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Anaerobiosis and acid-base status in marine invertebrates: a theoretical analysis of proton generation by anaerobic metabolism

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Summary. In animals, various organic acids are accumulated during hypoxia or anoxia as products of anaerobic energy metabolism. The diversity of such acids is largest in marine invertebrates where succinate, propionate, acetate, lactate, alanine, octopine, strombine, and alanopine, are produced mainly from glycogen and aspartate. The effect of these substances on the acid-base status was assessed by a theoretical analysis of the respective metabolic pathways. This resulted in a general rule which was applied to evaluate the proton balance of the reactions in energy metabolism: net changes in the number of carboxyl groups or changes in the degree of dissociation of other groups (e.g. phosphate or ammonia) determine the net amount of H^+ ions released or bound by the substrates and the metabolic end products.

For marine invertebrates the results of the analysis can be summarized as follows: In glycogenolysis one mol of protons per mol of end products is released during cytosolic glycolysis, independent of the type of metabolic end product (lactate, octopine, alanopine, strombine, or alanine). The same applies for mitochondrial production of propionate and acetate, whereas formation of succinate results in dissociation of two mol H^+ per mol. Fermentation of aspartate, however, diminishes the amount of protons which is produced in the succinate-propionate pathway. Net metabolisation of Mg ATP^{2-} yields extra protons, whereas the cleavage of phosphagens (e.g. creatine phosphate, arginine phosphate) consumes protons.

Additionally the break-down of energy-rich phosphates to inorganic phosphate has to be taken into account because of the shift of the intracellular buffer curve caused by changes of the respective effective pK values.

A lively discussion concerning proton generation during anaerobic glycolysis leading to the accumulation of lactate has developed during the last few years. Two main approaches to the analysis of the mechanism of proton release can be distinguished:

The first considers the degree of dissociation of the generated metabolites. For lactic acid ($pK \cong 3.9$) the underlying hypothesis was provided by J. Berzelius, C. Bernard, and E. du Bois-Reymond, early in the 19th century (quoted by Needham 1971). It was accepted by Meyerhof and Lohmann (1926), Lipmann and Meyerhof (1930), and many other authors, and recently defended by Wilkie (1979). The essential idea is that lactic acid produced in glycolysis results in an acidification of the cell by equimolar dissociation of protons.

The second approach has been provided by Krebs et al. (1975), Gevers (1977, 1979), and Zilva (1978). These authors proposed that the generation of protons is not related to the dissociation of lactic acid, but to the simultaneous hydrolysis of ATP formed during glycolysis. The quantitative correlation between proton generation and lactate formation, however, has not been questioned in their model.

Lactic acid is the main anaerobic end product in the Arthropoda and Vertebrata. Lower marine invertebrates such as annelids and molluscs produce a number of different metabolic end products in addition; succinate, propionate, acetate, alanine, octopine, alanopine, and strombine, are

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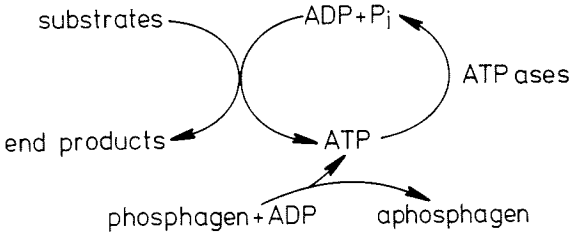

Fig. 1. Interrelationship between ATP-synthesizing and ATP-consuming processes during anaerobiosis. ATP, which is hydrolyzed by ATPases, is regenerated by catabolism and by cleavage of the phosphagen

formed mainly from degradation of glycogen and aspartate. In this realm, the first hypothesis mentioned above was adopted by several authors. Zammit (1978) concluded that the accumulation of the weak acid octopine has almost no acidifying effect, and Hochachka (1980) and Hochachka et al. (1983) assumed that hydrolysis of phospho-L-arginine would affect cell pH because of arginine accumulation.

Recently the method of Gevers (1977) was applied by Hochachka and Mommsen (1983). They sharply distinguished between metabolism, which generates ATP, and hydrolysis of ATP. They finally concluded that "essentially all animals in anoxia generate 2 H^+ per mol of glycosyl unit" when ATP formed by metabolism is hydrolyzed.

A complete analysis of proton balance during anaerobic metabolism in invertebrates has never been performed. The generalization by Hochachka and Mommsen (1983) seems to neglect some peculiarities of the metabolites formed. The present study is intended to completely describe the factors essential in a quantitative correlation between the accumulation of anaerobic metabolites and the release of protons. Part of the analysis presented has already been described by Pörtner (1982).

The following theoretical analysis is influenced by the ideas of Gevers (1977), and accordingly the basic equations of catabolism were written including the adenylate system. The aim of this procedure was to evaluate to what extent the adenylate system itself influences the proton release. Since a close interrelationship exists between adenylates, phosphagens, and inorganic phosphate (Fig. 1), the influence of all these substances was taken into account.

