6.1 Thermodynamic Considerations of Invertebrate Anoxibiosis

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ECOLOGICAL AND BIOCHEMICAL BACKGROUND

In no case was it unequivocally demonstrated that any metazoan organism is capable of performing its whole life cycle under strictly anoxic conditions "(105)". There are, however, ecological indications of persistent, if not permanent, anoxic animal life, especially abundant in marine sediments containing hydrogen sulfide "(24,73,77,78,107,108,114,115)" and to a lesser extent in anoxic strata of limnetic muds "(3, 63, 84, 99)" and meromictic lakes "(4,90)".

Under these conditions glycogen is commonly the prime source of energy, whereas the metabolic integration of amino acids and lipids is still a matter of speculation and contradiction "(14,32,35,45,135)". Pathway and control of glucose degradation are comparable to the classical Embden-Meyerhof-Parnas route to the level of phosphoenol pyruvate (PEP) "(85,101)". A detailed literature deals with the aerobic-anoxic control of CO_2-fixation at the PEP-branchpoint and the formation of succinate via the reversed tricarboxylic-acid-cycle sequence (Fig.1). Most exentsively studied in parasitic helminths "(6,7,93,123)" and intertidal bivalves "(34,37,38,46,81, 135)" the same basic frame of the anoxic pathway leading to succinate was also demonstrated in fresh-water bivalves "(30)", gastropoda "(70,71,76,103,104)", polychaeta "(89,

121)", oligochaeta "(20,21,100,101)" and chelicerata "(23)". Additional studies indicate the existence of these metabolic pathways in other phyla "(32,39,41,50,67)", and support the view of its general importance in anoxibiotic invertebrates, and possibly in some vertebrates "(53,58,59,82)".

A principal criterion which must be fulfilled by any scheme of anoxic energy metabolism is the maintenance of redox balance, i.e. a constant NAD/NADH ratio. In lactic fermentation NADH, generated by the oxidation of glyceraldehyde-3-phosphate, is fed into the reductive lactate dehydrogenase step. As a peculiarity of molluscs, lactate dehydrogenase may be partially replaced by octopine dehydrogenase "(26,28,29,36,87)". In animals with high anoxic capacity, however, the cytoplasmatic malate dehydrogenase takes over the function of lactate dehydrogenase and thereby plays an important role in the regulation of the PEP-branchpoint "(11,19,46,64,68,69,112,122)" - quite analogous to the glycerol phosphate shuttle preventing aerobic formation of lactate (Pasteur effect).

Oxidation of an additional molecule of NADH proceeds concomitantly with the formation of succinate (Fig.1). As a mechanism sustaining a constant NAD/NADH ratio in Ascaris, Saz and his coworkers described a "malate dismutation system" giving rise to equal amounts of succinate and pyruvate by the combined avtivity of mitochondrial fumarate reductase (succinate dehydrogenase) and malic enzyme, respectively "(93,94,95,102)", but the NADH requirement along the metabolic path leading to other end products is not accounted for.

In molluscs the same dismutation system would be operative if succinate and alanine accumulate in equimolaramounts, alanine being formed from pyruvate by transamination reactions "(33,70,106)". Hochachka's group postulated that during anoxibiosis the tightly linked catabolism of carbohy-

drate and amino acids is responsible for the maintenance of redox equilibrium, whereby one glucose unit, two molecules of aspartate, and two molecules of α-ketoglutarate must be mobilized simultaneously, and end products must be formed in constant ratios "(45)". Experimental evidence, however, suggests anoxic formation of amino acids rather than amino acid consumption "(1,2,32,70,101,106,121,132,136)", an exception being Fasciola hepatica "(51,60)" a parasite exposed to a high-protein diet "(86)". This, together with the fact that anoxic end products do not accumulate at a fixed ratio (see below), supports the scheme given by de Zwaan and his colleges "(55,132,135,137)": By slow action in the forward direction the tricarboxylic acid cycle generates NADH which is consumed by the fumarate reductase step. Therefore anoxic carbohydrate and protein catabolism are not connected by an obligatory link, but, rather, are related to ecological and physiological parameters like quality of food, starvation, and time of anoxibiosis. This sets the stage for a numerical evaluation of anoxibiotic processes and comparison of different biochemical strategies of carbohydrate catabolism on quantitative grounds.

