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The Pasteur effect in facultative anaerobic metazoa

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Abstract. The existence and the regulatory mechanisms of the Pasteur effect in facultative anaerobic metazoa are discussed. There are three reasons for the controversy surrounding this phenomenon. *1)* The different definitions of the Pasteur effect, *2)* the antagonistic effect of metabolic depression and its species specific response to hypoxia, as well as *3)* the laboratory-specific differences in the experimental procedures for analyzing the Pasteur effect and its regulation. This review aims to clarify the confusion about the existence of the Pasteur effect in facultative anaerobic metazoa and to offer possible molecular mechanisms.
Key words. Facultative anaerobes; metabolic depression; Pasteur effect; phosphofructokinase; glycogen phosphorylase.

Abbreviations. $F2,6P_2$ = fructose 2,6-bisphosphate; F6P = fructose 6-phosphate; $G1,6P_2$ = glucose 1,6-bisphosphate; G6P = glucose 6-phosphate; GPase = glycogen phosphorylase; P_i = inorganic phosphate; PFK = 6-phosphofructokinase.

The definitions of the Pasteur effect

In honour of Louis Pasteur (1822–1895) who described the fermentation of yeast in dependence on oxygen, Otto Warburg[85] defined the 'Pasteursche Reaktion' whereby ethylcarbylamine abolishes the inhibition of fermentation by respiration (fig. 1) in slices of different tissues. This was the first clear proof of the link between fermentation (carbohydrate oxidation with organic compounds as electron acceptors) and respiration (oxygen consumption) in animal tissues. This was taken to be responsible for what subsequently came to be called the Pasteur effect, the regulatory mechanisms of which challenge biochemists up to the present. Since Warburg quantified the 'Pasteursche Reaktion' using Meyerhof's quotient ([anaerobic fermentation–aerobic fermentation]/respiration) his definition does not necessarily mean a higher carbohydrate consumption in absence of oxygen. This, however, was the essence of Pasteur's observation with yeast[56]. Pasteur reported in 1861 that glucose uptake is increased in the absence of oxygen and is accompanied by enhanced ethanol production but lowered cell proliferation[54]. Pasteur's description of his experiments limited the Pasteur effect to cells taking up glucose and consequently excluded tissues using glycogen for fermentation. It also influences the thinking about the site at which the control operates (glucose uptake versus lactate or ethanol production, see ref. 68).
Another interpretation of the Pasteur effect was derived from the discovery of a molecular mechanism stimulating lactate production in frog muscles during work. In 1920, Meyerhof found that lactate production is the energy source for muscle contraction[47] and his observation was the beginning of the energetic concept of the Pasteur effect. Its molecular basis was elucidated when Mitchell proposed the chemiosmotic gradient as a mechanism to produce ATP by respiration[51] and Passonneau and Lowry[53] described the inhibition of phosphofructokinase (PFK) by ATP and its deinhibition by inorganic phosphate (P_i) or AMP (see refs 42, 58 for history). Stimulation of fermentation by work load is independent of respiration (fig. 1) and, therefore, is not a Pasteur effect in the strict sense according to Warburg's definition or the interpretation of Pasteur's experiments. The Pasteur effect has often been used independent of whether work load or reduced oxygen supply stimulate carbohydrate degradation. But it has to be noted that ATP turnover is dramatically enhanced during work while it is rather reduced in facultative anaerobes during hypoxia (see below).
According to Sols[68] and De Zwaan and Wijsman[10] we defined the Pasteur effect as the stimulation of carbohydrate consumption by reduced oxygen tension. Carbohydrate consumption is represented by the flux between glucose 6-phosphate (G6P) and phosphoenolpyruvate and measured by the different endproducts derived from this central part of the Embden-Meyerhof-Parnas pathway. This more general definition extends the quantitative aspect of Pasteur's observations and is independent of the carbohydrate (glucose or glycogen) used either for respiration or fermentation, as well as of the different endproducts which are produced in facultative anaerobes (see ref. 19 for review).

