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Microcalorimetric monitoring of ischemic tissue metabolism: Influence of incubation conditions and experimental animal species

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Abstract

Methodological problems arising in microcalorimetric measurement of ischemic tissue samples are discussed. Following a short description of the course of ischemic metabolism, usually represented as a decaying exponential function, a mathematical formulation of the resulting thermogram, suitable for simulation purposes, is given. Emphasis is placed upon the fact that, due to varying degrees of aerobiosis, biopsies do not reflect strictly ischemic conditions. Since, however, in larger tissue slices metabolic rate decreases very rapidly, methods of retarding decline of ischemic metabolism, including organ preservation, are presented. In this context, special attention is paid to possible mistakes in interpretation of microcalorimetric records obtained from ischemic tissue samples. Furthermore, the great influence exerted by the experimental animal species on the course of ischemic metabolism in homologous organs is stressed. From the results so far obtained, it is concluded that in ischemic tissue metabolism, deviations from a strictly exponential course in the form of intermediate "plateaus" are indicative of exogenous or endogenous protective effects.

Introduction

Structural and functional integrity of mammalian tissues depends on continuous blood supply. In the case of impaired or interrupted perfusion, called ischemia, progressive metabolic disturbances occur, leading finally to tissue death. In this paper, some of the methodological problems arising in microcalorimetric measurement of non-perfused tissue samples will be dealt with. Moreover, the specific contribution of microcalorimetry to the investigation of ischemic tissue metabolism will be outlined.

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In contrast to indirect calorimetry, requiring maintenance of tissue perfusion, and to biochemical sampling, yielding rather intermittent information, direct calorimetry allows continuous monitoring of ischemic tissue metabolism. This is of special interest in organ preservation which, in a broader sense, means all methods of protecting organs during transitory interruption of blood supply (e.g., in heart, liver, and kidney surgery or transplantation). In this field, monitoring of tissue heat output offers new experimental insights into the course of ischemic metabolism and may be of clinical use as a kind of "viability assay" [1,2].

However, the methodological problems to be presented here are perhaps of still more general relevance, there being various further imaginable indications for medical microcalorimetry of tissue samples (e.g., in diagnosis of muscular metabolic disorders or in grading tumor malignity). Unless provided with special experimental devices, such tissue samples will generally not be perfused. Thus, although in this case, the properties of the tissue itself are of interest rather than its ischemic behavior, nevertheless, for a correct interpretation of the results obtained, a knowledge of the rules of ischemic metabolism is an important prerequisite.

Typical course of ischemic tissue metabolism

In order to go into the problems of ischemia somewhat further, we shall first explain what happens in the case of interrupted tissue perfusion and what is measured by microcalorimetry.

In Figure 1, the turnover rate of ischemic tissues is plotted against time. Following interruption of blood supply, the oxygen reserves (physically dissolved oxygen, oxyhemoglobin, and, in muscle tissues, oxymyoglobin) are exhausted first so that for a very short time, the metabolic rate remains unchanged. After this free interval or "aerobic latency", tissue metabolism switches over to anaerobic energy production, i.e., lactic fermentation. Since, however, anaerobic glycolysis, at least at normal body temperature, is unable to meet the high energetic requirements of homeothermic tissues, a decline of metabolism results, in the course of which the organ functions are soon lost and, subsequently, structural damage develops. The total interval from the onset of acute ischemia to the manifestation of severe structural damage is called "resuscitation time". This does not necessarily mean that afterwards, a resuscitation of the tissue is not at all possible, but it does mean that after this critical time has elapsed, irreversible structural and functional lesions will remain [3–5]. Altogether, the ischemic metabolism is commonly represented as a decaying exponential function [6,7].

