The biochemical consequences of hypoxia

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Unlike plants which can utilize the energy obtained from the sun through photosynthesis man requires to oxidize substrates in order to obtain energy. This energy is required for the many energy-utilizing or exergonic reactions which take place in the body and are necessary to sustain life (table I). Energy exists in

Syntheses
Active transport
Muscular contraction
Nerve function

Table I *Energy-utilizing (exergonic) reactions*

the body in several forms. First there is potential energy contained in macromolecules and second there is a group of compounds which have been termed high-energy phosphates which act as the energy currency of the body. The phrase 'high energy' is used because the free energy of hydrolysis of the terminal phosphate is -7000 cal/mol compared with -4000 cal/mol for ordinary ester phosphates. The major high energy phosphates are shown in table II. These include ATP and the other nucleo-

Nucleotides	Others
Adenosine triphosphate (ATP)	Creatine phosphate
Guanosine triphosphate (GTP)	1,3-diphosphoglycerate (1,3-DPG)
Inosine triphosphate (ITP)	Phosphoenolpyruvate (PEP)
Cytidine triphosphate (CTP)	
Uridine triphosphate (UTP)	

Table II *High-energy phosphates*

tide phosphates. ATP in particular is ubiquitous and is involved in exergonic reactions throughout the body. Its energy is used, for example, in muscular contraction, in nervous excitation, for active transport and in the synthesis of compounds such as fatty acids. Other high-energy phosphates with more limited roles also exist (table II). These include creatine phosphate whose role is restricted purely to muscle, and 1,3-diphosphoglycerate and phosphoenolpyruvate which are parts of the glycolytic pathway. It should be remembered that other biologically important compounds also fall into the high energy class. These include acetyl CoA, certain amino acid esters, S-adenosyl methionine and uridine diphosphate glucose.

In hypoxia it is the synthesis particularly of ATP

and creatine phosphate which is impaired. The rest of this review will be concerned with the production of ATP aerobically and anaerobically, examining first glycolysis, then the tricarboxylic acid cycle, and finally the cytochrome system. The biochemical consequences of hypoxia to the whole organism will then be discussed using two models in particular: exercise where oxygen delivery remains normal but tissue requirement greatly exceeds delivery and hypoxia in relation to the whole organ. The final section will deal with possible methods of assessing tissue hypoxia in clinical situations.

Glycolysis

The three parts of the carbohydrate oxidation system take place in three different parts of the cell. Glycolysis is a cytoplasmic process: the tricarboxylic acid cycle is localized within the matrix of the mitochondrion while the electron transport chain occupies the inner mitochondrial membrane. Glycolysis itself thus functions primarily as a preliminary step in the breakdown of glucose, although it should be remembered that it also has important functions as a means of converting an acute energy source, glucose, into a storage form of energy, triglyceride. Compared with the tricarboxylic acid cycle the energy yield of glycolysis is small but it does have one unique function. This is the ability to produce ATP anaerobically.

The major energy-utilizing and producing steps of glycolysis are shown in the figure. It can be seen that a high-energy phosphate is used up in admitting glucose to the pathway. A further ATP molecule is required to phosphorylate fructose-6-phosphate to fructose-1,6-diphosphate. Thereafter, however, energy is produced rather than used. ATP is produced at two stages: the breakdown of 1,3-diphosphoglycerate and of phosphoenolpyruvate. As two of each of these molecules is made from one glucose molecule four ATP molecules will be produced per passage of one glucose molecule down the pathway. This gives a net yield of two ATPs between glucose and pyruvate. This is equivalent to 15 000 cal/mol of glucose and in many senses is trivial in that it represents just over 2 per cent of the total potential

Fig Energy-utilizing and producing reactions of glycolysis.

energy of glucose (686 000 cal/mol). This amount of energy is, however, sufficient to sustain certain cells in the resting state when hypoxia or anoxia is present.