Pasteur effect versus metabolic depression

Metabolic depression is generally defined as the reduction of ATP turnover below the standard metabolic rate

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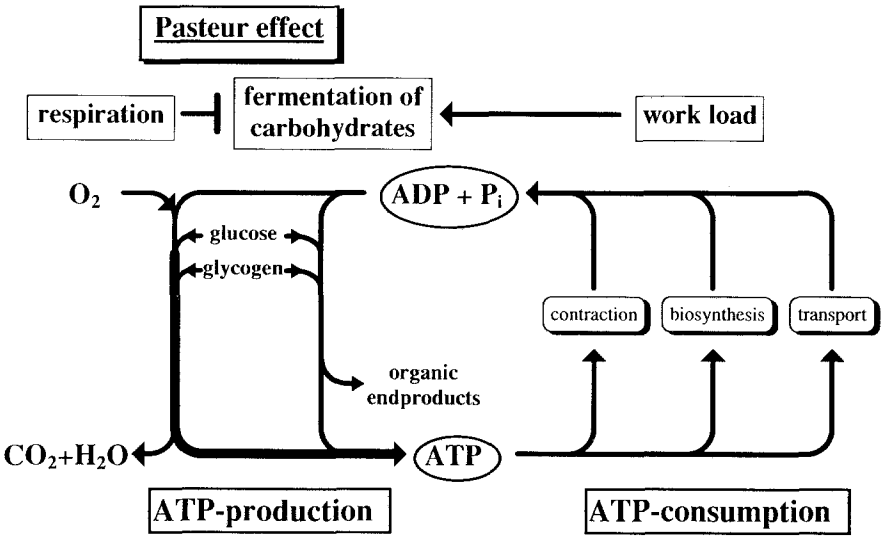

Figure 1. Scheme of metabolic turnover and its relation to the Pasteur effect. In the presence of oxygen, respiration enables ATP production from carbohydrate oxidation with high efficiency (broad line) and fermentation is inhibited (—|). When oxygen is limited, fermentation can replace respiratory ATP production but with lower efficiency. A complete compensation requires an elevation of carbohydrate consumption. Fermentation is also stimulated when work load enhances ATP consumption.

(SMR), which means the aerobic metabolic rate at 'rest' (ref. 19 for review). The advantages of such a depressed metabolic rate include conservation of fuel and minimization of accumulation of endproducts under anaerobiosis, thus extending the length of time that the animal is able to sustain hypoxic stress[26].

Metabolic rate can be estimated by measuring heat dissipation, which is reduced during hypoxia to 20% of the normoxic value in the peanut worm *Sipunculus nudus*[22], to 10–15% in the limnic oligochaete *Tubifex sp.*[16], to 13% in the limnic hirudine *Hirudo medicinalis*[63], to 5–10% in marine mollusc *Mytilus edulis*[15] and to 15% in the freshwater turtle *Pseudemys scripta*[30]. However, there is no consistent correlation between heat dissipation, ATP turnover and carbohydrate consumption during transition from aerobiosis to anaerobiosis[18]. Therefore, biochemical analysis is necessary when metabolic depression is related to a reduction in energy requirements. For many facultative anaerobes, lack of oxygen results in a dramatic decrease in calculated ATP turnover rates (fig. 2, right panel). The reduced energy output during hypoxia has led to the proposal that a general feature of facultative anaerobes is a lack of, or even a reversed, Pasteur effect[24,75]. This conclusion is based on calculation of carbohydrate utilization from either oxygen consumption or anaerobic endproduct accumulation during prolonged periods of hypoxia, especially in molluscs. An evaluation of data from the literature enlarges this picture for several species (fig. 2, left panel). When hypoxic periods were subdivided into short intervals, a remarkable increase in carbohydrate consumption became visible during the initial period of hypoxia in most species (fig. 2A–D) including molluscs[6,80]. This initial Pasteur effect is not in conflict with the concomitant decrease in ATP turnover due to the lower efficiency of carbohydrate fermentation compared with the oxidation to CO_2 and H_2O. An initial increase and subsequent reduction of glycolysis during hypoxia was not observed in *S. nudus*, which shows an extremely low glycosyl and ATP turnover (fig. 2E). No Pasteur effect was observed in the marine mollusc *M. edulis* (fig. 2F), but it should be mentioned that normoxic carbohydrate consumption is lower than estimated from respiration because catabolism of lipids and amino acids contributes to oxygen consumption. Therefore an initial Pasteur effect cannot be excluded for either *S. nudus* or for *M. edulis*.