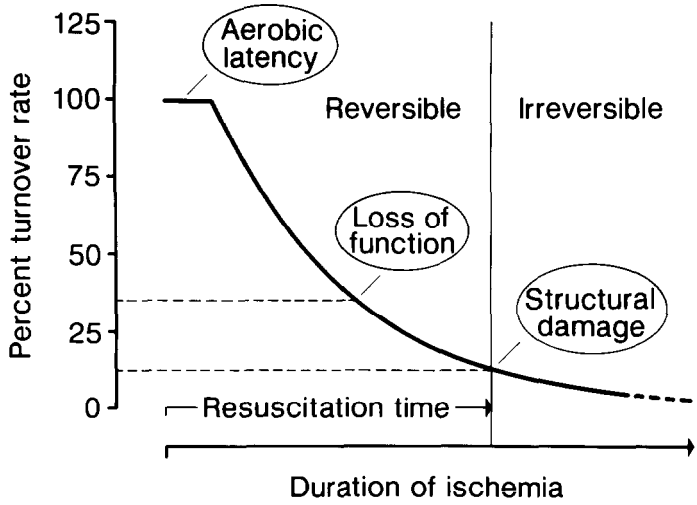
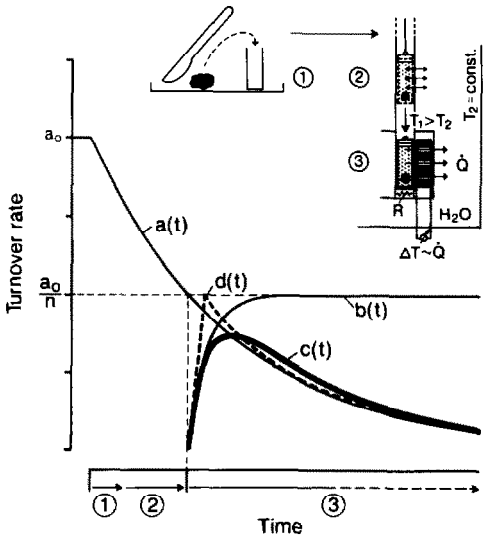


Figure 1. Course of tissue metabolism in the case of interrupted perfusion (ischemia). Following a short free interval, an exponential decay of turnover rate is assumed to occur. The time span from the onset of ischemia to the limit of irreversibility is called "resuscitation time".

In Figure 2, this curve is displayed again. In microcalorimetric investigation of ischemic tissues, however, a certain time is necessary for taking and preparing the sample as well as for filling, closing and cleaning the measuring ampoule. After this preparatory period which, under favorable conditions, does not last more than ten minutes, the measuring ampoule has to be suspended in the pre-thermostating position of the microcalorimeter[1] [8,9]. In this position, thermal equilibration takes place between the ampoule and the surrounding precisely thermostated water bath, requiring another twenty minutes. Only then can the ampoule be lowered into the measuring chamber where all the heat produced in the tissue sample passes into the large surrounding heat sink through a Peltier thermopile. Thereby, a thermal gradient arises which is directly proportional to the heat flow and allows continuous monitoring of the same. As this thermal gradient, although very small, takes some time to build up, the calorimeter has typical time response characteristics which can be seen, in electrical calibration, by the slow climbing of the signal up to the plateau. From this calibration curve, the time constant of the microcalorimeter (for reasons of simplicity, only *one*

[1] 2277 Thermal Activity Monitor (TAM™), ThermoMetric AB, Järfälla, Sweden



Decline of ischemic metabolism

$$a(t) = a_0 \cdot e^{-\lambda_1 \cdot t}$$

Time response characteristics of the microcalorimeter

$$b(t) = \frac{a_0}{n} (1 - e^{-\lambda_2 \cdot t})$$

Resulting thermogram (BATEMAN's function)

$$c(t) = \frac{a_0}{n} \cdot \frac{\lambda_2}{\lambda_2 - \lambda_1} \cdot (e^{-\lambda_1 \cdot t} - e^{-\lambda_2 \cdot t})$$

Dynamic correction

$$d(t) = c(t) + \frac{1}{\lambda_2} \cdot c'(t) = \frac{a_0}{n} \cdot e^{-\lambda_1 \cdot t}$$

Figure 2. Diagram of experimental procedure in microcalorimetric measurement of ischemic tissue slices, showing the time lag between onset of ischemia and start of measurement. The characteristic thermogram results from a combination of the decline of ischemic metabolism and the time response curve of the instrument. Only partial dynamic correction was performed in the graph to avoid the initial "overshoot" typical of the correction algorithm applied here [14].