Phosphates and protons

Phosphate has an important effect on the acid-base status because of the shift of the dissociation constants when it is bound to or released from organic

Table 1. Mg^{2+}-binding constants and H^+-dissociation constants of inorganic and organic phosphates (from Netter 1959; Phillips et al. 1965, 1966; Curtin and Woledge 1978). Most of the constants are given for $\mu = 0.1$

Phosphate compound	log K′ (Mg^{2+})	pK′ (H^+)
P_i	1.7	6.81 (pK'_2)
ATP^{4-}	4.63	–
ADP^{3-}	3.44	–
$MgATP^-$	–	5.21
MgADP	–	5.30
ATP^{3-}	2.86	6.95
ADP^{2-}	2.0	6.78
AMP^-	1.69	6.45
IMP^-	2.1	6.44

intermediates: The secondary acidity constant pK'_2 of uncomplexed free phosphate, which is the prevailing species in any tissue (Veloso et al. 1973; Burt et al. 1976; Dawson et al. 1977; Curtin and Woledge 1978) is close to the cell pH (6.81 at $\mu = 0.1$; Netter 1959). The value quoted tallies with the constant found for the intracellular fluid of marine invertebrates (cf. the $^{31}P_{NMR}$ data of Barrow et al. 1980). When phosphate is bound pK'_2 is lowered specificly for the molecule formed. Protons are therefore released when Mg ATP is hydrolyzed to form Mg ADP and inorganic phosphate, or consumed when phospho-L-arginine is cleaved to form L-arginine and inorganic phosphate (see Eqs. 1 and 5). Thus, as demonstrated below, inorganic phosphate determines the proton balance of adenylates and phosphagens.

Adenylates and protons

The following prerequisites have to be considered in the analysis of proton balance of the adenylates. The binding of Mg determines the dissociation of the phosphate groups both in ATP and ADP (Table 1). Hoult et al. (1974) and Dawson et al. (1977) demonstrated by $^{31}P_{NMR}$ that intracellular ATP in vertebrate muscle tissue is almost entirely bound to Mg. This was also demonstrated for other tissues (brain, liver, kidney) by Veloso et al. (1973) and for a marine bivalve by Barrow et al. (1980). There are no experimental data for Mg ADP because the concentration of free ADP is very low in intact muscle (Barrow et al. 1980; Dawson et al. 1980). The apparent binding constant of $Mg\,ADP^-$, however, like the binding constant of $Mg\,ATP^{2-}$ indicates an equilibrium towards the Mg^{2+} complex, being log $K' = 3.44$ at $\mu = 0.1$ and 25 °C (Phillips et al. 1966, Table 1). This is especially true in muscle tissues (Burton 1980), whereas in other tissues a larger part of ADP may

not be complexed because of the lower Mg^{2+} concentration (3 mmol l^{-1} in muscle tissue, Cohen and Burt 1977; 0.6–1.3 mmol l^{-1} in liver, brain, and kidney, Veloso et al. 1973). It is, however, still unclear how far the binding constant for Mg mentioned is applicable to living tissues, so that the percentage of Mg-bound ADP cannot definitely be estimated at present.

At cell pH most of the Mg complexes are present as $MgATP^{2-}$ and $MgADP^{-}$, because the acidity constants pK'_a of $MgATP^{-}$ and MgADP are 5.21 and 5.30 ($\mu = 0.1$, 25 °C), respectively (calculated from Phillips et al. 1966, cf. Table 1). At rest cell pH ranges from 6.9 to 7.5, depending on temperature, in striated vertebrate muscle (Hoult et al. 1974; Burt et al. 1976; Heisler 1975, 1980; Roos and Boron 1978). Few reliable data are available for marine invertebrates. Barrow et al. (1980) and Ellington (1983a, b) found the pH to be 6.9 to 7.2 at 22 to 24 °C in isolated tissues of different species of molluscs. In intact invertebrates, intracellular pH was 7.4 to 7.5 at 28 °C in an amphibious crab (Cameron 1981) and 7.32 ± 0.14 at 15 °C in the body wall of *Sipunculus nudus* (Pörtner et al. 1984a).

The turnover of protons by means of the adenylates can be estimated by consideration of the behaviour of ATP, ADP, and AMP in metabolism. Energy metabolism tends to keep the concentrations of the adenylates constant; under aerobic conditions, no net change occurs in the adenylate system. Only when there is a net change in ATP content the specific turnover of protons during synthesis and cleavage of ATP becomes important.

The following equation describes hydrolysis or formation of ATP at 3 mmol Mg^{2+} l^{-1} and pH = 7.32 in muscle tissue:

$$\begin{aligned} &0.982\ MgATP^{2-} + 0.007\ MgATP^{-} \\ &+ 0.008\ ATP^{4-} + 0.003\ ATP^{3-} + H_2O \\ &\rightleftharpoons 0.864\ MgADP^{-} + 0.008\ MgADP \\ &+ 0.105\ ADP^{3-} + 0.024\ ADP^{2-} + 0.685\ P_i^{2-} \\ &+ 0.10\ MgP_i + 0.215\ P_i^{-} + 0.753\ H^{+} \\ &+ 0.017\ Mg^{2+}. \end{aligned} \quad (1)$$

Constants were adopted from Curtin and Woledge (1978) or calculated from data obtained in vitro by Phillips et al. (1966) ($\mu = 0.1$; Table 1). At decreasing Mg concentrations Mg^{2+} will be liberated to a larger extent during hydrolysis (Burton 1980) and subsequently, because of the higher pK'_a of ADP^{2-}, the yield of protons will be diminished, especially at lower pH values.

As follows from Eq. (1) and as could be demonstrated by $^{31}P_{NMR}$, Mg^{2+} barely influences the dissociation of inorganic phosphate (Burt et al. 1976; Dawson et al. 1977). Therefore Eq. (1) may be rewritten for muscle tissue as follows, with only negligible inaccuracy:

$$\begin{aligned} &MgATP^{2-} + H_2O \\ &\rightleftharpoons MgADP^{-} + 0.764\ P_i^{2-} + 0.236\ P_i^{-} + 0.764\ H^{+} \end{aligned} \quad (2)$$

As mentioned above this reaction becomes significant for the acid-base status only when a net change in the ATP concentration occurs. A drop of the ATP content may happen during anaerobiosis and muscular activity and an increase may occur during recovery. Under such conditions the contribution of ATP to changes in the acid-base status can be calculated using an equation similar to Eq. (2) (for muscle tissue) or similar to Eq. (1) (for tissues with lower Mg concentrations).