Regulation of phosphofructokinase activity

Strong evidence exists that the phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F1,6P_2) catalyzed by the enzyme 6-phosphofructokinase (PFK; EC 2.7.1.11) is the central site of the Pasteur effect (see refs 42, 58, 81 for review). Extensive studies on PFK regulation have revealed complex mechanisms, including allosteric effects, covalent and assembly state modifications (see refs 27, 70, 82 for review).

Allosteric modulations: Respiratory ATP-production and PFK activity are considered to be under the control of adenosine phosphates (ATP, ADP and AMP) and P_i[53]. This energetic coupling can activate carbohydrate utilization when the ATP level is reduced while ADP, AMP and P_i levels are elevated either as a consequence of work load or of hypoxia. For many facultative anaerobes of different phyla, respective metabolic changes are reported during hypoxia. In *A. marina*, for instance, ADP, AMP and P_i concentrations are in-

creased several fold, while ATP is slightly reduced during initial hypoxia[38,65]. Similar results are reported for *H. medicinalis*[62,89], *Tubifex sp.*[66], and for *S. nudus*[23] and *M. edulis*[86]. A transient decrease in ATP during initial hypoxia is reported for turtle brain[40,45].

This basic concept of allosteric PFK control was modified when additional effectors of PFK were included, e.g. citrate as inhibitor, NH_4^+, $F1,6P_2$, glucose 1,6-bisphosphate ($G1,6P_2$) and fructose 2,6-bisphosphate ($F2,6P_2$) as deinhibitors. It was argued that numerous allosteric effectors all contribute to integrate a variety of metabolic signals[69]. In yeast, aerobic and anaerobic glycolytic flux is fully explicable by control of PFK, when physiological changes in the levels of $F2,6P_2$, $F1,6P_2$, ATP, AMP, P_i, and in pH were considered[59]. An increase of $F2,6P_2$ upon hypoxia markedly contributes to stimulation of glycolysis in yeast. However, in facultative anaerobic metazoa, a decrease rather than an increase of $F2,6P_2$ has been reported (see below) and initial acceleration of glycolysis seems not to be induced by $F2,6P_2$. In mammalian hepatocytes it was also concluded that $F2,6P_2$ is not involved in the mechanism of the Pasteur effect[29].

Covalent modification: Evidence for reversible phosphorylation of PFK was clearly established for vertebrates and invertebrates, when incorporation of phosphate could be demonstrated in vitro and in vivo (see ref. 27 for review). However, the role of phosphorylation in glycolytic control in vertebrates has remained unclear. Foe and Kemp reported an inactivation of rabbit muscle PFK upon phosphorylation[17], but allosteric properties are only slightly changed and the physiological relevance is controversial[27]. In contrast, phosphorylation of PFK from the parasitic nematode *Ascaris suum*[28] and other helminths[33,72] resulted in distinct activation of the enzyme. Similar results were reported for PFK of the molluscs *M. edulis* and *Helix pomatia*[1]. Incubation of purified PFK from the limnic annelid *H. medicinalis* in the presence of catalytic subunits of cAMP-dependent protein kinase, Mg^{2+} and ATP resulted in clear alterations in the kinetic properties of the enzyme[62]. The substrate saturation curve is shifted to lower F6P concentrations and the sensitivities to the main activators AMP and $F2,6P_2$ are significantly enhanced (fig. 3). Although there is no experimental evidence in vivo, phosphorylation of PFK would therefore