It can be seen that NADH is also produced during glycolysis. This represents a form of potential energy and if fully oxidized will yield six further ATPs per mole of glucose. For this to occur, the NADH must first enter the mitochondrion. Mitochondria are, however, impermeable to NADH and a rather roundabout route is followed, termed the 'malate-oxaloacetate shuttle' (equations 1-4). First of all the reducing equivalent of NADH is transferred to oxaloacetate yielding malate.

(1) IN CYTOPLASM:

Malate then traverses the mitochondrial membrane and is oxidized back to oxaloacetate restoring NADH:

(2) IN MITOCHONDRION

The oxaloacetate is then converted to aspartate.

(3) IN MITOCHONDRION

Both aspartate and 2-oxoglutarate diffuse out of the mitochondrion.

(4) IN CYTOPLASM

The net result therefore is the translocation of NADH from the cytoplasm to the mitochondrion.

If all reactions in the shuttle were at equilibrium the pyridine nucleotides would equilibrate between cytoplasm and mitochondria. This would be unfavourable in that a high cytosolic NAD is required to drive glyceraldehyde 3-phosphate dehydrogenase in the direction of glycolysis while in the mitochondria a high NADH is required to drive the electron transport chain. This state is achieved by making one of the reactions of the malate shuttle irreversible. This is either malate transport into the mitochondria or aspartate transport out (Newsholme and Start, 1973). In hypoxia the transport system will rapidly break down. NADH will accumulate within the mitochondrion. Malate and NAD will rapidly accumulate and malate will no longer get in. As a result cytosolic malate and NADH will accumulate and the reaction sequence will cease.

Obviously glycolysis would cease entirely if NADH accumulated in an uncontrolled way in the cytoplasm in that no NAD would remain for glyceraldehyde 3-phosphate dehydrogenase. If this were the case even the anaerobic formation of ATP would cease and cells would die. The NAD is in fact restored by the reduction of pyruvate with NADH yielding lactate and NAD. Thus an anaerobiosis lactate will accumulate in large amounts and will spill out of the hypoxic cells into the circulation to be re-used when oxygen is again available.

Regulation of glycolysis occurs at one main step: the conversion of fructose-6-phosphate to fructose 1,6-diphosphate. The enzyme, phosphofructokinase, is inhibited by ATP and citrate as well as by hydrogen ions. Inhibition by ATP and citrate means that when energy is plentiful and the tricarboxylic acid cycle is saturated glycolysis will cease so that substrate is not passed unnecessarily down the glycolytic pathway. The inhibition by hydrogen ion is interesting in that this will limit lactic acid production during exercise and will prevent intracellular pH dropping to unacceptably low levels. In hypoxia glycolysis is accelerated due to the stimulation of phosphofructokinase by AMP and lack of ATP (Newsholme and Start, 1973).

Tricarboxylic acid cycle

This forms the final common pathway for all oxidative energy-yielding reactions. The glycolytic end product pyruvate enters the cycle as acetyl CoA by condensing with oxaloacetate. During the passage of the acetyl CoA round the cycle two CO₂ molecules are generated so that there is no net gain of carbon compounds. There are, however, several steps at which energy is unleashed, generally in the form of NADH. These energy yielding steps are shown in equations 5-10.

- (5) Pyruvic acid + NAD^+ + CoA.SH $\longrightarrow$ acetyl CoA + $\text{NADH} + \text{H}^+$
- (6) Isocitric acid + NAD^+ $\longrightarrow$ Oxalosuccinic acid + $\text{NADH} + \text{H}^+$
- (7) 2-Oxoglutaric acid + NAD^+ + CoA.SH $\longrightarrow$ Succinyl CoA.SH
- (8) Succinyl CoA + $\text{GDP} \longrightarrow$ succinic acid + $\text{GTP} + \text{CoA.SH}$
- (9) Succinic acid + $\text{FAD} \longrightarrow$ Fumaric acid + FADH_2
- (10) Malic acid + NAD^+ $\longrightarrow$ oxaloacetic acid + $\text{NADH} + \text{H}^+$

Each NADH formed will yield three ATP molecules. There is also one ATP equivalent formed in reaction 8 in the shape of GTP and the FADH_2 produced in reaction 9 will yield two ATP molecules. There will thus be 15 ATP molecules formed from the passage of each pyruvate around the cycle. As one glucose molecule produces two pyruvates there will be 30 ATPs formed, making a total of 38 for the oxidative breakdown of one glucose molecule. This is equivalent to 42 per cent of the total potential energy of glucose, the rest being released in the form of heat which is obviously a useful form of energy in mammals.