CHANGES OF FREE ENERGY ASSOCIATED WITH CATABOLISM OF GLUCOSE AND ANOXIC ATP GENERATION

The catalytic action of both pyruvate kinase in classical glycolysis and phosphoenolpyruvate carboxykinase "(110)" in the anoxic pathway, yields a net production of high-energy phosphate bonds by well known substrate level phosphorylation reactions. In the anoxic pathway, however, this is not the only ATP generating system. (The different nucleotide phosphates are treated as thermodynamically identical.) NADH-dependent phosphorylation by an anoxic electron transport chain with fumarate - analogous to oxygen - acting as terminal electron acceptor was demonstrated in parasitic hel-

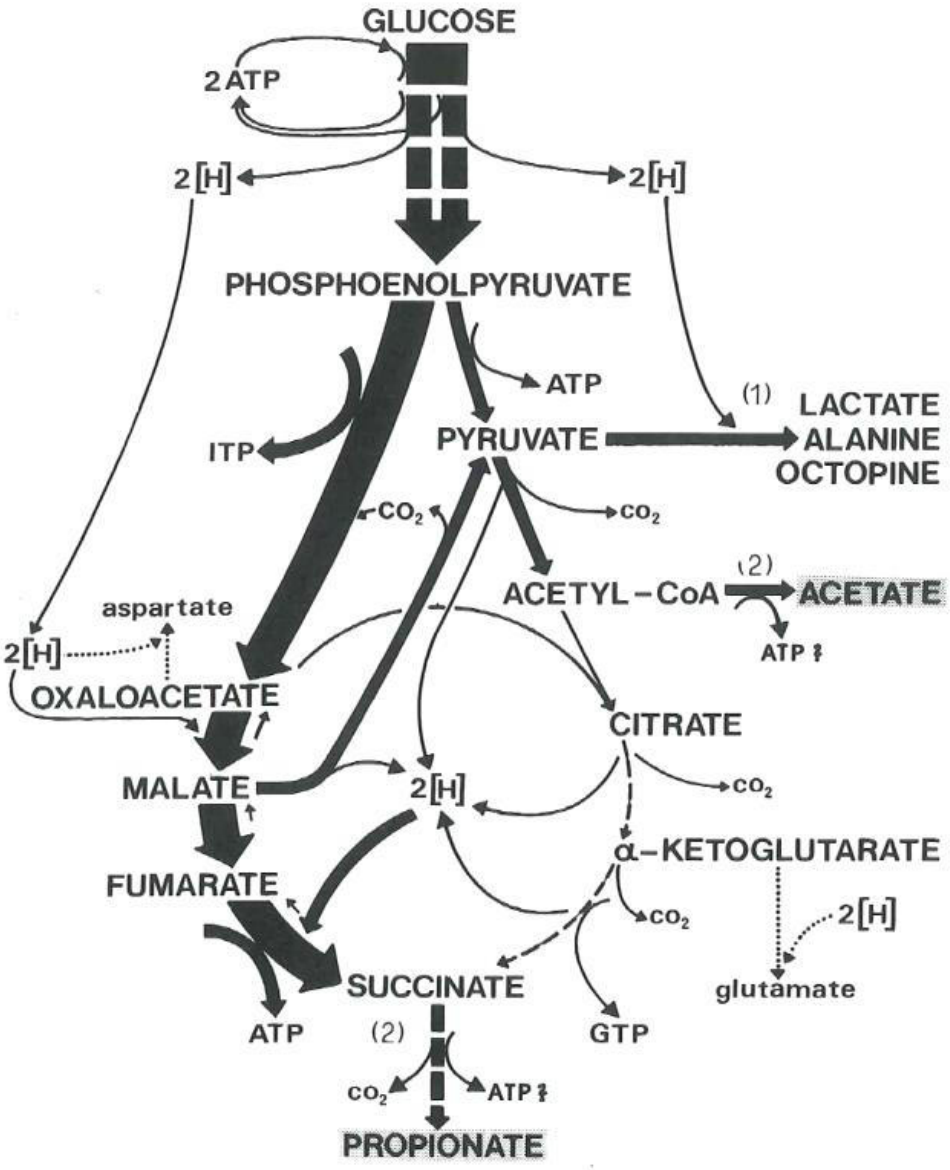

Fig.1. The basic frame of the anoxic pathways, and formation of primary (1) and secondary (2) end products in invertebrates.

minths "(15,16,17,18,57,98,102,124)" and seems to operate in other invertebrates "(40)" and in vertebrates as well "(43, 44,88,92,119)". As the reaction

(1) $Fumarate^{2-} + NADH + H^+ = Succinate^{2-} + NAD^+$

proceeds with $\Delta G^{o\prime}$ (change of free energy in standard state conditions at pH 7) of -67.7 kJ $mole^{-1}$ as calculated from standard tables "(66)", the free energy retained in ATP (30.5 kJ or 7.3 kcal $mole^{-1}$; "(62)") equals 45 %. The same thermodynamic efficiency is given for the aerobic electron transport chain (42 %;"(62)"). At "near physiological conditions"(see Tab.1) ΔG of reaction (1) amounts to -67.8 kJ $mole^{-1}$, and thermodynamic efficiencies are 70 % and 65 % for the anoxic and aerobic electron-transfer-coupled phosphorylations respectively.