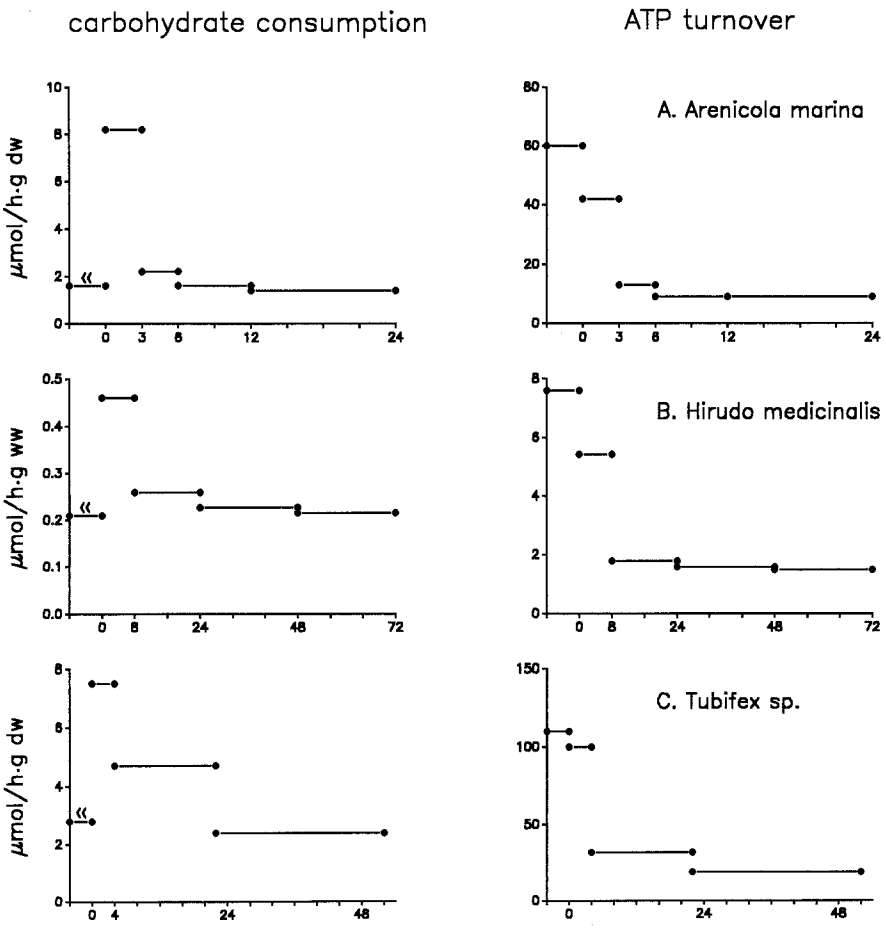

Figure 2.