A net decrease of the ATP concentration leads to an accumulation of ADP. Part of the ADP, however, is converted to AMP and ATP by adenylate kinase:

$$2\ ADP \rightarrow ATP + AMP \quad (3)$$

Some of the AMP may be withdrawn by AMP deaminase, and thereby a pronounced change in the ratio of the adenylates resulting in a dramatic drop of the energy charge (Atkinson and Walton 1967; Atkinson 1968) is avoided:

$$AMP^{2-} + H_2O \rightarrow IMP^{2-} + NH_3 \quad (4)$$

Since the adenine residue in adenosine is not protonated at cell pH (cf. Smith and Martell 1975) the reaction consumes protons due to subsequent ionization of the ammonia.

If AMP or IMP are accumulated to a considerable extent it has to be taken into account that they bind Mg^{2+} as weakly as inorganic phosphate and thus less strongly than ATP (Table 1). The amount of protons released by net hydrolysis of ATP will then be diminished, because pK'_a of AMP^{-} is 6.45 at $\mu = 0.1$. This pK'_a value is virtually the same for all nucleoside monophosphates (Phillips et al. 1965). Accordingly, AMP formation from $MgADP^{-}$ (Eq. 3) results in proton consumption.

Phosphagens and protons

The phosphagens (e.g. phospho-L-arginine or phosphocreatine) are utilized to buffer the ATP concentration (Newsholme et al. 1978; Wilson et al. 1981). High concentrations of phosphagens are mainly found in muscle tissue which is per-

forming anaerobically (Beis and Newsholme 1975). Considering that the ATP content is kept constant by cleavage of the phosphagen, hydrolysis and resynthesis of a phosphagen may be written as follows (for muscle $pH_i = 7.32$):

$$R-PO_3^{2-} + H_2O + 0.24\ H^+ \rightleftharpoons R-H + 0.24\ P_i^- + 0.76\ P_i^{2-} \quad (5)$$

As indicated, the residue R is affected only by replacing the phosphate group, which is bound to the guanidino group of creatine or arginine, with hydrogen.

Comparing the acidity constants (pK_a) of phospho-L-arginine (2.8; 4.5; 9.6; 11.2; Sober 1973) and L-arginine (1.85; 9.27; 12.48; Sober 1973) it is obvious that the pK_a of the phosphate group has vanished in L-arginine after hydrolysis. It has changed from $pK = 4.5$ (organic) to $pK = 6.81$ (inorganic). This implies uptake of H^+ by phosphate during hydrolysis. The alkalizing effect may facilitate a higher glycolytic rate because it counteracts the accumulation of protons during muscular activity.

There is no change of the pK_a values of L-arginine which could be relevant for proton balance. Since L-arginine in phospho-L-arginine is in dissociation equilibrium with cell water before hydrolysis, cell pH is not affected by the accumulation of L-arginine as has been supposed by Hochachka (1980) and Hochachka et al. (1983). The characteristics of the phosphagens presented here have already been elaborated by Meyerhof and Lohmann (1928) and Lipmann and Meyerhof (1930).

Depletion of the phosphagens leads to the accumulation of inorganic phosphate. This has been demonstrated for phosphocreatine by Dawson et al. (1978, 1980), and for phospho-L-arginine by Barrow et al. (1980) and Ebberink and de Zwaan (1980). The amount of accumulated inorganic phosphate may, however, be diminished by the formation of phosphorylated glycolytic intermediates (Ebberink and de Zwaan 1980; Sahlin et al. 1981) or by the release of inorganic phosphate into the extracellular fluid as has been suggested by Aragon and Lowenstein (1980).

Inorganic phosphate, apart from binding protons after hydrolysis of the phosphagen, will in addition influence the acid-base status by increasing the amount of intracellular buffers. The buffer value of inorganic phosphate (P_i) can be calculated according to the following formula:

$$\beta_{P_i} = \frac{2.303\,[H^+] \times K_a' \times [P_i]}{(K_a' + [H^+])^2}\ (\text{mmol}\ l^{-1}\ pH^{-1}). \quad (6)$$

A change in β_{P_i} will directly affect the non-bicarbonate buffer value β_{NB} which usually is determined in isolated tissue at rest (Heisler and Piiper 1971). The effective buffer value β_{NBeff} which results after activity or long term anaerobiosis can be calculated by:

$$\beta_{NBeff} = \beta_{NB} + \Delta\beta_{P_i}\ (\text{mmol}\ l^{-1}\ pH^{-1}). \quad (7)$$

Thus the inorganic phosphate which is released from phosphagens contributes by two different mechanisms to avoid a lethal drop in intracellular pH: firstly by consuming protons during hydrolysis of the respective phosphagen, and secondly by increasing the amount of intracellular buffers.

Generation of protons in the anaerobic catabolism of carbohydrates and aspartate

In invertebrates glycogen is the main substrate of anaerobic energy metabolism. Two pathways of glycogenolysis can be distinguished in lower invertebrates. The first one is the monocompartmental classical glycolysis (Embden-Meyerhof pathway) ending with the reduction of pyruvate (Fig. 3). In some marine invertebrates pyruvate is converted by reductive condensation with different amino acids, mostly glycine, alanine, and L-arginine yielding strombine [N-carboxymethyl-D-alanine], alanopine [*meso*-N-(1-carboxyethyl)alanine] and octopine [N^2-(D-1-carboxyethyl)-L-arginine], respectively (Grieshaber 1982; Livingstone 1982).