time constant is presumed) can easily be derived. Since in measuring ischemic tissues, the heat production rate of the sample continues to decline while the thermal gradient is still building up, a combination of the two curves results. This combined function, originally formulated by Bateman for the activity of a radionuclide descending from a parent isotope and decaying itself, is also known in pharmacology where it is used for the blood concentration of substances which, while being resorbed from the intestine, are already excreted by the kidney [10,11]. In the case of microcalorimetry, it may be dynamically corrected for the time constant of the instrument with the aid of a simple but sufficient algorithm [12–14]. Since, however, the latency of the calorimeter is small in comparison to the course of ischemic tissue metabolism, and the resulting time error amounts to but a few minutes for a process monitored over six to ten hours, this can be largely dispensed with. Accordingly, a dynamic correction is not routinely performed, and in the following, only original curves are demonstrated. In these curves, an initial maximum and a subsequent long-lasting decrease of heat output can be distinguished.

Differences between biopsies and tissue slices

Whereas in organ preservation research, large tissue slices are preferentially used for analytical purposes, a few microcalorimetric investigations have been performed with small biopsies [2,15]. Because there are certain differences apparent in the results obtained, we decided to undertake a systematic comparison between tissue slices and biopsies on ischemic rat liver tissue. The results of this comparison are presented in the next two figures, i.e., in Figure 3 for the maximum values and in Figure 4 for the decline of ischemic metabolism.

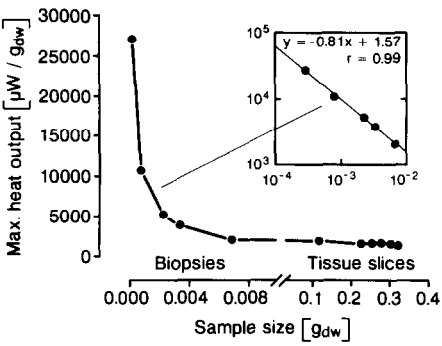


Figure 3. Comparison between biopsies and tissue slices: summit heat output vs. sample size (Tutofusin® incubated rat liver samples at 25 °C).

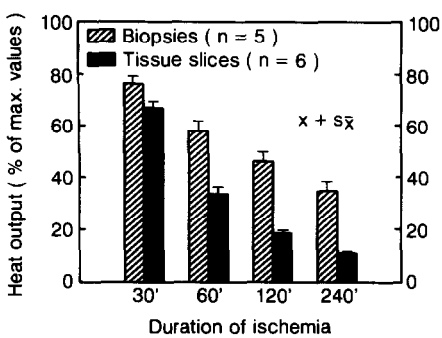


Figure 4. Comparison between biopsies and tissue slices: relative decrease in heat output with time (Tutofusin® incubated rat liver samples at 25 °C).

To begin with, the initial maximum of heat output, expressed in microWatts per gram dry weight, is always the same, as long as large tissue slices are used (Fig. 3). Of course, this is only true when exactly the same time protocol is followed, i.e., ten minutes of preparation and twenty minutes of thermal equilibration are invariably observed. Under these conditions, the reproducibility of the measurement is excellent. However, when tissue samples of biopsy size are used, the summit heat output becomes the higher, the smaller the sample is. As demonstrated by the linear fit in the double-logarithmic plot, this relationship can be expressed by a potency function with an exponent of -0.8 (the significance of which remains to be explained). This phenomenon is obviously due to the fact that the biopsies are so small and have such a large surface area in relation to their volume that they can at least partly be supplied by the oxygen physically dissolved in the incubation medium, thus retaining a higher metabolic rate.

Since the possibility of aerobiosis in small tissue samples is often underestimated, this will now be explained in more detail:

The solubility coefficient of oxygen, at 25 °C, amounts to 2.831 ml O_2 per 100 ml H_2O and 760 mmHg pressure [16]. Since the measuring ampoule has a filling volume of 4 ml, and the partial pressure of oxygen under atmospheric conditions is 150 mmHg, the incubation medium contains up to 22 µl O_2.