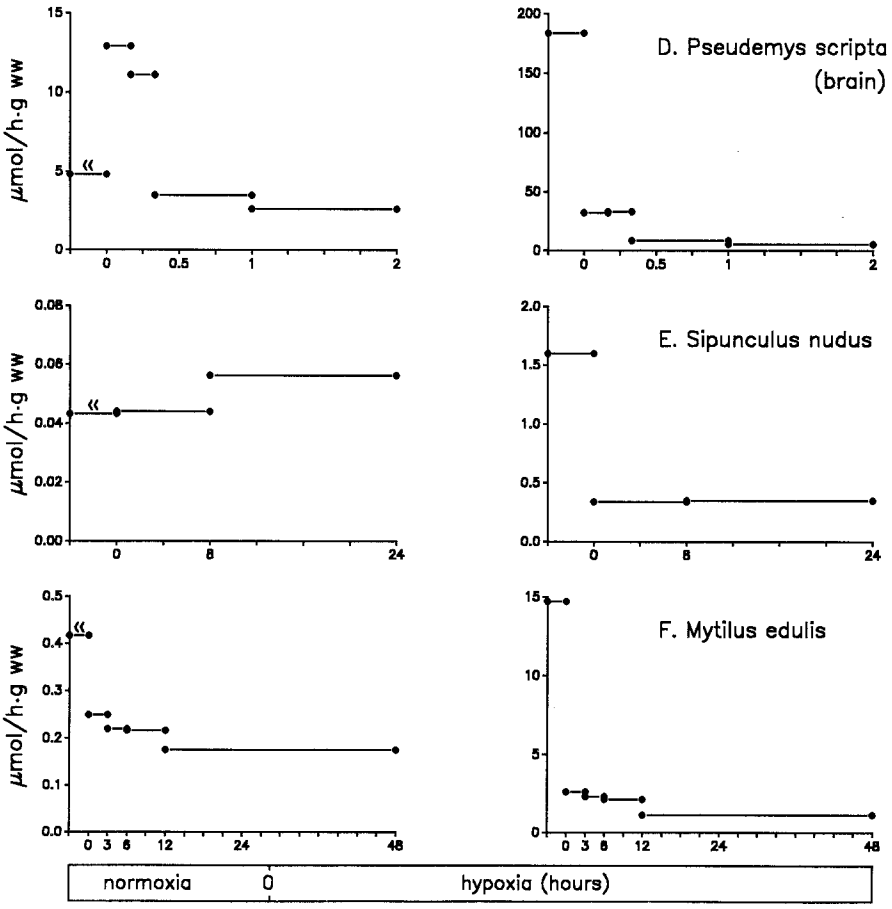

Figure 2 cont'd. Carbohydrate consumption and ATP turnover during normoxia and hypoxia in several facultative anaerobes. Carbohydrate consumption is given in µmol glycosyl units per hour and gram wet weight (ww) or dry weight (dw), as indicated in the figure. ATP turnover is given in µmol ATP produced per hour and gram, respectively. The data were either directly taken from literature or calculated. Normoxic ATP turnover was calculated from oxygen consumption at rest, except for turtle brain where the data are based on oxygen consumption by tissue slices[60]. Anaerobic values were calculated from endproduct formation, regarding the different ATP yields of the various pathways. Normoxic carbohydrate consumption was calculated from oxygen consumption, assuming that only carbohydrates are utilized. Since other substrates (amino acids or lipids) may be oxidized, the values correspond to the maximal possible rate. Anaerobic carbohydrate consumption was calculated from anaerobic endproduct formation regarding alternative anaerobic substrates (e.g. aspartate and malate). For *Tubifex sp.* values are based on glycogen breakdown. Data for *Arenicola marina* (A) are from Kamp[35], for *Hirudo medicinalis* (B) from Schmidt and Nieczaj[62], for *Tubifex sp.* (C) from Seuß et al. 1983[66], for the brain of *Pseudemys scripta* (D) from Robin et al., 1979[60] and Lutz et al.[45], for *Sipunculus nudus* (E) from Hardewig et al.[23] and for *Mytilus edulis* (F) from de Zwaan and Wijsman[10] and Shick et al.[67].

stimulate carbohydrate consumption and support the induction of a Pasteur effect.

Assembly state modification: In recent years protein assembly (binding of proteins to subcellular structures) has attracted much attention and is considered to be a powerful mechanism in metabolic control (see refs 71, 73 for review). For vertebrate muscle, association of PFK to F-actin increases catalytic activity[5,44] and a shift between the soluble to the bound fraction may contribute to the regulation of PFK. Although some reports of PFK assembly are available especially for marine molluscs, its physiological relevance during hypoxia is still obscure, because the observed changes were rather transient and not always correlated to the carbohydrate flux[2,43].

Depression of glycolysis

Since carbohydrate consumption in facultative anaerobes is suppressed during prolonged hypoxia (fig. 2), initial activation of PFK should be compensated for or even reversed. As mentioned above a decrease in the level of the non-covalent activator $F2,6P_2$ may be involved, but the data vary with the tissue and species and a general function has not been established. For instance, a drop in the $F2,6P_2$ level occurs in soft tissues and foot muscle but not in adductor muscles from *M. edulis*[76] while levels were stable in foot muscle and dramatically decreased in ventricle of the whelk[78]. No change in $F2,6P_2$ was detected in the body wall of *S. nudus*[23], while a significant decrease was observed in the body wall of *H. medicinalis* (Schmidt et al., unpublished).