Summarizing, half a glucose unit catabolized via the anoxic pathway (reaction 2) yields a net of 2 ATP. Redox balance is maintained if this reaction proceeds at a rate five times the rate of succinate formation via the tricarboxylic acid cycle (reaction 3): Two moles of phosphoenolpyruvate are converted into one mole of oxaloacetate and pyruvate respectively, giving rise to citrate by a condensation reaction (see Fig.1). In this way one mole of $succinate^{2-}$ (Suc) is derived from one mole of glucose (Glc), accompanied by three substrate level phosphorylation reactions.

(2) $0.5\ Glc + HCO_3^- + NADH = Suc + 2\ H_2O + NAD^+$

(3) $Glc + 4\ H_2O + 5\ NAD^+ = Suc + 2\ HCO_3^- + 5\ NADH + 9\ H^+$

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(4) $Glc + 0.86\ HCO_3^- = 1.71\ Suc + 1.71\ H_2O + 2.57\ H^+$

A resultant reaction (4) proceeds with a net yield of 3.714 mole of ATP $mole^{-1}$ glucose. Compared to lactic fermentation the "succinic" fermentation produces 85 % more ATP per mole

Tab.1. Changes of free energy and heat production in catabolism of glucose by anoxic pathways in invertebrates as compared with aerobic respiration.

Metabolic Pathway	$-\Delta G^{o}$ (kJ)	$-\Delta G$ (kJ)	$-\Delta G$ (kcal)	ATP per Glc	Thermod. Efficiency	$-\Delta H$ (kJ)	$-\Delta H$ (kcal)	Caloric Efficiency
aerobic	2604	2903	693.9	36	58.6	2816	673	1.28
a) lactic	117	208	49.8	2	45.4	88	21	2.28
b) alaninic	140	209	50.0	2	45.2	267	63.9	0.75
c) succinic	171	273	65.3	3.7	64.2	255	60.9	1.46
d) propionic		(330)	(78.9)	3.7 - 5.4	53.2 - 77.7	181	43.6	3.00
e) acetic-succinic	204	288	68.9	3.3 - 4.0	54.1 - 65.6	240	57.3	1.67
f) acetic-propionic		(364)	(87.0)	3.3 - 5.3	43.3 - 69.3	183	43.8	2.89