The second pathway, part of which is located within the mitochondria, leads to the formation of succinate, propionate, and acetate. In this type of anaerobic metabolism, which is characteristic for long-term lack of oxygen, aspartate may serve as an additional substrate during the first hours of anaerobiosis. There may be an inverse correlation of aspartate utilization and alanine accumulation (Fig. 3; Schöttler 1980; de Zwaan et al. 1982).

A correct method for the analysis of the proton balance cannot easily be defined. Of the two approaches cited in the Introduction, the analysis of the degree of dissociation of a given metabolite appears to be doubtful, because the importance of the substrate and of the metabolic pathway for proton generation is neglected. The other approach, the quantification of protons produced during hydrolysis of ATP formed by a pathway is incomplete. We prefer to consider the consumption and generation of carboxyl groups in metabolism. The pK' values of these groups are generally low enough to consider them as completely dissociated at cell pH. Their way of formation as well as the participation of ATP are taken into account

for each metabolite in order to see how and at which step of the pathway protons are released or consumed.

Energy metabolism requires that the redox status remains more or less unchanged. Therefore, the analysis of proton balance can be conducted assuming that steady state conditions are realized by metabolic regulation. If, however, net changes in the concentration of $NADH+H^+$ occur, their influence on acid-base balance can be considered separately, as already pointed out for the adenylates (see above).

The assumption of steady state conditions simplifies the analysis, as it implies that there is no net change in the concentrations of ATP and ADP, in other words that any $MgATP^{2-}$ synthesized is hydrolyzed instantaneously. Under these conditions no error is introduced by using only whole numbers to write equations. Synthesis and hydrolysis of MgATP can then be described by

$$MgADP^- + P_i^{2-} + H^+ \rightleftharpoons MgATP^{2-}. \quad (8)$$

The Embden-Meyerhof pathway

In the glycolytic pathway the following two reactions are subject to these considerations:

$$\text{P-glyceraldehyde}^{2-} + NAD^+ + P_i^{2-} \rightarrow 1,3\text{-}P_2\text{-glycerate}^{4-} + NADH + H^+ \quad (9)$$
(in steady state: $NADH + H^+ \rightarrow NAD^+ + 2H$).

$$1,3\text{-}P_2\text{-glycerate}^{4-} + MgADP^- \rightarrow 3\text{-P-glycerate}^{3-} + MgATP^{2-} \quad (10)$$
(in steady state: $MgATP^{2-} \rightarrow MgADP^- + P_i^{2-} + H^+$).

The net process therefore is:

$$\text{P-glyceraldehyde}^{2-} \rightarrow 3\text{-P-glycerate}^{3-} + H^+. \quad (11)$$

The proton required for ATP synthesis is delivered in reactions (9) and (10) by the oxidative formation of the carboxyl group. Consequently the proton which eventually is released by ATP hydrolysis does not originate from ATP but from the pathway.

Following anaerobic glycolysis to the end, the only net processes of this pathway are the accumulation of end products, whose carboxyl groups have been formed de novo in reactions (9) and (10), and the accumulation of the corresponding protons liberated via simultaneous hydrolysis of ATP. This conclusion is valid for all cytosolic end products of anaerobic glycolysis, including also the products of the reductive condensation of pyruvate with amino acids (Fig. 2).

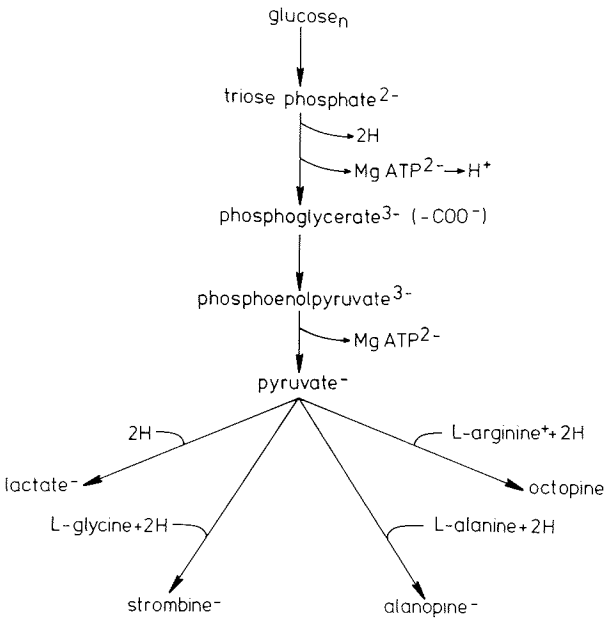

Fig. 2. Pathway of anaerobic glycolysis leading from glycogen (glucose$_n$) to different end products. Steps in the pathway, where protons are released via hydrolysis of $MgATP^{2-}$ and where carboxyl groups are formed de novo, are indicated by $MgATP^{2-} \rightarrow H^+$ or ($-COO^-$), respectively. 2H: reduction equivalents; for further explanations, see text

As a corollary, the accumulation of strombine, alanopine, and even octopine (which actually is a weak acid delivering 0.035 mol protons per mol of octopine at pH 7.32; cf. Zammit 1978) delivers one mol of protons per mol of end product formed. The essential point is that the amino acids reacting with pyruvate have been in dissociation equilibrium with the cell water before and that their state of dissociation is not affected by the condensation reaction. For the production of octopine, alanopine, and strombine, break-down of glycogen can be described as follows (Fig. 2):

$$\text{glucose}_n + 3\ P_i^{2-} + 2\ \text{L-arginine}^+ + 3\ MgADP^- + H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{octopine} + 3\ MgATP^{2-} \quad (12)$$

$$\text{glucose}_n + 3\ P_i^{2-} + 2\ \text{glycine} + 3\ MgADP^- + H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{strombine}^- + 3\ MgATP^{2-} \quad (13)$$

$$\text{glucose}_n + 3\ P_i^{2-} + 2\ \text{alanine} + 3\ MgADP^- + H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{alanopine}^- + 3\ MgATP^{2-} \quad (14)$$