The maximum heat output of the rat liver tissue slices was 1500 $\mu W/gram_{dw}$. At a mean dry weight of 0.2 g, this corresponds to an absolute heat production of 0.3 mW or, assuming a respiratory quotient (RQ) of 1 and a caloric equivalent of 21 J/ml O_2, to an oxygen consumption of 0.857 µl/min. To meet only these needs, the O_2 physically dissolved in the incubation medium would last 26 minutes. Thus, if aerobic, the tissue slice would consume the whole O_2 reserve while still in the thermal equilibration position. For the biopsies, however, the dry weight was around 0.002 g so that, despite the higher corresponding heat output of 5000 $\mu W/g_{dw}$, a much lower absolute O_2 consumption of 0.029 µl/min results, which could be satisfied for as long as 13 hours. Thus, for the energy metabolism of biopsies, the O_2 reserves in the ampoule are not a limiting factor.

However, the ability of a tissue sample to be supplied with oxygen, at a given partial pressure, depends on its thickness and its turnover rate. Following Warburg's formula for the maximum thickness of tissue slices to be used in microrespiration trials [3,17] (corresponding to the considerations of Krogh and Meyerhof on oxygen delivery to tissue [6,18,19]), the "critical depth" is given by the square root of the term $2K(pO_2/\dot{V}O_2)$ where pO_2 is the oxygen partial pressure of the incubation medium, $\dot{V}O_2$ the oxygen consumption of the tissue, and K Krogh's diffusion constant. The latter is known, depending slightly on temperature and tissue type, to be around $2x10^{-8}$ ml O_2 per min per cm per mmHg [16,18]. Thus, assuming the above-mentioned maximum turnover rates, aerobically supplied tissue depths can be calculated ranging from 450 µm (for the biopsies) to 850 µm (for the slices).

Hence, in tissue slices, there is an aerobic border zone of less than one millimeter which, as compared to the bulk of the sample, is relatively negligible (at still lower temperatures and further reduced oxygen consumption rates, however, it becomes of increasing importance!). Biopsies, in contrast, lie in the range of the critical tissue thickness so that the rise in energy turnover with decreasing sample size is well explainable by increasing aerobiosis (at 37 °C, the "transitional zone" is shifted to smaller sample sizes!). This does not exclude the possibility that the accumulation of metabolic end products in an unstirred fluid layer may lead, also in tissue samples [20–22], to inhibitory effects as are known from experiments with blood cell suspensions [23,24]. In the case of biopsies, however, the "crowding effect", i.e. the decrease of metabolic rate with increasing sample size, seems to be primarily due to lower degrees of partial aerobiosis.

In other words, biopsies are not representative for strictly anaerobic or ischemic conditions. Moreover, and still more important, they can hardly be standardized, since a difference in the tissue size as small as one thousandth of a gram may result in a metabolic difference of a factor of two or more which has nothing to do with the tissue itself but is only due to methodological reasons. In order to give reproducible results, biopsies would have to be small enough to ensure complete aerobiosis. This, however, precludes their use in ischemia research!

Besides the difference in the maximum heat output, there is also a considerable difference in the rapidity of metabolic decline between tissue slices and biopsies (Fig. 4). Whereas in the latter, it takes place rather slowly, in large and "truly ischemic" samples, there is a much more rapid decline of turnover rate.

Methods of retarding decline of ischemic metabolism

This rapidity may be, however, a problem in itself. If sufficiently large tissue slices were measured at 37 °C, i.e., at normal body temperature, then the decline of ischemic metabolism would be so fast that at the moment the measurement begins, the heat output would already have reached its terminal "baseline".

Therefore, it is important to know what methods are available to retard the ischemic decline of tissue metabolism. The two most important ones are illustrated in the next figures, namely in Figure 5 the cooling and in Figure 6 the additional pretreatment by protective solutions. The underlying investigations were performed on the left ventricle of the rat heart which is large enough to guarantee, at 25 °C, predominantly anaerobic conditions.