Fatty acids enter the cycle through acetyl CoA. They are first broken down by beta-oxidation, each step yielding an acetyl CoA. In the formation of each acetyl CoA one NADH and one reduced flavoprotein molecule is formed so that five ATP equivalents are produced per acetyl CoA. A further 12 ATPs will be formed by passage of the acetyl CoA around the cycle. Allowing for the initial activation, a typical fatty acid such as palmitate will yield 980 000 calories which amounts to 41 per cent of the potential energy of the molecule. In the absence of oxygen no energy will be released from fatty acids. Thus when oxygen is available there is a vastly greater energy yield than under anaerobic conditions.

The electron transport chain

If NADH is to yield any ATP at all, then the reducing equivalents must be passed down the electron transport chain, finally yielding water. The components of the chain are now fairly well established and comprise flavo-protein, coenzyme Q and the cytochrome system. For electrons to flow down the chain the components must be arranged in order of increasing redox potential with the terminal cytochrome a_3 responsible for the combination of the reducing equivalents with molecular oxygen. The components and their order are shown in equation 11.

It is known that three ATP molecules are formed from the free energy released during passage of the electron. It has been calculated that there must be a free energy change of approximately 9000 calories between two components of the chain if an ATP molecule is to be formed at that point. Four sites fulfil these requirements and in fact three are used as shown in equation 11. The coupling of ATP formation to the respiratory chain is known as oxidative phosphorylation. It can be seen that the passage of reducing equivalents from NADH will result in the formation of three ATPs while electron transport from a reduced flavoprotein will result in the formation of only two ATPs.

The actual mechanism whereby ATP formation is coupled to the electron transport chain remains controversial. For the purposes of this review it is sufficient to say that there are two main hypotheses, a chemical hypothesis postulating direct chemical coupling (Ernster, Lee, and Janda, 1967) and the chemi-osmotic hypothesis which invokes the transport of hydrogen ions across the mitochondrial membrane (Mitchell, 1972).

The localization of the electron transport chain has attracted much attention. It is suggested that repeating units exist on the inner mitochondrial membrane. These comprise a base piece which is said to contain the electron transport chain, a stalk, and a headpiece containing the ATP synthetic system (MacLennan, 1970).

The regulation of the electron transport chain is obviously of interest when considering hypoxia. One major point is that cytochrome oxidase has an extremely high affinity for oxygen which allows the respiratory chain to function even when the mitochondrion is virtually anoxic. This is obviously crucial in protecting tissues against low oxygen tensions. The activity of the cytochrome system is regulated by the availability of ADP, of substrate and of oxygen. Obviously when oxygen is totally lacking the system cannot operate. The closest regulation will come from ADP concentration so that when energy utilization is high or hypoxia is present and ATP concentrations are low with a raised ADP

formation, then the activity of the chain will be stimulated. This will tend, therefore, to restore ATP formation. Build up of NADH will also tend to drive the chain. This could be relevant following a period of hypoxia or exercise when NADH concentrations will be raised and ATP concentrations low. Further information on biochemical mechanisms can be found in papers by Newsholme and Start, and by Harper, 1973; 1975).

Muscle and hypoxia

It is relevant to consider muscle first in relation to hypoxia in that this tissue is most often exposed to low oxygen tensions in normal man. This occurs during exercise. Muscle is in fact one of the most resistant tissues to hypoxia. In experiments in our own laboratory we showed that total occlusion of blood supply to an arm for five minutes gave no biochemical evidence of hypoxia (Braybrooke *et al*, 1976). Only if the arm was exercised at the same time was the effect of hypoxia apparent. This resistance to hypoxia is well known to surgeons who use tourniquets for lengthy periods during amputations of limbs.