Assuming steady state conditions as in Eq. (8) the net process is as follows (e.g. for octopine):

$$\text{glucose}_n + 2\ \text{L-arginine}^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{octopine} + 2\ H^+. \quad (12a)$$

In contrast to Hochachka and Mommsen (1983) we do not conclude that the binding of opines to inorganic cations (e.g. to Ca^{2+}) is relevant for the proton production by glycolysis since the dissociation of the carboxyl groups of glycolytic end products is not influenced by complex formation with Ca^{2+} at cell pH.

Succinate-propionate pathway and acetate formation compared to aerobic metabolism

During long term anaerobiosis the amount of resynthesized ATP is not equivalent to the amount of carboxyl groups formed. Moreover in marine invertebrates, namely in annelids, in bivalves, and in *Sipunculus nudus*, aspartate is utilized during the first hours of anaerobiosis as a second substrate until the anaerobic metabolism has been shifted to a carboxylation reaction occurring in the Embden-Meyerhof pathway (Schöttler 1980; Schöttler and Wienhausen 1981; de Zwaan et al. 1983; Pörtner et al. 1984b). The different intermediary and end products of this type of anaerobic metabolism are alanine, malate, succinate, propionate, and acetate (Fig. 3). In addition to these pathways which have been described in detail by Zebe et al. (1980) and Zandee et al. (1980), de Zwaan et al. (1982) have recently found that the inverse correlation between aspartate utilization and alanine accumulation may include a decarboxylation of aspartate. This possibility has been taken into account in the present analysis of the proton balance of anaerobic metabolism.

In carboxylation and decarboxylation reactions bicarbonate is assumed to participate in the respective conversion, although CO_2 may be the actual partner as has been proposed for phosphoenolpyruvate carboxykinase reaction (Cooper et al. 1968). Proton balance is identical, notwithstanding whether CO_2 or bicarbonate are used or liberated in the reaction. Carboxylation under participation of bicarbonate causes a net production of protons because the removal of bicarbonate is equivalent to an equimolar release of protons. On the other hand protons are released during fixation of CO_2 as well. Therefore it is not required to write equations with fractional numbers for CO_2 or bicarbonate. The consideration of the CO_2/bicarbonate system which is indispensable for the analysis of proton generation by metabolic pathways is one reason why some of our conclusions are at variance with those of Hochachka and Mommsen (1983).

In the following the metabolic reactions occurring during anaerobiosis are compiled. For the sake of convenience phase I (glycogen and aspartate are used as substrates) and phase II (glycogen is the only substrate) are considered separately. For reasons of redox balance the citric acid cycle takes part in the anaerobic metabolism (Zebe et al. 1980; Zandee et al. 1980) and, therefore, has to be taken into account. The participation of H_2O has no influence on the proton balance and may be neglected in the following equations:

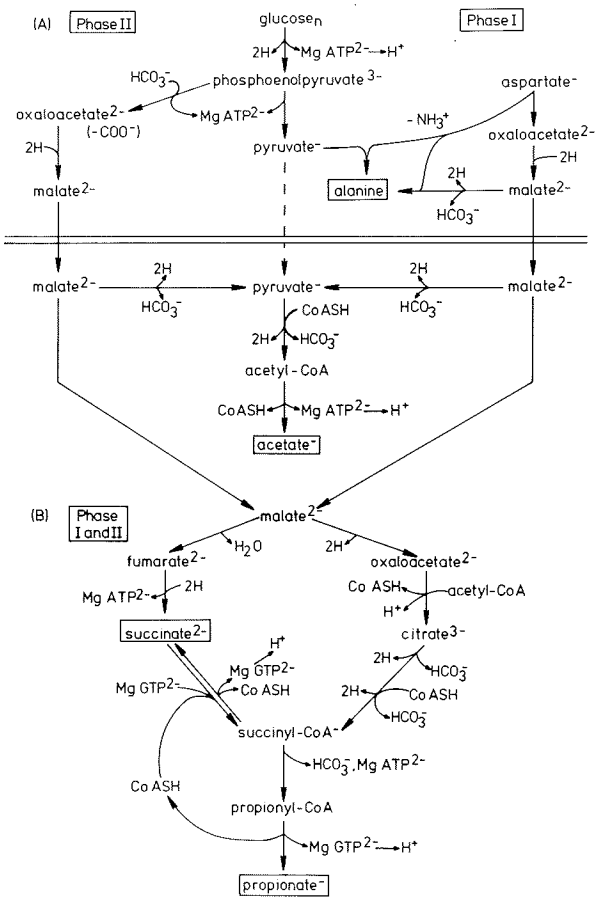

Fig. 3A, B. Succinate-propionate pathway and acetate formation starting from glycogen (glucose$_n$, phase I and II) and aspartate (phase I). Phase I: early anaerobiosis, phase II: late anaerobiosis. **A** Cytosolic formation of malate in phase I and II of anaerobiosis; formation of acetate from malate or pyruvate inside of mitochondria. **B** Mitochondrial conversion of malate via fumarate reductase and via citric acid cycle. Both directions of the pathway may finally result in propionate formation. Steps in the pathway, where protons are released directly or via hydrolysis of $MgATP^{2-}$ (or $MgGTP^{2-}$) are indicated by H^+ or $MgATP^{2-} \rightarrow H^+$, respectively. (-COO$^-$) symbolizes carboxyl groups formed de novo. Carboxylation and decarboxylation reactions are given as consumption or release of bicarbonate (HCO_3^-). 2H: reduction equivalents, CoASH: Coenzyme A; for further explanations, see text

Phase I: Substrates are glycogen and aspartate.