As Figure 5 shows, successive cooling alone produces an increasing slowing down in the energy turnover and, hence, in the decline of ischemic

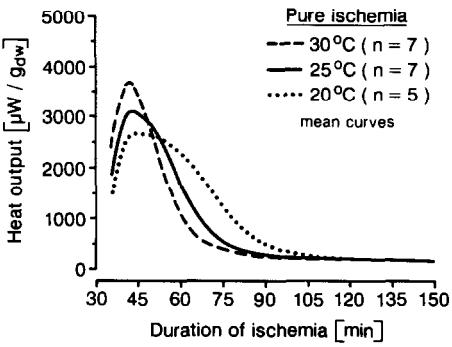


Figure 5. Thermograms of Tutofusin® (balanced electrolyte solution) incubated left ventricular rat myocardium at different storage temperatures. The retardation of metabolic decline with decreasing temperatures is evident.

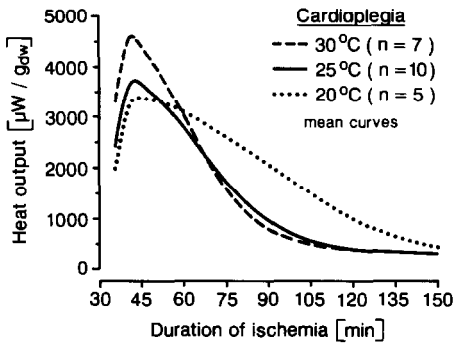


Figure 6. Thermograms of Custodiol® perfused left ventricular rat myocardium at different storage temperatures. The further retardation of ischemic decline leads to higher metabolic rates at the start of measurement.

metabolism [7,25]. In Figure 6, the supplementary pretreatment by an organ-preservative solution is illustrated which, in the case of the heart, is called cardioplegia [26]. Cardioplegia means artificial arresting of the heart by flushing the coronary vessels with a cold solution which, due to its ionic composition, leads to an electrical and mechanical inactivation of the myocardium. With the HTK (Histidine-Tryptophane-Ketoglutarate) solution (Custodiol®) used in our experiments, this is mainly achieved by a withdrawal of sodium and calcium [4,5]. Additionally, this solution has a large buffering capacity so that the reduction of metabolism produced by the "-plegia" itself is combined with a higher efficiency of anaerobic glycolysis [27]. The result is an impressive slowing down in metabolic decline as compared with the purely ischemic samples. Thus, it becomes clear what organ preservation in a narrower sense means, namely a retardation of ischemic injury which exceeds the protective effect of temperature reduction alone [28,29].

However, in these two figures, some apparently paradoxical effects can also be seen. In Figure 5, although cooling leads to a reduction of metabolism, the summit values of heat output are only slightly different. Thus, the steepness of the curve, at a given point, seems to be a more suitable indicator of the protective effect than its absolute level. Similarly, in Figure 6, the heat output values of the tissue, although pretreated by cardioplegic solution, are higher than in the purely ischemic samples. This is obviously due to the fact that the energy turnover, at the moment the measurement begins, has not fallen so far in the pretreated as in the purely ischemic tissue. These reflections thus point to some possible mistakes in interpretation of microcalorimetric curves from ischemic tissues which will be described in the following.

Possible mistakes in interpretation of thermograms from ischemic tissues

The most important sources of error are illustrated in a more general manner in Figure 7 and 8 in which the microcalorimetric curves were computer-simulated with the aid of the above-mentioned Bateman's function.

In Figure 7 an important risk of momentary measurements is demonstrated. Let us assume that someone has had the idea of shortening the procedure by determining the summit values of ischemic metabolism alone. In this case, following the thermal equilibration period, the measuring ampoule would be left in the measuring chamber only until the initial maximum of heat output had passed and would then, for instance, be replaced by other samples. What can go wrong under such circumstances is illustrated in this simulation.