Muscle has indeed several mechanisms whereby it is protected against mild hypoxia. First, oxygen is stored attached to myoglobin, particularly in muscles designed for sustained contraction such as the postural muscles. The oxygen dissociation curve for myoglobin is a rectangular hyperbola which is placed to the left of that for haemoglobin. Myoglobin can take up oxygen from haemoglobin and will release it only when oxygen tensions within the muscle cells become very low. It therefore serves as an emergency reserve. In terms of whole body oxygen utilization myoglobin plays only a minor role, but it is said to be important for cardiac muscle.

Muscle also contains important energy reserves in the form of glycogen and creatine phosphate. Considering first creatine phosphate, this is formed from ATP and creatine when ATP supplies are plentiful.

The relative reserves of ATP, creatine phosphate and glycogen have been calculated by Hultman and Bergström (1973). They have shown that there are 10 μmol of high-energy phosphate per gram dry weight of muscle as ATP, 61 μmol as creatine phosphate and 1060 μmol as glycogen for anaerobic metabolism, and 14 200 μmol high-energy phosphate per gram dry weight from glycogen for aerobic metabolism. It is obvious that the amount of creatine phosphate is relatively small, but this is

nonetheless extremely important at the beginning of exercise and for short periods of severe exercise. In 90 per cent maximal exercise the creatine phosphate has virtually all disappeared at between two and five minutes (Karlsson, 1971; Hultman and Bergström, 1973). At 50 per cent maximal exercise there is an initial drop in creatine phosphate to about half of resting values but then the concentration remains constant, suggesting that sufficient ATP is being produced to re-phosphorylate creatine.

Most of the body's glycogen is to be found in muscle. This represents a total energy reserve of some 1000 kilocalories. It should be remembered that this glycogen can only be used for muscles and their requirements in that glucose-6-phosphatase is lacking in muscle so that glucose cannot be released into the circulation. If exercise is totally anaerobic then this glycogen reserve would last for approximately 30 minutes.

The role of fat in providing fuel for muscle should not be underestimated (Felig and Wahren, 1975). At rest fatty acids provide 90 per cent of energy requirements for muscle. About three quarters of this comes from endogenous triglyceride and the rest from circulating free fatty acids. During exercise there is a triphasic sequence of fuel utilization. Initially there is a rapid phase of glycogenolysis in which muscle glycogen is used. In the second phase of exercise blood glucose provides substrate, this coming from liver glycogen. In the third phase free fatty acids provide most of the fuel. However, if exercise is severe then the muscle will become anaerobic, fatty acids cannot be used and muscle becomes entirely dependent on anaerobic metabolism. The amount of exercise will then be determined by the ability of muscle to go on producing sufficient ATP and many authorities have shown that this depends on the starting concentration of glycogen. Experiments in which muscle glycogen has been increased by feeding regimens before exercise have shown that this increases exercise tolerance (Rennie and Johnson, 1974; Bergström *et al*, 1967).

During severe exercise muscle lactate concentrations rise to very high levels, amounting to as much as 25 mmol/kg wet weight. Concentrations within muscle may be several fold higher than those in the circulation where maximum levels achieved are in the order of 20 mmol/l, but more usually nearer 10 mmol/l. Pyruvate concentrations do not rise proportionately and may increase by as little as twofold in muscle tissue. This means that the [lactate]/[pyruvate] ratio becomes very high (see below).