– Formation of opines (Eqs. 12 to 14).
– Formation of malate and alanine:

$$2\ \text{aspartate}^- + \text{glucose}_n + 3\ P_i^{2-} + 3\ \text{MgADP}^- + H^+ \rightarrow 2\ \text{malate}^{2-} + \text{glucose}_{n-1} + 2\ \text{alanine} + 3\ \text{MgATP}^{2-}. \quad (15)$$

– Formation of succinate by fumarate reductase:

$$2\ \text{aspartate}^- + \text{glucose}_n + 5\ P_i^{2-} + 5\ \text{MgADP}^- + 3\ H^+ \rightarrow 2\ \text{succinate}^{2-} + \text{glucose}_{n-1} + 2\ \text{alanine} + 5\ \text{MgATP}^{2-}. \quad (16)$$

– Formation of propionate via fumarate reductase:

$$2\ \text{aspartate}^- + \text{glucose}_n + 7\ P_i^{2-} + 7\ \text{MgADP}^- + 5\ H^+ \rightarrow 2\ \text{propionate}^- + \text{glucose}_{n-1} + 2\ \text{alanine} + 7\ \text{MgATP}^{2-} + 2\ HCO_3^-. \quad (17)$$

– Formation of propionate out of aspartate and glycogen via citric acid cycle:

$$\text{glucose}_n + 2\ \text{aspartate}^- + 10\ P_i^{2-} + 10\ \text{MgADP}^- + 2\ H^+ \rightarrow \text{glucose}_{n-2} + 2\ \text{propionate}^- + 2\ \text{alanine} + 10\ \text{MgATP}^{2-} + 8\ HCO_3^-. \quad (18)$$

– Formation of acetate:

$$2\ \text{aspartate}^- + \text{glucose}_n + 5\ P_i^{2-} + 5\ \text{MgADP}^- + H^+ \rightarrow 2\ \text{acetate}^- + \text{glucose}_{n-1} + 2\ \text{alanine} + 5\ \text{MgATP}^{2-} + 4\ HCO_3^-. \quad (19)$$

– Decarboxylation of aspartate:

$$\text{aspartate}^- \rightarrow \text{alanine} + HCO_3^-. \quad (20)$$

Phase II: Glycogen is the only substrate

– Formation of malate:

$$\text{glucose}_n + 3\ P_i^{2-} + 3\ \text{MgADP}^- + H^+ + 2\ HCO_3^- \rightarrow \text{glucose}_{n-1} + 2\ \text{malate}^{2-} + 3\ \text{MgATP}^{2-}. \quad (21)$$

– Formation of succinate by fumarate reductase:

$$\text{glucose}_n + 5\ P_i^{2-} + 2\ HCO_3^- + 5\ \text{MgADP}^- + 3\ H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{succinate}^{2-} + 5\ \text{MgATP}^{2-} \quad (22)$$

– Formation of propionate via fumarate reductase:

$$\text{glucose}_n + 7\ P_i^{2-} + 7\ \text{MgADP}^- + 5\ H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{propionate}^- + 7\ \text{MgATP}^{2-}. \quad (23)$$

– Formation of propionate via citric acid cycle:

$$\text{glucose}_n + 10\ P_i^{2-} + 10\ \text{MgADP}^- + 2\ H^+ \rightarrow \text{glucose}_{n-2} + 2\ \text{propionate}^- + 10\ \text{MgATP}^{2-} + 6\ HCO_3^-. \quad (24)$$

– Formation of acetate:

$$\text{glucose}_n + 5\ P_i^{2-} + 5\ \text{MgADP}^- + H^+ \rightarrow \text{glucose}_{n-1} + 2\ \text{acetate}^- + 5\ \text{MgATP}^{2-} + 2\ HCO_3^-. \quad (25)$$

Table 2 summarizes the turnover of $MgATP^{2-}$ and the delivery or consumption of bicarbonate and protons during the formation of intermediary and end products. The most important point to note with regard to the influence of anaerobic metabolism on the acid-base status is that in all cases net changes in the amount of H^+ are stoichiometric to the amount of carboxyl groups generated or consumed. This means for the two phases of anaerobiosis (Fig. 3):

Phase I. The accumulation of opines as end products of anaerobic glycolysis leads to a stoichiometric accumulation of protons. Formation of alanine from glycogen and aspartate also yields one mol of protons per mol of alanine because no net change occurs in the protonation of the amino group transferred from aspartate to pyruvate (Eq. 15). Formation of alanine by decarboxylation of aspartate, however, will consume a stoichiometric amount of protons, because the amount

Table 2. Net production of protons by formation of metabolites produced from glycogen (glyc.) and aspartate (asp.) as substrates. Accumulation of opines takes place essentially during the first hours of anaerobiosis (de Zwaan and Zurburg 1981; Pörtner et al. 1984b). Total ΔH^+ (ΔH^+_{tot}) was calculated from the turnover of protons by means of ATP (ΔH^+_{ATP}) and from the changes in the amount of bicarbonate (ΔHCO_3^-)

