,0	87,6						
-9	100,0	100,7	100,0	104,0	100,0	96,8	6,9	9,1	-9	9310,3	9119,7	-9	93,1	90,6						
-7	99,7	105,3	100,0	103,2	100,0	100,6	7,8	8,6	-7	9220,4	9494,4	-7	92,4	90,2						
-5	97,9	104,5	101,2	102,4	104,9	98,6	7,1	8,2	-5	9889,7	9266,0	-5	101,0	88,7						
-3	99,8	104,5	100,8	106,5	102,4	100,7	6,3	7,4	-3	9686,3	9939,0	-3	97,1	95,1						
-1	99,2	104,4	111,9	110,1	103,0	99,2	6,0	7,3	-1	10856,4	10110,1	-1	109,4	96,9						
1	0,0	0,0	108,8	110,6	16,9	29,3	5,8	6,0	1	1206,2	2580,8	1	0,0	0,0						
3	0,0	0,0	30,3	22,8	0,0	0,0	6,5	7,5	3	0,0	0,0	3	0,0	0,0						
5	0,0	0,0	0,0	0,0	0,0	0,0	7,2	7,4	5	0,0	0,0	5	0,0	0,0						
7	0,0	0,0	0,0	0,0	0,0	0,0	7,2	7,4	7	0,0	0,0	7	0,0	0,0						
9	0,0	0,0	0,0	0,0	0,0	0,0	7,3	7,4	9	0,0	0,0	9	0,0	0,0						
11	0,0	0,0	0,0	0,0	0,0	0,0	7,4	7,9	11	0,0	0,0	11	0,0	0,0						
13	0,0	0,0	0,0	0,0	0,0	0,0	7,6	10,8	13	0,0	0,0	13	0,0	0,0						
15	0,0	0,0	0,0	0,0	0,0	0,0	11,4	19,0	15	0,0	0,0	15	0,0	0,0						
17	0,0	0,0	0,0	0,0	0,0	0,0	35,5	32,7	17	0,0	0,0	17	0,0	0,0						
19	0,0	0,0	0,0	0,0	0,0	0,0	53,1	41,2	19	0,0	0,0	19	0,0	0,0						
21	0,0	0,0	0,0	0,0	0,0	0,0	50,8	43,8	21	0,0	0,0	21	0,0	0,0						
23	0,0	0,0	0,0	0,0	0,0	0,0	47,0	42,9	23	0,0	0,0	23	0,0	0,0						
25	0,0	0,0	0,0	0,0	0,0	0,0	44,3	40,3	25	0,0	0,0	25	0,0	0,0						
27	0,0	0,0	0,0	0,0	0,0	0,0	42,2	38,3	27	0,0	0,0	27	0,0	0,0						
29	0,0	0,0	0,0	0,0	0,0	0,0	40,7	36,5	29	0,0	0,0	29	0,0	0,0						
31	50,6	62,5	96,4	128,7	83,5	72,1	70,6	63,1	31	1244,8	1161,1	31	24,6	18,6						
33	53,2	62,2	90,5	164,7	104,6	89,3	88,3	82,5	33	1470,7	1121,0	33	27,6	18,0						
35	56,5	60,7	121,3	123,2	112,1	99,4	92,6	91,8	35	2362,7	933,5	35	41,8	15,4						
37	57,2	61,4	139,7	86,0	104,2	98,0	92,0	90,8	37	1702,2	620,5	37	29,8	10,1						
39	56,6	62,3	128,5	102,4	100,5	97,0	88,3	84,8	39	1564,6	1247,2	39	27,7	20,0						
41	57,8	61,0	120,3	84,3	99,1	95,0	90,4	84,5	41	1040,6	887,0	41	18,0	14,5						