Legend to Tab. 1. Units are in kJ or kcal $mole^{-1}$ glucose; Temp. 25°C; ΔG^0 values are for unit activity and CO_2 (aq); ΔG values are for 0.2 atm O_2, 0.05 atm CO_2 (aq), pH 7 and 0.01 M concentration of other reactants. Values of free energy are calculated from data according to Burton and Krebs "(12)" unless otherwise stated. ΔH values are calculated from heats of combustion "(5)" unless otherwise stated, and no corrections are made for heats of solution and dissociation of the acids. Thermodynamic efficiency is the percentage of ΔG retained in phosphorylation reactions (endergonic) per ΔG of the exergonic reaction. The caloric efficiency is the percentage of maximal ATP turnover in mole per kJ heat produced.

a) $Glc = 2\ Lactate^- + 2\ H^+$

b) $Glc + 2\ NH_4^+(aq) = 2\ Alanine^- + 2\ H_2O + 4\ H^+$

Alanine production is assumed to proceed by NH_3 fixation onto pyruvate and therefore with maintenance of redox balance in the overall reaction. Transaminase reactions, however, proceeding without changes of free energy, may also play an important role. ΔH was calculated from heats of formation.

c) $Glc + 0.857\ HCO_3^- = 1.714\ Suc^{2-} + 1.714\ H_2O + 2.571\ H^+$

d) $Glc = 1.714\ Prp^- + 0.857\ HCO_3^- + 2.571\ H^+$

Production of proprionate (Prp) from succinate (Suc) via the reversal of a reaction sequence known from catabolism of isoleucine and methionine was demonstrated in parasitic helminths "(96, 123)". Therefore the same considerations apply to "propionic" and "succinic" fermentation (see text), but an additional substrate level phosphorylation is probably driven by decarboxylation of succinate. As calculated from data of Wood "(120)", the biotin linked reaction

$$Methylmalonyl\text{-}CoA^- + H_2O = Propionyl\text{-}CoA + HCO_3^-$$

proceeds with $\Delta G = -33$ kJ. As the methylmalonylmutase reaction and the transfer of CoA from propionate to succinate are readily reversible "(120)", the same value may be taken for approximating the overall decarboxylation of succinate to yield propionate.

e) $Glc + 0.667\ HCO_3^- =$
$= 0.667\ Act^- + 1.333\ Suc^{2-} + 1.333\ H_2O + 2.667\ H^+$

f) $Glc = 0.667\ Act^- + 1.333\ Prp^- + 0.667\ HCO_3^- + 2.667\ H^+$

Formation of acetate delivers reducing equivalents for two fumarate reductase reactions. Therefore the NAD/NADH ratio

is maintained, if acetate and succinate or propionate are produced in a ratio of 1 : 2. ATP production by acetate kinase is known from micro-organisms, but has not yet been proved in invertebrates.

of glucose, "propionic" fermentation probably 170 % more. In the latter case thermodynamic efficiency would approximate 80 %. Higher yields of ATP seem thermodynamically unfeasible. Increased efficiencies of phosphorylation in anoxic pathways are due not only to an increased thermodynamic efficiency, but also to larger increments of free energy in the respective reactions (Tab.1).

PRIMARY AND SECONDARY END PRODUCTS

The relative importance of the different anoxic pathways determines the overall efficiency of the anoxic energy metabolism. Due to short experimental acclimation periods (in the range of a few hours) the quantitative importance of lactate, alanine, and of succinate as end products of the anoxic energy metabolism has been largely overestimated. In these studies different sections were taken through a dynamic biochemical transitory process and described as different patterns of anoxic intermediary metabolism. This led to a confusing picture of various traits of metabolic strategies. Abundant evidence now exists that in invertebrates resistant to anoxia, lactate and alanine are initial end products only, accumulating in relatively small amounts during the first 12 to 24 hours of anoxibiosis "(10,27,55,113)". As time of anoxibiosis proceeds "propionic" and/or "acetic-propionic" fermentation become increasingly more important (Tab.2), "(30,56,135)", whereby in some cases the two volatile fatty acids are excreted after condensation to methylbutyrate "(21,97)".