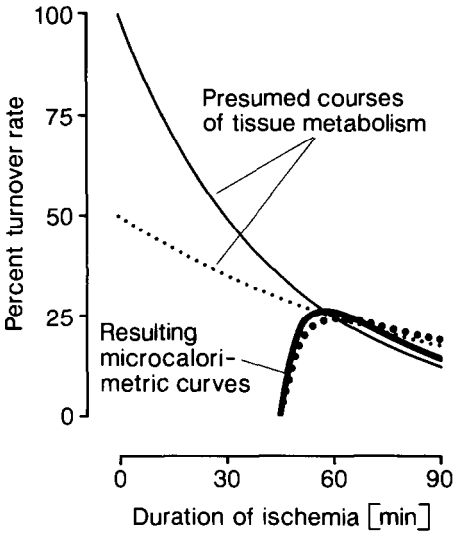


Figure 7. Possible error in short-term microcalorimetry of ischemic tissue samples: The difference in kinetic behavior is ignored.

Two fictitious courses of ischemic metabolism are shown, the first one with a high initial metabolic rate and a consecutively rapid decline of metabolism, and the second one with a reduced metabolic rate and an accordingly retarded metabolic decline. It is assumed that after a preparation period of 45 minutes, the microcalorimetric measurement is performed for another 45 minutes. Interestingly enough, the resulting microcalorimetric curves fail to exhibit any difference! In other words, inherent in the recording of "single values", there is a risk of completely overlooking a difference in the kinetic behavior of two samples. Thus, short-term measurements are not sufficient for a full characterization of the course of ischemic metabolism and need to be completed by an analysis of the slope of the curve.

When, however, long-term measurements are performed, a second danger results from a direct comparison between several curves. This is demonstrated in Figure 8, in the lower part of which two pairs of typical (simulated) thermograms from ischemic tissues are plotted. In both cases, one curve with a higher and another one with a lower metabolic rate are to be distinguished. If, however, the underlying metabolic courses as displayed in the upper part of the figure are compared, it becomes evident that only in the right hand example is this interpretation correct in so far as the metabolic rate of the one sample is always higher than that of the other one. In the left hand example, on the contrary, the situation is exactly inverse. Here, there is one sample with a very high metabolic rate which, consequently, shows a fast decline of ischemic metabolism and, therefore, falls rapidly below the other one which, because of its lower metabolic rate,

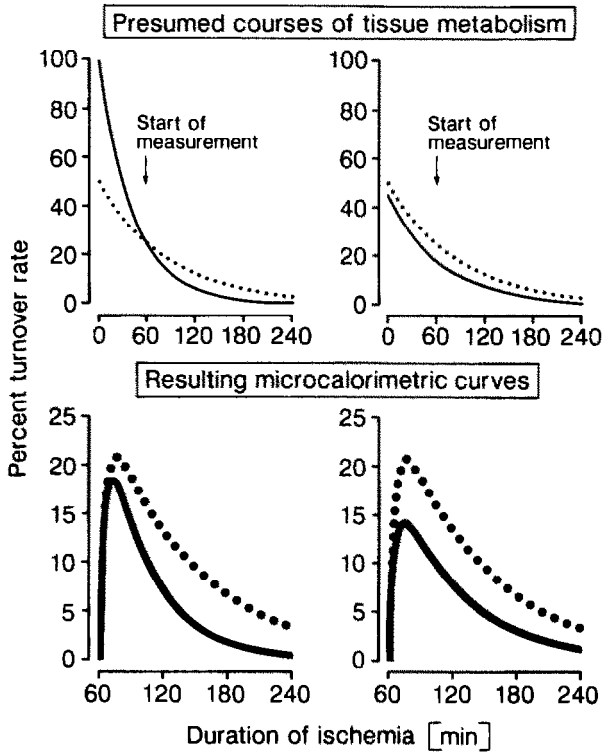


Figure 8. Possible error in comparing microcalorimetric thermograms from ischemic tissues: The metabolic difference between two samples can be misinterpreted.

has a much slower decline of metabolism. Since the measurement only begins at the cross-over point, in this case, the sample with the higher heat output actually is the one with the lower initial metabolic rate and vice versa. Hence, it is necessary to be very careful in the interpretation of the absolute level of microcalorimetric records from ischemic tissues, especially if, as in this example, the preparation time is somewhat longer and a rather late part of the metabolic decline is monitored. However, the same would be true, even with short preparation times, if the incubation temperature were kept at high, e.g., at normal body levels. To avoid such problems, temporary cooling of the sample (e.g., by putting it in ice during transportation) may be helpful although it is not sure that the subsequent rewarming to the measuring temperature without reestablishment of tissue perfusion would have no detrimental effects of its own.