43	58,2	60,9	127,6	94,2	98,3	93,5	89,4	83,2	43	1130,2	972,4	43	19,4	16,0						
45	58,4	60,7	102,9	98,2	97,7	92,9	88,7	82,3	45	923,3	1041,3	45	15,8	17,2						
47	58,6	60,2	118,1	93,4	97,0	92,6	84,8	81,6	47	1434,3	1023,2	47	24,5	17,0						
49	58,1	60,1	114,4	85,3	96,5	91,4	87,6	80,7	49	1014,0	911,5	49	17,5	15,2						
51	57,6	59,9	109,1	92,7	96,1	91,6	87,1	79,3	51	982,6	1134,0	51	17,1	18,9						
53	57,5	59,8	121,8	82,0	95,9	91,8	86,1	78,4	53	1199,3	1097,7	53	20,9	18,3						
55	57,3	59,7	128,5	86,4	94,8	92,1	85,6	77,1	55	1192,4	1299,4	55	20,8	21,8						
57	57,2	59,6	107,8	83,0	94,7	93,6	84,6	76,1	57	1093,2	1456,1	57	19,1	24,4						
59	57,2	59,4	95,8	82,4	94,7	94,9	83,9	75,0	59	1033,6	1642,5	59	18,1	27,6						
61	57,1	59,4	87,3	87,4	94,3	96,5	83,1	74,1	61	980,3	1959,1	61	17,2	33,0						
63	57,1	58,9	89,6	86,0	93,9	97,5	82,6	73,4	63	1013,2	2074,0	63	17,7	35,2						
65	57,0	58,8	101,3	83,1	92,9	98,2	82,2	72,4	65	1090,3	2148,3	65	19,1	36,5						
67	56,3	58,5	85,3	83,6	93,7	99,1	81,4	71,7	67	1054,4	2295,6	67	18,7	39,2						
69	56,1	59,1	82,9	79,2	93,4	100,1	80,7	70,9	69	1046,9	2315,2	69	18,7	39,2						
71	56,0	61,8	90,5	79,4	92,8	100,4	79,9	70,2	71	1168,4	2400,4	71	20,9	38,8						
73	55,8	62,2	87,9	87,9	93,5	100,9	79,2	69,7	73	1256,8	2738,8	73	22,5	44,0						
75	55,2	61,2	81,5	76,9	93,8	101,0	78,3	69,1	75	1258,0	2454,6	75	22,8	40,1						
77	54,9	61,1	83,2	76,1	93,9	101,4	77,9	68,4	77	1333,0	2507,3	77	24,3	41,1						
79	54,9	60,8	83,0	77,0	94,1	101,6	77,2	68,0	79	1402,6	2594,3	79	25,6	42,6						
81	54,5	60,0	87,0	75,4	94,0	101,6	76,1	67,6	81	1550,8	2567,0	81	28,5	42,8						
83	54,3	60,0	87,2	75,6	93,5	102,3	76,0	67,5	83	1520,6	2630,9	83	28,0	43,9						
85	53,5	60,0	85,5	77,0	93,2	102,2	75,2	67,1	85	1533,9	2702,9	85	28,7	45,1						
87	53,4	59,9	121,4	89,7	92,9	102,2	68,0	67,1	87	3013,9	3142,6	87	56,4	52,4						
89	53,2	59,9	79,8	89,7	93,2	87,1	67,9	61,8	89	2014,9	2270,4	89	37,9	37,9						