Metabolite	Substrate	Stoichiometric turnover of ATP	ΔH^+_{ATP}	ΔHCO_3^-	ΔH^+_{tot}
Opines	glycogen	1.5	+1	–	+1
Alanine	glycogen	1.5	+1	–	+1
	aspartate	–	–	+1	−1
Malate	aspartate	0	0	–	–
	glycogen	1.5	+1	−1	+2
Succinate	aspartate	1	0	–	–
	glycogen	2.5	+1	−1	+2
Propionate	aspartate	2	0	+1	−1
	glycogen	3.5	+1	–	+1
	glyc. + asp., citric acid cycle	3.5	+1+2	+4	−1
	glyc., citric acid cycle	5	+2+2	+3	+1
Acetate	aspartate	1	+1	+2	−1
	glycogen	2.5	+2	+1	+1

of acidic groups is diminished (Eq. 20). The same stoichiometric relationship exists for propionate and acetate originating from the carbon skeleton of aspartate (Eqs. 17 to 19), whereas succinate produced by aspartate degradation is accumulated without any influence on the acid-base status (Eq. 16).

Cytosolic glycolysis is therefore the only process during the early phase of anaerobiosis which will lead to a production of protons. This process is probably a function of the degree of muscular activity and, therefore, may vary considerably. Since protons are consumed to a certain amount by the depletion of a phosphagen during early anaerobiosis there is some doubt regarding the occurrence of an acidosis during this period, especially in tissues exhibiting a high phosphagen content. In fact, *Sipunculus nudus* L. does not develop an acidosis until after 12 h of experimental anaerobiosis (Pörtner et al. 1984a).

Phase II. If glycogen is the sole source of succinate, propionate, and acetate formation the amount of protons accumulated per mol of end products is increased. Production of succinate, propionate, and acetate, yields 2 or 1 mol of protons, respectively. The carboxyl groups of succinate for instance are generated by the oxidation reaction in glycolysis (Eqs. 9 and 10) and by the carboxylation of phosphoenolpyruvate (Eq. 22).

It should be emphasized that the proton balance of anaerobic metabolism is specific for the respective metabolite, depending on the substrate and net changes in the amount of carboxyl groups.

Turnover of ATP formed in the mitochondria does not increase total ΔH^+ because there is no further net generation of carboxyl groups in this compartment. This is true not only for anaerobic but also for aerobic conditions. The mechanisms of ATP production shall be discussed in detail in order to clarify these reflections (Fig. 3):

Fumarate reductase reaction. During anaerobiosis the succinate dehydrogenase reaction of the aerobic citric acid cycle is reversed. Fumarate is used as an electron acceptor, the transfer of reduction equivalents being accompanied by synthesis of ATP. The fumarate reductase system is part of the respiratory chain. The process of ATP formation is analogous to aerobic oxidative phosphorylation (Schroff and Schöttler 1977). In accordance with aerobic metabolism there is no net production of protons by this type of ATP generation. This is evident because no acidic group is formed by the reduction of fumarate.

Decarboxylation of succinate. Propionate is formed anaerobically from succinate which must be activated by coenzyme A (Schroff and Zebe 1980; Schulz and Kluytmans 1983). Succinate may be delivered by fumarate reductase as well as by the citric acid cycle (Fig. 3). The chain of reactions may be described as follows:

$$\text{succinate}^{2-} + \text{CoASH} + \text{MgGTP}^{2-} \rightarrow \text{succinyl-CoA}^{-} + \text{MgGDP}^{-} + P_i^{2-} \quad (26)$$

$$\text{succinyl-CoA}^{-} \rightarrow \text{methylmalonyl-CoA}^{-} \quad (27)$$

$$\text{methylmalonyl-CoA}^{-} + \text{biotin} \rightarrow \text{propionyl-CoA} + \text{biotin-CO}_2^{-} \quad (28)$$

$$\text{propionyl-CoA} + \text{MgGDP}^{-} + P_i^{2-} \rightarrow \text{propionate}^{-} + \text{CoASH} + \text{MgGTP}^{2-} \quad (29)$$

$$\text{biotin-CO}_2^{-} + \text{MgADP}^{-} + P_i^{2-} + H_3O^{+} \rightarrow \text{biotin} + \text{MgATP}^{2-} + HCO_3^{-}. \quad (30)$$

Since $MgGTP^{2-}$ is cycled from reaction (29) to reaction (26) there is no net release of protons via hydrolysis of $MgGTP^{2-}$. The net process of these reactions is the conversion of a carboxyl group into bicarbonate which is equivalent to the consumption of a proton:

$$\text{succinate}^{2-} \rightarrow \text{propionate}^{-} + HCO_3^{-}. \quad (31)$$

The citric acid cycle and acetate formation. In Table 2 part of the propionate is assumed to be formed via the citric acid cycle. The amount of protons which is turned over via hydrolysis of ATP has been split up into two separate numbers. The first one refers to ATP formed in glycolysis, and the second one to ATP generated by the reactions of the citric acid cycle and by the formation of propionate.

When glycogen and aspartate are stoichiometrically utilized as substrates, 1 mol of H^+ (via ATP) per mol of propionate is generated during glycolysis (Eqs. 9 and 10) which also delivers pyruvate. Pyruvate is converted into acetyl-CoA. The utilization of aspartate renders oxaloacetate for citrate formation (Eq. 18). The amount of protons originating from glycolysis (via ATP) increases to 2 mol H^+ per mol propionate when glycogen alone is used as a substrate. Both oxaloacetate and acetyl-CoA have to be delivered from glucose (Eq. 24), since there is no cyclic regeneration of oxaloacetate during anaerobiosis.