In summary, correct interpretation of microcalorimetric records from ischemic tissue samples requires exact knowledge of the underlying kinetic peculiarities. In order to obtain more information about the "true" metabolic course, it may be useful either to compare the heat output curves at different incubation temperatures, as shown above, or to perform a mathematical extrapolation back to the sampling time (in the latter case, a dynamic correction of the initial part of the curve may be necessary to eliminate steepness errors).

Dependence of ischemic tissue metabolism on experimental animal species

Besides the above-mentioned methodological aspects, the animal species is another very important factor to be considered in microcalorimetry of ischemic tissues. It is not uncommon practice to draw conclusions directly from rats and mice to humans. However, as in other fields, also in calorimetry, this is not automatically allowed! The marked influence exerted by the animal species on the ischemic metabolism of homologous tissues is demonstrated in Figure 9 and 10.

An especially important point in this context is the body size [30]. Its influence is due to the fact that large mammals have a considerably lower metabolic rate per unit of mass than smaller species. Although this "law of metabolic reduction" is found throughout the whole animal kingdom including poikilothermic organisms, for mammals, it is commonly explained by the needs of homeothermy. Following this explanation, smaller organisms because of their greater surface to volume relationship lose more heat and, therefore, need a higher endogenous heat production to maintain the same body temperature as larger animals (hence "surface rule"). According to the mathematical formulation of this rule given by Kleiber [17], the specific metabolic rate of a rat is three to four times that of a dog. As was first described by Krebs in his classical microrespiration experiments [31], this metabolic difference is also more or less to be found in many organs. In fact, its relevance at the tissue level is further confirmed by microcalorimetry. This is shown in Figure 9 by the example of the cardioplegically arrested heart at 25 °C in rats and dogs. In the rat heart, the decline of ischemic metabolism occurs much faster than in the dog heart. Remarkably, when one compares the time of the terminal metabolic decline shortly before reaching the final "baseline", one finds a difference of a factor of 3–4, i.e., the species difference in the metabolic level seems to be converted, in ischemia, into a time factor of metabolic decline! Moreover, in the dog heart, the metabolic situation obviously is so favorable that by cardioplegia, an intermediate "plateau" is reached, indicating that in this case, anaerobic

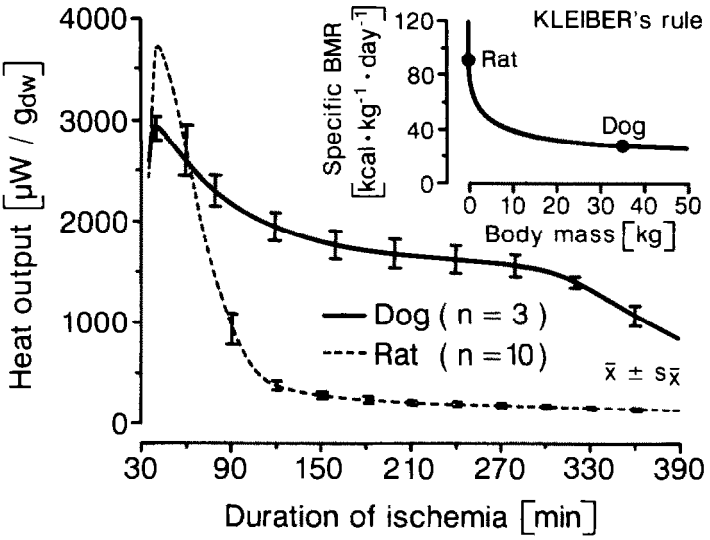


Figure 9. Thermogram of cardioplegically arrested dog left ventricular myocardium in comparison to identically treated rat heart (Custodiol^{R}, 25 °C). Inset: The so-called "surface rule", showing the increase of specific basal metabolic rate (BMR) with decreasing body mass. This allometric relationship seems to be converted, under optimal preservation conditions, into a time factor of anaerobic metabolism.

glycolysis is able to meet the energetic requirements for a limited time, whereas in the rat heart, although its metabolic decline is also retarded as compared with purely ischemic conditions (see above), no such equilibrium is to be seen at 25 °C [7,32].