Tab.2. Production of organic acids in µmol/g dry weight/h by Tubifex during successive periods of anoxic acclimation. The percentage of total organic acids is given in brackets. Calculated from data of Schöttler and Schroff "(101)".

hours	0 - 14	14 - 24	24 - 38	38 - 48
lactate	0.58 (4.2)	--	--	--
alanine	1.62 (11.8)	0.44 (4.8)	0.06 (0.6)	-0.06
succinate	4.33 (31.5)	3.33 (36.7)	2.48 (26.4)	1.20 (12.0)
acetate	1.64 (11.9)	1.90 (21.0)	2.36 (25.1)	2.30 (23.0)
propionate	5.57 (40.5)	3.40 (37.5)	4.50 (47.9)	6.50 (65.0)
glucosyl-equivalents [a]	7.42	4.78	4.89	5.26
glycogen-consumption [b]	6.29	5.59	4.77	3.52

a) The rate of consumption of glucosyl-equivalents is calculated from the rate of formation of end products under the assumption that glycogen is the sole source of energy and redox balance is maintained. The difference between this and the sum of organic acid production x 2^{-1} is proportional to the rate of the tricarboxylic acid cycle functioning in its forward direction.
b) Measured glycogen consumption in µmol glucosyl-equivalents/g dry weight/h.

The significance of lactate and alanine as primary end products of anoxibiosis is therefore seen in their influence on the activities of regulatory enzymes during the aerobic-anoxic transition of intermediary metabolism "(46,47,65,75,

74,116,130,131,132,133,134)". Accumulation of these primary end products, the formation of which is less efficient in generating high-energy phosphate bonds, initiates a new state of anoxi-metabolic equilibrium. Essentially, the observed trend can be interpreted as the general tendency to stabilize anoxic pathways with the highest yield of ATP (Fig.2).

APPLICATION OF DIRECT CALORIMETRIC METHODS

The confusing variety of species-specific patterns of organic acid production not only during anoxic acclimation, but in different tissues "(1,14,31,64)", developmental stages "(48, 91,113)", different sexes "(9)", and seasons "(121)" makes the biochemical assessment of the level of anoxic energy metabolism a tedious task. The variable ratio of accumulated and excreted end products "(22,72,101)" necessitates the consideration of both. Discontinuous carcass analysis is therefore unavoidable, whereby subtractions of estimated values measured on different individuals will introduce substantial errors due to considerable inhomogeneities, especially when large quantities of analytical substances (e.g.glycogen) are involved "(27,30,101,138,139)".

Heat production is an unspecific measure of the enthalpy changes accompanying all metabolic reactions of an organism. In long-term experiments continuous registration of heat production is possible, until a new steady state of anoxic energy metabolism - if ever - is reached. Turnover of ATP comprises the essential parameter of interest, since the scope of energy metabolism is the generation of high energy phosphate bonds as intermediate driving forces for all energy requiring biological processes. Provided that net synthesis is negligible during the experimental period "(49)", the caloric efficiency of ATP production may be calculated from the relative contribution of the different anoxic pathways