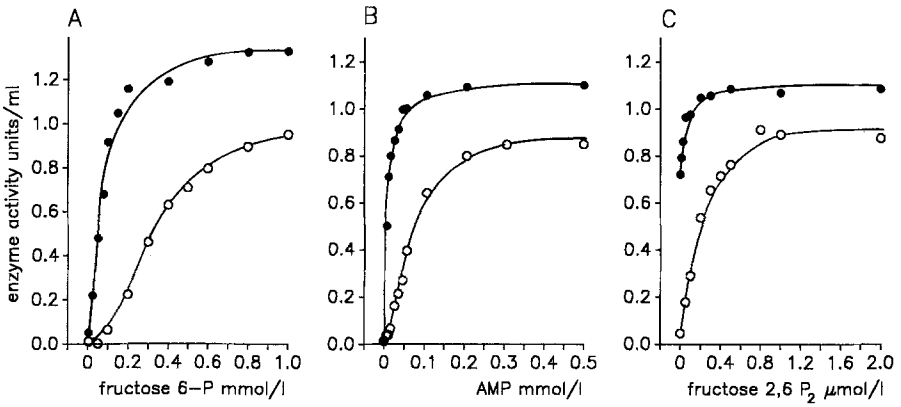

Figure 3. Effects of in vitro phosphorylation on kinetic properties of phosphofructokinase. PFK was purified from the body wall of *Hirudo medicinalis* in the absence of any phosphatase inhibitor (dephosphorylated form). After incubation (30 min, 20 °C) with 1.5 mM ATP and 5 mM $MgCl_2$, either in the presence (closed symbols, phosphorylation) or absence (open symbols, control) of the catalytic subunit of cAMP-dependent protein kinase, the activity was assayed at physiologically relevant concentrations of ATP (3 mM) and P_i (2 mM) at pH 7.2. In (A) AMP was present (10 µM), in (B) F6P concentration was 0.1 mM, and in (C) F6P was 0.1 mM and AMP was 10 µM. Data are from Schmidt et al., unpublished.

Covalent modification of PFK by phosphorylation/dephosphorylation has been suggested for tissues of several molluscs as a consequence of hypoxia (see ref. 79 for review). PFK extracted from anaerobic animals showed a reduced affinity to F6P, a stronger inhibition of ATP and lower sensitivity to allosteric activators than PFK extracted from aerobic animals. Kinetic properties of the aerobic enzyme were restored by incubation with alkaline phosphatase and it was concluded that the modification during hypoxia was due to phosphorylation of PFK[74,79,87]. However, this hypothesis presumed inactivation by phosphorylation and it is not consistent with the finding that phosphorylation of the purified enzyme clearly results in an activation in several invertebrates from different phyla (see above). Therefore it was questioned whether the kinetic measurements in extracts were really due to enzyme phosphorylation[1]. Correspondingly, it was argued that PFK modulation is due to dephosphorylation in muscle tissue from *M. edulis*[50].

PFK from several vertebrates is inactivated by acidification which is induced by reversible dissociation of active tetramers into inactive dimers and has been considered to be a mechanism of PFK inactivation under physiological conditions (see ref. 70 for review). A pH-mediated shift in the tetramer-dimer equilibrium was indeed demonstrated in ischaemic rat heart under in vivo conditions[3]. In vertebrates, reduction of pH seems to have two different effects on PFK, leading to opposite effects on activity (increased assembly to F-actin = activation, increased dissociation to dimers = inactivation). Therefore, Somero and Hand proposed that a moderate reduction in pH_i would increase the particulate fraction of PFK and enhance glycolytic flux, but further acidification would result in dissociation of tetramers into dimers and reduce glycolytic flux[70]. There are only a few reports of pH-induced inactivation of PFK in facultative anaerobes but PFK from muscle of *M. edulis* was shown to be pH-sensitive, and acidosis during hypoxia was considered as an important mechanism in depression of glycolysis[12]. A considerable decrease in intracellular pH during hypoxia is observed in many facultative anaerobes[14,36,55] (fig. 4) and there is evidence for the contribution of acidosis to the depression of carbohydrate consumption[32]. Consequently, the moderate acidification (from pH 7.24 to pH 7.08, 24 h) during prolonged hypoxia in *S. nudus*[23] may explain the persistent acceleration of glycolysis.

Glucose and glycogen mobilization

Glucose uptake and its metabolism during hypoxia have been demonstrated in tissues of facultative anaerobes by in vivo application of radioactive labelled glucose (e.g. *Mytilus edulis*[9], *Arenicola marina*[88], *Tubifex tubifex*[64]. On the other hand, isolated tissues produce the same anaerobic endproducts without addition of glucose[11,48], indicating their energetic autonomy due to abundant glycogen stores[25]. Consequently, considering the Pasteur effect in tissues of facultative anaerobic metazoa, both glycogen and glucose have to be regarded as fuels for carbohydrate fermentation[83] but glucose uptake and its regulation have been rather neglected in the past.