Also within the group of small rodents, however, there may be some important differences in the behavior of ischemic metabolism depending on species specific adaptation features. This is demonstrated in Figure 10 by the example of the purely ischemic liver of rat and golden hamster. The latter is even smaller than the rat and should therefore have a still faster metabolic decline. But the reverse is true. In the hamster liver in comparison to the rat liver, an intermediate elevation of heat output is found which shows slight oscillations (these are markedly reinforced at 15 °C whereas the rat liver exhibits no such phenomenon). This temporary "metabolic increment" can be correlated, following our own biochemical investigations, with a more profound acidification of the interstitial space despite a nearly identical total lactate production and with a higher formation of alanine leading to a greater efficiency of anaerobic glycolysis in the time interval in question.

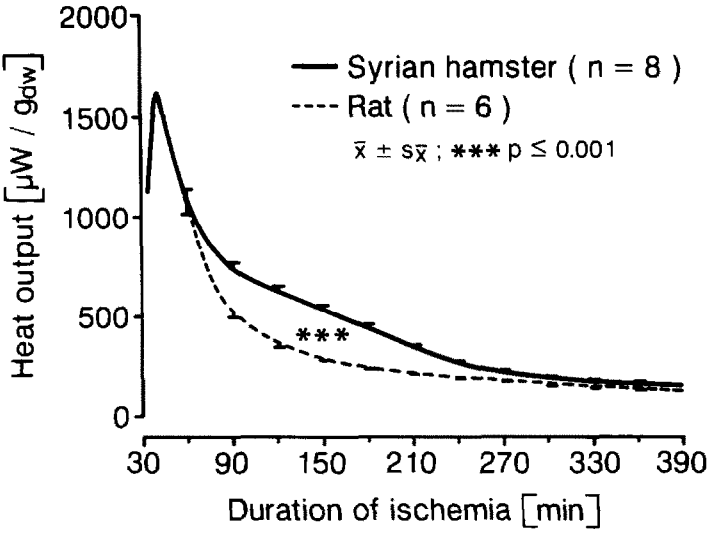


Figure 10. Thermograms of purely ischemic, Tutofusin® incubated liver slices from rat and Syrian (golden) hamster at 25 °C. In the hamster, there is an intermediate, slightly oscillating increment of heat output as compared to the rat. (Significance was determined by Student's t-test for independent samples).

Apparently, this species is somewhat more successful in gaining energy from anaerobic pathways and in extruding H^+-ions from the cells [32,33]. This, perhaps, may reflect a special adaptation. Golden hamsters, namely, are hibernators displaying regulating capabilities at much reduced metabolic levels. Thus, they are known to maintain, in the deeply hibernating state, a relatively uniform minimal metabolic rate around 0.1 kcal x kg^{-1} x h^{-1} [7, 34,35]. Most remarkably, this is exactly the same level at which in liver ischemia the "last regulating effort" can be microcalorimetrically detected.

Final remarks

The last two figures do not only illustrate the importance of animal species, but also one of the most interesting insights into ischemic tissue metabolism so far obtained by microcalorimetry. They clearly show that the turnover rate does not necessarily take the course of a decaying exponential function, but may well have some discontinuities. Such discontinuities in the form of intermediate metabolic increments apparently always arise when protective

mechanisms, be they artificial or natural, are effective [7]. This is a phenomenon which by conventional intermittent biochemical analyses would not have been demonstrable in such a clear manner, but was only detectable by a method which allows continuous monitoring of ischemic tissue metabolism – as is the case for microcalorimetry.

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