The rate at which muscle becomes hypoxic will depend not only on the rate of oxidative metabolism within the tissue but also on the supply of oxygen

in the circulation. The circulation is fully adapted to increasing blood flow to exercising muscle (Mitchell and Blomqvist, 1971). Thus cardiac output increases from 6 to 24 litres/min with blood flow to active muscle increasing from 0.65 litres to 20.9 litres/min. At the same time pulmonary ventilation increases twelve-fold from 10 litres/min to 120 litres/min with oxygen uptake going up from 0.3 to 3.8 litres/min. Extraction in the tissues is also greatly increased so that the arteriovenous oxygen difference rises from 5.6 to 15.8 ml/100 ml blood. This serves not only to increase oxygen and substrate supply to muscle, but also to remove lactic acid and to expire increased amounts of CO₂ thus helping to preserve acid-base balance. It is obvious that any disease which interferes with any of these processes will render muscle hypoxic more rapidly. Thus severe bronchopulmonary disease or myocardial disease will be associated with a greatly reduced exercise potential. Similarly anaemia will cause a decrease in exercise capacity. This is true as well for any disease associated with diminished tissue glycogen stores.

One special aspect of limited oxygen supply is that of high altitude. This could be re-defined as a state of chronic hypoxia. It is known that persons living all the time at high altitude have a decreased ability to produce lactic acid during exercise and a decreased exercise capacity. It is thought that this decreased ability to accumulate lactic acid is due to a diminished alkali reserve, which implies less buffering capacity so that pH will fall more quickly per mole of lactic acid produced. This will then inhibit glycolysis and ATP production will be diminished. It is probably not due to hypoxia *per se* in that acute replacement of oxygen has little effect on this process (Cerretelli, 1967). In contrast, experiments with acute hypoxia have shown no impairment in the production of lactic acid.

The regulation of muscle metabolism in hypoxia may be summarized as follows. Initially creatine phosphate and ATP concentrations will fall. AMP concentrations will rise. This will result in acceleration of glycolysis from glycogen through stimulation of phosphofructokinase. Lactic acid will accumulate in order to regenerate NAD. The fall in intracellular pH will have two effects: first, phosphofructokinase will tend to be inhibited but also muscle permeability to glucose will be enhanced (Randle and Smith, 1958). Hypoxia will also have a more distant effect in that catecholamine secretion will be stimulated. This will directly increase glycogen breakdown in muscle thus further enhancing glycolysis (Cain, 1969).

(The reader is referred to Pernow and Saltin, 1971, for further discussion of muscle metabolism during exercise.)

Other tissues and hypoxia

The ability of tissues to resist hypoxia appears to depend on their glycogen content. Brain cells contain little glycogen while their supporting cells, the astrocytes, contain only sufficient for some 15 seconds' anaerobic ATP production. Brain cells are notoriously sensitive to hypoxia with irreversible changes occurring within minutes. In contrast a tissue such as liver may withstand anaerobiosis for lengthy periods. Woods and Krebs (1971) have shown this elegantly in experiments with livers perfused under anaerobic conditions. They showed that livers from well fed rats perfused anaerobically could function normally for two to three hours with stoichiometric conversion of glycogen to lactate. They were also able to use glucose from the perfusion medium. In contrast livers from starved rats were much more sensitive showing cellular oedema and decrease in bile flow in an hour or so. These livers were unable to use added glucose, presumably because glucokinase activity was decreased due to the starvation. In less severe hypoxia, liver probably continues to obtain most of its energy supply from fatty acid degradation, thus allowing the various essential synthetic processes to continue.

Clinical assessment of tissue hypoxia

It is easy to discuss theoretical or experimental aspects of hypoxia. It is more difficult to convert this into clinically useful diagnostic methods. An obvious method for assessing hypoxia has been to measure blood lactic acid concentration. This can be done easily and rapidly so could be available in most centres. Certainly in severe exercise or severe hypoxia, the increase in lactic acid concentration in the blood is proportional to the degree of hypoxia. Thus, lactic acidosis is a known complication of shock, low cardiac output states, septicaemia and poor tissue perfusion (Cohen and Simpson, 1975). In most of these situations, however, the diagnosis of what is known as type A lactic acidosis is obvious and chemical confirmation does little to help the patient. In milder degrees of hypoxia, measurement of lactate alone is of questionable value. There are many reasons for an elevation in blood lactate other than hypoxia. These include liver disease, therapy with drugs such as biguanides, ingestion of alcohol or intravenous feeding with substances such as fructose. Lactate also rises in physiological circumstances (Huckabee, 1958) such as in eating meals where the resultant insulin secretion acutely inhibits gluconeogenesis and the liver and lactate uptake by that organ is temporarily prevented. Thus a small rise in blood lactate concentration is not specific

(Krebs *et al.*, 1975).