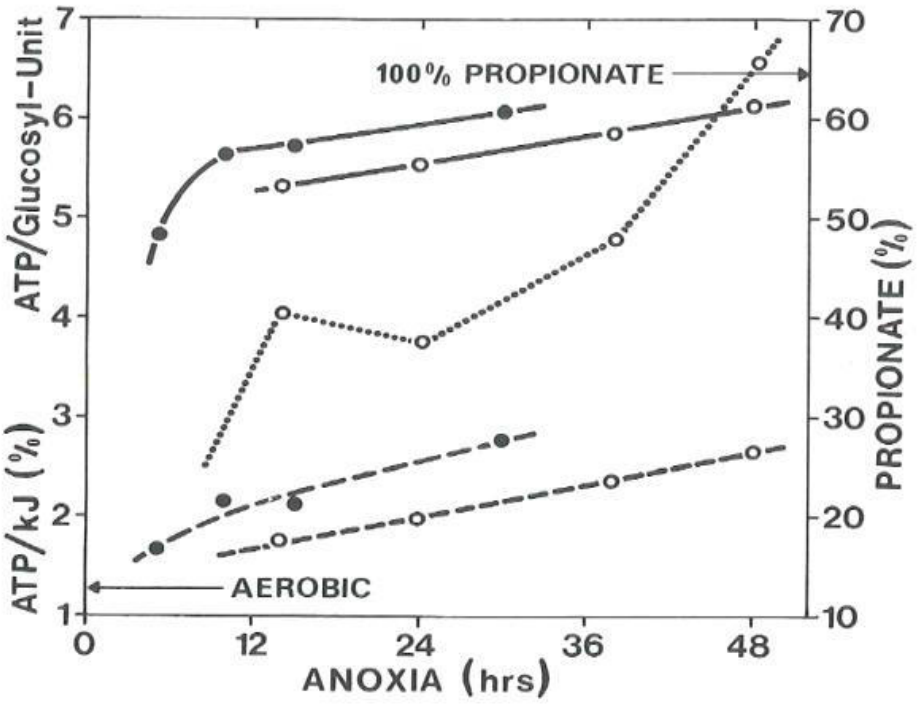

Fig.2. Anoxic acclimation of anoxibiotic invertebrates: Tubifex (open circles; calculated from data in Tab.2) and Anodonta (full circles; calculated from data of Gäde et al. "(30)". Solid line: Molar efficiency of ATP production in catabolism of glycogen. For every mole of glucose derived from glycogen one mole of ATP is saved, which is added to the maximal ATP production in the different anoxic pathways (Tab. 1). The arrow "100 % propionate" indicates the maximal molar efficiency of ATP production in pure "propionic" fermentation. Dashed line: Caloric efficiency. Values of Tab.1 are used in their proportional contribution to the anoxic metabolism without correcting for glycogen degradation. The calculated mean of total heat production is 85 µW/g dry weight of Tubifex and 97 µW/g dry weight of Anodonta. The arrow "aerobic" indicates the caloric efficiency level of aerobic metabolism. Dotted line: Percentage of propionate on total organic acid production.

to the overall metabolism (Tab.1). The biological interpretation of calorimetric data is difficult especially for the transitory period, but may be approximated by supplementary biochemical investigations (Fig.2). However, various side reactions "(25)", or energy production by "anoxic endogenous oxidation" "(42,54,125,126,127,128)" and net utilization of energy reserves "(109,117,118,129)" may obscure the calculated values.

Anoxic metabolism of diving vertebrates "(52,83)" and of whole benthic communities "(79,80)" was studied using direct calorimetric methods, but data of heat production of anoxibiotic invertebrates are not available so far. Inconsistencies of the presented calculations with future experimental investigations may reveal some gaps in our understanding of the biochemical mechanisms promoting anoxic animal life. Incorporating recent refinements of microcalorimetric techniques "(8,13,61,111)", the application of direct calorimetry to the study of ecological energetics will therefore contribute to better insight into the quantitative relationships of invertebrate anoxibiosis.

SUMMARY

New insight into the biochemical mechanism of invertebrate anoxibiosis made possible the calculation of the free-energy changes associated with the generation of high-energy bonds in nucleoside triphosphates (ATP, GTP, ITP) under anoxic conditions. The values obtained are compared with thermodynamic data of aerobic and fermentative energy production, and indicate a selection towards increased energetic efficiency of biochemical pathways leading to less toxic and readily excretable end products in anoxibiotic invertebrates. The thermodynamic model is mainly based upon a metabolic scheme elaborated on intertidal bivalves by de Zwaan et al. "(135)",

benthic oligochaetes "(101)" and fresh-water bivalves "(30)". It may provide a general hypothesis for the energetic processes which operate in a variety of ecological and taxonomic groups of anoxibiotic animals.

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