Furthermore 2 protons are released (via ATP) by the following mitochondrial reactions:

$$\text{pyruvate}^{-} + \text{CoASH} \; (+\text{NAD}^{+}) \rightarrow \text{acetyl-CoA} + HCO_3^{-} \; (+\text{NADH} + H^{+}) \quad (32)$$

$$\alpha\text{-ketoglutarate}^{2-} + \text{CoASH} \; (+\text{NAD}^{+}) \rightarrow \text{succinyl-CoA}^{-} + HCO_3^{-} \; (+\text{NADH} + H^{+}) \quad (33)$$

Equations (32) and (33) describe oxidative decarboxylation reactions leading to the CoA-esters of acetate and succinate, respectively. Cleavage of these CoA-esters results in production of nucleoside triphosphates. Synthesis of GTP from succinyl-CoA is well known during aerobiosis. ATP synthesis from acetyl-CoA may occur during anaerobiosis (Wienhausen 1981).

$$\text{succinyl-CoA}^{-} + \text{MgGDP}^{-} + P_i^{2-} \rightarrow \text{succinate}^{2-} + \text{MgGTP}^{2-} + \text{CoASH} \quad (34)$$

$$\text{acetyl-CoA} + \text{MgADP}^{-} + P_i^{2-} \rightarrow \text{acetate}^{-} + \text{MgATP}^{2-} + \text{CoASH}. \quad (35)$$

Protons are released, since during the steady state ATP is hydrolyzed simultaneously.

During prolonged anaerobiosis succinyl-CoA is converted into propionate (see above), and accordingly the CoA-ester to be hydrolyzed is propionyl-CoA instead of succinyl-CoA. Under both aerobic and anaerobic conditions acetyl-CoA also condenses with oxaloacetate with a concomitant release of one proton by the liberation of the acidic group:

$$\text{oxaloacetate}^{2-} + \text{acetyl-CoA} + H_2O \rightarrow \text{citrate}^{3-} + \text{CoASH} + H^{+}. \quad (36)$$

Net production of protons by means of oxidative decarboxylation of pyruvate and α-ketoglutarate (Eqs. 32 to 35) is, however, fictitious, because the same reactions release base equivalents in form of bicarbonate, so that net total ΔH^+ for these two reactions is zero (Table 2). In contrast, oxidative decarboxylation of isocitrate results in release of bicarbonate only:

$$\text{isocitrate}^{3-} \; (+\text{NAD(P)}^{+}) + H_2O \rightarrow \alpha\text{-ketoglutarate}^{2-} + HCO_3^{-} \; (+\text{NAD(P)H} + H^{+}) \quad (37)$$

Therefore, the overall balance of the citric acid cycle including decarboxylation of pyruvate is as follows:

$$\text{oxaloacetate}^{2-} + \text{pyruvate}^{-} \rightarrow \text{oxaloacetate}^{2-} + 3HCO_3^{-} + 2\,H^{+}. \quad (38)$$

According to this equation net ΔH^+ for the reactions taking place in the mitochondria is actually -1. A net consumption of protons in the matrix of mitochondria, however, occurs only if pyruvate enters these organelles in dissociated form. This mechanism could support the generation of a pH-gradient between the mitochondrion and the cytosol, which is assumed to be relevant for ATP synthesis by oxidative phosphorylation (Boyer et al. 1977).

The overall process of glucose oxidation, which occurs in both compartments and which results in the end products CO_2 and water, has a net pro-

ton production of zero because the protons formed by cytosolic glycolysis will be consumed by intramitochondrial decarboxylation reactions. This statement is valid as long as steady state gas exchange conditions for the release of CO_2 prevail, preventing a build-up of CO_2 along with hydration and dissociation of H^+ ions in the organism.

Conclusions

I. The proton generation by metabolic pathways can most accurately be assessed first by the quantitative evaluation of net changes in the amount of carboxyl groups or other groups with dissociation patterns relevant for the acid-base balance; and secondly, by the evaluation of changes in dissociation of some groups whose pK values vary between substrates and products of a reaction (e.g. phosphate or ammonia in phosphagen hydrolysis, or AMP deamination, respectively). The proton balance of metabolism is different and specific for each individual combination of a certain metabolite produced from degradation of a certain substrate.

II. The end products of cytosolic glycolysis deliver a stoichiometric amount of protons during their respective accumulation, ATP possibly being involved in the release of those protons. The amount of protons generated per mol of metabolites in the succinate-propionate pathway and during acetate formation is less than the amount of ATP turned over because aspartate is temporarily utilized as a substrate. Moreover, some reactions are involved which are also typical for aerobic metabolism: Decarboxylation reactions may deliver base equivalents in form of bicarbonate; oxidative decarboxylation may deliver bicarbonate and protons together which after combination to CO_2 and water are eliminated by respiratory gas exchange. The oxidative phosphorylation, which is correlated to fumarate reduction during anaerobiosis, is neutral for the acid-base status.

III. Carboxylation and decarboxylation reactions influence the proton balance of metabolic pathways since the removal or delivery of CO_2 or bicarbonate are equivalent to the generation or consumption of protons, respectively. This becomes important when C_2- and C_4-compounds are generated from glucose or when carbonic acids are metabolized.

IV. The contribution of the adenylate system and any ATP regenerating pathway may be regarded separately. If there is a net production of protons via hydrolysis of ATP, this is caused
a) by net consumption of ATP or
b) by generation of acidic groups in the pathway with concomitant dissociation of protons, which are temporarily consumed by ATP synthesis.

V. Changes in the concentration of phosphagens have a twofold effect on the acid-base status. Firstly, protons are consumed during hydrolysis. Secondly, inorganic phosphate, which is released during hydrolysis, increases the intracellular non-bicarbonate buffer value. This second effect may also be observed during consumption of ATP.

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