Tabella VIII ( Table 1, p 147; Figures 3, p 150 and 7, p 154, Part 1)

TABLE OF DATA. LONG PERFUSION. MAXIMUM SYSTOLIC PRESSURE ( ABSOLUTE, % VALUES AND THEIR STATISTICAL EVALUATIONS).

Table with 24 columns for time points (Nov24a98, Nov24b98, Nov25a98, Nov27a98, Nov27b98, Jul19a99, Jul20a99, Jul20b99, Jul28a99, Jul28b99, Nov26b98, Dec1a98, Dec2a98, Oct29a99, nov4a99, nov4b99) and 4 columns for statistical parameters (Tim, Means, S.D., Contr.St.Err. Cor). It contains multiple rows of numerical data representing systolic pressure values and their statistical evaluation.





















Table XVIII (Figure 8, Part 2)

TABLE OF DATA SHORT PERFUSION, NAD(P)H FLUORESCENCE. % VALUES OF EACH HEART.

Time (min)	Nov30a98	Dec08c98	Dec09a98	Nov26c98	Oct28b99	Oct29b99	Control 1	Control 2	Control 3	Capsaicin	Capsaicin	Capsaicin	Time	Average C.S.D.	Contr.	Stand	Erro	Average C.S.D.	Caps.	Stand	Erro	Test T (P)
-15	-2.6	-0.4	7.3	-24.4	14.9	-0.5	-15	1.4	5.19	2.99	-3.3	19.78	11.42	0.72								
-13	-2.6	-0.4	3.9	-5.9	40.7	0.0	-13	0.3	3.31	1.91	11.6	25.35	14.64	0.52								
-11	-2.6	-0.4	0.5	-4.7	30.7	4.8	-11	-0.8	1.59	0.92	10.3	18.31	10.57	0.40								
-9	-0.9	-0.4	8.6	-6.0	23.2	2.9	-9	2.4	5.34	3.08	6.7	14.99	8.66	0.68								
-7	-0.8	-0.4	0.0	-2.3	10.9	11.3	-7	-0.4	0.42	0.24	6.6	7.70	4.45	0.25								
-5	3.9	-0.4	6.3	0.4	4.9	9.8	-5	3.2	3.40	1.96	5.0	4.72	2.73	0.62								
-3	4.4	-0.4	4.5	-1.3	3.6	-2.7	-3	2.8	2.81	1.62	-0.1	3.30	1.91	0.30								
-1	5.9	-0.4	2.7	0.0	0.0	-1.3	-1	2.7	3.14	1.81	-0.4	0.74	0.43	0.22								
1	68.2	61.3	73.1	51.4	94.9	86.0	1	67.5	5.95	3.43	77.4	23.00	13.28	0.54								
3	81.5	85.1	90.0	85.1	100.0	95.9	3	85.5	4.28	2.47	93.7	7.69	4.44	0.20								
5	95.7	94.8	91.4	91.0	99.3	92.0	5	94.0	2.28	1.32	94.1	4.53	2.62	0.96								
7	97.1	92.9	95.9	92.0	98.6	94.5	7	95.3	2.12	1.23	95.0	3.34	1.93	0.91								
9	96.6	100.0	95.9	93.3	97.3	98.5	9	97.5	2.20	1.27	96.4	2.74	1.58	0.61								
11	96.3	91.2	91.9	97.7	97.3	95.5	11	96.1	2.78	1.61	96.8	1.20	0.69	0.13								
13	93.2	96.3	100.0	95.2	97.3	100.0	13	96.5	3.39	1.96	97.5	2.42	1.40	0.70								
15	95.0	91.4	88.5	90.3	98.4	97.3	15	91.6	3.27	1.89	95.3	4.41	2.55	0.31								
17	93.9	96.7	85.7	98.2	96.5	97.2	17	92.1	5.74	3.31	97.3	0.88	0.51	0.25								
19	96.4	90.8	85.2	92.0	94.2	94.9	19	90.8	5.60	3.23	93.7	1.48	0.86	0.47								
21	95.5	95.6	84.6	100.0	92.3	96.1	21	91.9	6.30	3.64	96.1	3.87	2.23	0.39								
23	95.8	93.6	84.9	99.9	93.9	95.3	23	91.4	5.74	3.31	96.4	3.11	1.80	0.28								
25	100.0	92.6	87.7	95.7	90.2	95.1	25	93.4	6.19	3.57	93.7	2.99	1.73	0.96								
27	99.9	90.3	90.6	92.1	87.7	96.9	27	93.6	5.46	3.15	92.3	4.62	2.67	0.76								
29	95.0	86.6	83.5	93.4	84.0	94.2	29	88.4	5.99	3.46	90.6	5.66	3.27	0.67								
31	50.0	52.4	29.6	14.4	54.8	13.9	31	44.0	12.56	7.25	27.7	23.49	13.56	0.37								
33	32.3	41.9	17.9	-1.1	16.6	8.7	33	30.7	12.05	6.96	8.1	8.86	5.12	0.06								
35	20.9	42.1	3.0	-8.6	2.3	8.6	35	22.0	19.57	11.30	0.8	8.67	5.01	0.19								