A significant decrease in glycogen content was demonstrated in various species of different phyla but only after a relatively long period of hypoxia. This is because the consumption is small compared to the glycogen stores and its individual fluctuations do not allow a sensitive detection of short term degradation. Studies on the activation mechanisms of the regulatory enzyme glycogen phosphorylase (GPase) during hypoxia in various facultative anaerobes resulted in a very confusing situation. The interpretation of the experimental results varied between negation of any activation[75,78], activa-

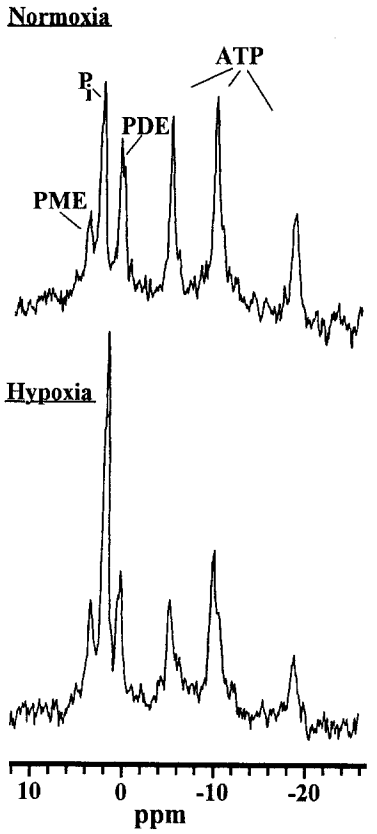

Figure 4. In vivo ^{31}P-NMR spectra of the leech *H. medicinalis*. The spectra were obtained from a leech kept in a NMR tube (10 mm diameter) with fresh water and recorded with a Bruker AM 270 spectrometer operating at 109.35 MHz according to Kamp and Juretschke[36]. The animals investigated were without food for approximately half a year. After recording the normoxic spectrum the leech was kept in the closed NMR tube for 12 h at 20 °C and monitored again (hypoxia). The shift of the P_i signal during hypoxia indicates an intracellular acidosis from pH 7.3 to pH 6.8.

tion by enzyme phosphorylation[78], or activation by phosphorus metabolites like AMP or P_i[13,34], depending on the tissue used for experiment. Indeed, the relatively slight decrease in the glycogen content during the initial hours of hypoxia can be explained without any activation of GPase if one assumes that the glycogen turnover (glycogen synthesis and glycogenolysis) is equivalent under normoxia and hypoxia and a decrease in the glycogen content is accounted for by reduced glycogen synthesis due to the lack of glucose which is preferentially used for fermentation. Only for the lugworm *A. marina* can this assumption be definitively excluded due to an in vivo ^{13}C-NMR spectroscopic experiment. When tissue glycogen was labelled with ^{13}C-1 glucose, this label was unchanged over 24 h of normoxia while a significant decrease became visible after only 5 h hypoxia[31]. This clearly shows that glycogen turnover under normoxia is distinctly lower than under hypoxia.

Covalent modification of glycogen phosphorylase

Determination of the ratio of the phosphorylated GPase (a-form, 'active form') and the unphosphorylated b-form ('inactive form'), dependent on AMP (criterion: only GPase b activity depends strictly on AMP), has often been used as the physiologically relevant parameter for the stimulation or inhibition of glycogenolysis[49,75,78]. This however may be questionable due to the following: (1) up to 90% of human muscle GPase can be converted into the active a-form by adrenaline infusion with only minimal glycogen breakdown[4], (2) during electrical stimulation of vertebrate muscles GPase a reverts back to the b-form despite continued contractile activity and glycogenolysis[7,8], (3) GPase kinase-deficient mice metabolize glycogen without GPase a[46,57], and (4) tissues of vertebrates and invertebrates contain GPase a at rest[20], or even during periods of glycogen synthesis[21]. In addition, the experimental procedure of GPase a and b determination is often beset with artefacts in tissue preparation due to slow freezing and inappropriate enzyme assay conditions[20]. In addition, the existence of phospho-dephospho hybrids has often been neglected when testing for dependence on AMP. For example, lugworm GPase measured in dependence on AMP shows 36% GPase a[34] but enzyme chromatography and the radioactive phosphorylation of the purified GPase b in vitro have revealed that more than 90% of the GPase exists as a phospho-dephospho hybrid[37]. Consequently, the determination of the GPase a to b ratio dependent on AMP is not sufficient for assessing the status of activation and the exact quantitation of GPase b or a. Studies of these problems with facultative anaerobes are few[61]. Considering all these complications, there is no proof of GPase activation by phosphorylation in muscles of facultative anaerobes during hypoxia.