One possible method for measuring whole body hypoxia of a temporary nature would be to measure the so-called oxygen debt. It is well known that after exercise, oxygen consumption remains elevated for a considerable period. This is purported to be the oxygen necessary for metabolism of the lactic acid which has accumulated anaerobically during the hypoxic phase, of, for example, exercise. Whether this is the correct explanation remains controversial. Its use clinically would only be of limited value in that it would be helpful only for temporary periods of hypoxia.

The most commonly used assessment method is that based on equations 13 and 14

$$(14) \frac{[\text{Lactate}]}{[\text{Pyruvate}]} = \frac{[\text{NADH}]}{[\text{NAD}]} \cdot \frac{1}{K} \cdot [\text{H}^+]$$

Thus by re-arranging the lactate dehydrogenase reactants into the mass action equation one can see that the ratio of lactate to pyruvate is proportional to the ratio NADH to NAD. A rise in the NADH:NAD ratio should therefore be reflected by a rise in the lactate:pyruvate ratio. It should be remembered immediately that this refers to events in the cell cytoplasm and is a reflection only of the cytoplasmic redox state. Nonetheless measurement of lactate:pyruvate ratios in blood has been used to assess hypoxia. The various assumptions inherent in this measurement must be remembered. These include the assumption that lactate and pyruvate are freely diffusible; that lactate and pyruvate are produced and used at the same rate; that the lactate:pyruvate ratio is the same in all tissues; that the cytoplasmic redox ratio will reflect whole cell redox state; and that the reaction has reached equilibrium. Exceptions to each of these assumptions have been recorded. In the absence of a better index, however, it is still possible to use this ratio as a crude guide to hypoxia. Two further points must be remembered. The first is that even if the L:P ratio is raised one has no indication which tissue is hypoxic. The second is that the ratio is influenced by pH. Examination of equation 14 shows that the hydrogen ion is involved and that a doubling of hydrogen ion concentration, which is equivalent to a fall in pH from 7.4 to 7.1, could result in a doubling of the lactate:pyruvate ratio without any change in redox state being implied. In most hypoxic states, some degree of acidemia is present so that interpretation of the L:P ratio must always be done in conjunction with pH measurements. The normal L:P ratio in blood is about 10 and this can rise to approximately 40 in a severe acidemia.

Values above this level probably do reflect some intracellular oxygen deficit.

The 3-hydroxybutyrate:acetoacetate ratio has been used in a similar way to give an indication of the mitochondrial redox state. It can be useful when measured together with the L:P ratio but all the assumptions made for the latter must also be applied to this ratio.

One can only suggest that newer and better methods for assessing tissue hypoxia are still required.

Summary

The various phases of energy production have been described. These include glycolysis which is unique in its ability to produce ATP anaerobically, the tricarboxylic acid cycle with its major contribution to ATP production coming through the generation of NADH, and the cytochrome system at which reducing equivalents are converted to water, the released energy being incorporated into high-energy phosphates. The regulation of these pathways has been briefly described and the importance of the small amount of ATP generated anaerobically emphasized. The adaptation of muscle to periods of hypoxia through the presence of myoglobin, creatine phosphate and large amounts of glycogen is then discussed. The role of pH in limiting anaerobic glycolysis in muscle and the importance of the circulation in providing oxygen for exercising muscle are outlined. The effects of hypoxia on certain other tissues such as liver and brain have been detailed and finally methods for assessment of tissue hypoxia in man such as the measurement of the lactate:pyruvate ratio in blood are presented.

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