37	13.1	32.3	-1.1	-7.6	0.3	10.1	37	14.8	16.77	9.68	0.9	8.86	5.12	0.30								
39	14.0	32.5	-4.7	-3.8	2.3	13.3	39	13.9	18.60	10.74	4.0	8.67	5.01	0.47								
41	8.7	33.3	3.0	-7.6	1.2	14.9	41	15.0	16.07	9.28	2.8	11.32	6.54	0.35								
43	10.7	31.2	3.0	-7.7	0.3	11.1	43	15.0	14.59	8.42	1.2	9.40	5.43	0.25								
45	8.7	34.1	7.5	-13.5	0.1	9.3	45	16.8	15.05	8.69	-1.3	11.47	6.62	0.18								
47	10.4	28.9	14.7	-8.6	0.6	12.4	47	18.0	9.68	5.59	1.4	10.53	6.08	0.12								
49	7.0	28.2	5.2	-5.6	-1.4	11.6	49	13.5	12.81	7.40	1.5	8.94	5.16	0.26								
51	2.8	28.8	5.1	-9.8	-2.3	14.8	51	12.2	14.38	8.30	0.9	12.58	7.27	0.36								
53	3.6	25.6	8.2	-6.8	-1.0	8.6	53	12.5	11.58	6.69	0.2	7.78	4.49	0.21								
55	-0.9	24.1	13.0	-8.5	-1.4	11.1	55	12.1	12.53	7.24	0.4	9.92	5.73	0.28								
57	-3.6	25.8	9.4	-10.2	-1.5	13.8	57	10.5	14.73	8.50	0.7	12.17	7.03	0.42								
59	0.2	22.5	10.7	-7.0	0.9	12.3	59	11.1	11.14	6.43	2.1	9.73	5.62	0.35								
61	-2.3	21.1	8.6	-1.7	-0.3	12.7	61	9.1	11.67	6.74	3.6	7.96	4.59	0.54								
63	-9.2	16.6	9.8	-4.2	-0.4	11.6	63	6.1	12.83	7.41	2.3	8.27	4.78	0.70								
65	-7.5	17.8	18.3	-2.6	-0.2	10.8	65	9.5	14.78	8.54	2.7	7.13	4.11	0.52								
67	-5.7	10.3	16.1	-1.1	-0.1	11.4	67	6.9	11.28	6.51	3.4	6.91	3.99	0.67								
69	-8.0	10.7	23.7	-2.8	-0.9	11.2	69	8.8	15.95	9.21	2.5	7.60	4.39	0.58								
71	-10.1	10.3	26.4	-0.3	1.1	11.5	71	8.9	18.30	10.57	4.1	6.46	3.73	0.71								
73	-13.4	12.8	21.4	-2.2	-0.6	11.2	73	6.9	18.16	10.49	2.8	7.35	4.24	0.74								
75	-14.8	3.1	22.7	1.2	-8.4	10.9	75	3.6	18.75	10.83	1.3	9.66	5.58	0.86								
77	-18.1	5.3	19.7	0.8	-10.5	9.7	77	2.3	19.08	11.02	0.0	10.08	5.82	0.86								
79	-20.4	3.5	30.9	-0.5	-11.3	9.5	79	4.6	25.68	14.83	-0.7	10.39	6.00	0.76								
81	-21.0	1.2	31.5	2.2	-9.9	10.0	81	3.9	26.34	15.21	0.7	10.01	5.78	0.86								
83	-22.3	-5.8	29.5	2.2	-8.9	9.5	83	0.5	26.44	15.27	0.9	9.28	5.36	0.98								
85	-26.8	-1.1	30.7	2.2	-18.3	9.7	85	0.9	28.82	16.64	-2.1	14.47	8.35	0.88								
87	-26.8	-0.4	29.0	2.2	-25.2	9.7	87	0.6	27.93	16.13	-4.5	18.39	10.62	0.81								
89	-26.8	-0.4	29.0	2.2	-25.2	9.7	89	0.6	27.93	16.13	-4.5	18.39	10.62	0.81								

5.3 FILES OF THE ORIGINAL PHOTON COUNTED FLUORESCENCE EMISSIONS (COUNTS  $\times 10^{-4}$  SECONDS) VERSUS TIME ( $10^{-3}$  SECONDS) FLUORESCENCE KINETICS

These are available at request as an Excel matrix of 23 columns of single hearts fluorescence emissions vs 1760 lines of each millisecc subsequent times (6 min sampled initial decays of the final oxygenated reperfusion). Ask to: [l.rossini@univpm.it](mailto:l.rossini@univpm.it).

The 23 K slopes of the semilog fittings, and the  $R^2$  correspondent variability values of all the hearts indicated in the B) column of the Table presented in the 5.1 subsection, are reproduced as evaluated according to the t test for the control vs capsaicin three groups in the Table 2, section 4. Results.

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