Non-covalent modifications of GPase

The most important non-covalent effectors which are discussed for in vivo regulation of mammalian GPase are the activators AMP and IMP, the inhibitors ATP and G6P, and the substrate P_i. Neither covalent nor non-covalent enzyme modifications can influence GPase activity if the substrate concentration is not sufficient. In contrast to glycogen, the cytoplasmic P_i level is low at rest and increases to the same extent as the phosphagen is mobilized. This was demonstrated for various facultative anaerobes in vivo by ^{31}P-NMR spectroscopy[14,36,84]. Cytoplasmic P_i level can also increase in tissues which do not contain a phosphagen like the leech *H. medicinalis* (fig. 4).

The determination of the different parameters (GPase phosphorylation, kinetic properties, calculation of cytoplasmic concentrations of P_i and AMP) and their changes in the lugworm *A. marina* during hypoxia have led to the proposal that activation of glycogenolysis in particular depends on the release of P_i and AMP as a consequence of phosphagen and ATP degradation[35]. Phosphagen degradation might be initiated by the in-

crease in cytoplasmic ADP level (near equilibrium reaction) when respiratory ATP production ceases due to lowered oxygen tension. In this metabolic cascade stimulation of glycogenolysis is directly connected to respiration like the classical mechanism proposed for PFK regulation (Pasteur effect).

Depression of glycogenolysis

In some cases a decrease of the GPase a to b ratio was reported during prolonged hypoxia, which was interpreted as a mechanism depressing glycogenolysis[77,79]. The mechanism which initiates GPase dephosphorylation is, however, unknown. Some workers suggested that the extent of metabolic reduction depends on the development of intracellular acidosis during hypoxia[41,52]. In hypoxic lugworms acidosis was compensated for in experiments by buffering the ambient seawater, and consequently the depression of glycogenolysis was significantly reduced[32]. The mechanism by which protons inhibit GPase activity is unknown. A direct protonation of the enzyme molecule or a shift of the substrate HPO_4^{2-} towards $H_2PO_4^{1-}$ [39] do not significantly reduce lugworm GPase activity in the range between pH 7.3 and 6.6 under conditions of simulated hypoxia (Kamp, unpublished results). Otherwise PFK is known as a pH-sensitive site of glycolysis (see above) and its inhibition would lead to an increase of F6P and G6P concentrations. The latter is known as a potent inhibitor of GPase b, and as a moderate inhibitor of the phospho-dephospho hybrid (GPase ab), while GPase a is less sensitive to G6P. This has been clearly demonstrated on fish white muscle GPase (Schmidt and Wegener, unpublished results). It might be of interest to clarify whether G6P accumulation inhibits GPase-like hexokinase as a response to PFK inhibition by acidosis during prolonged hypoxia and if it represents an important mechanism of metabolic depression.

In conclusion, there is evidence for the existence of the Pasteur effect in several facultative anaerobic metazoa. However, the effect can be reversed due to strong metabolic depression. The molecular mechanism of the Pasteur effect probably occurs at the cellular level in particular by concentration changes in the adenine nucleotides and P_i when respiration is prevented. The molecular mechanism(s) of reversing the Pasteur effect during prolonged hypoxia are still under discussion and the results are rather contradictory. Probably intracellular acidification contributes considerably to the conservation of carbohydrates during prolonged hypoxia and may have an important function in integration and coordination of metabolic depression.

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