POSITIVE FEEDBACK IN THE LIVING PROCESS: THE ROLE OF ATP IN ISCHAEMIC CELL DEATH

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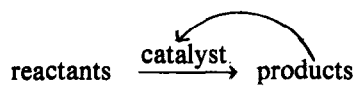
SUMMARY

Some essential functions of the organism—the continual replacement of its own component parts, the ability to make or to procure the things that it needs—are seen to be positive feedback effects. Because of its foundation of positive feedback, the process has an underlying tendency to become unstable. The borderline between life and death may be understood in terms of the opposition between destabilising (positive feedback) and stabilising (control) effects. A dynamic hypothesis of cell death in ischaemia is suggested, in which the critical change need involve no damage to enzymic machinery. It may be possible to revitalise dead cells by relatively simple measures.

POSITIVE FEEDBACK

Positive feedback is much more important in the organism than is generally supposed.

A chemical reaction may be said to show positive feedback if a product tends to increase the reaction rate—for example, by acting as a catalyst:



Now the cell, considered as a whole, is a chemical system which continuously generates its own catalytic conditions, in the form of its enzymes, coenzymes, cofactors etc. Accordingly, it can be demonstrated that the metabolic system as a whole has the layout of a network of positive feedback loops.

A simple example is shown in Fig. 1. Glucose is metabolised to form ATP, and ATP is used in the synthesis of enzymes, including those which catalyse ATP production.

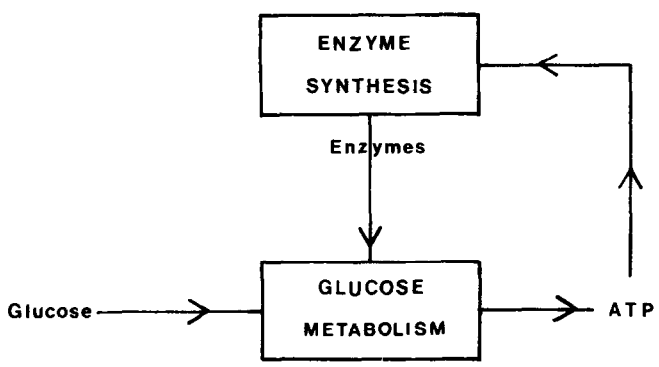


Fig. 1. The metabolism of glucose gives rise to ATP, which helps to produce the enzymes that catalyse glucose metabolism. This is a positive feedback loop.

The scheme shown in Fig. 2 is more elaborate, though by no means exhaustive. The enzymes, coenzymes and cofactors which produce ATP themselves require ATP for their own synthesis and accumulation. More directly, ATP is required for the initial phosphorylation of glucose, before further progress to ATP production occurs. Again, the enzymes which produce the pentose sugars, and convert them to nucleic acids, themselves require nucleic acids for their synthesis.

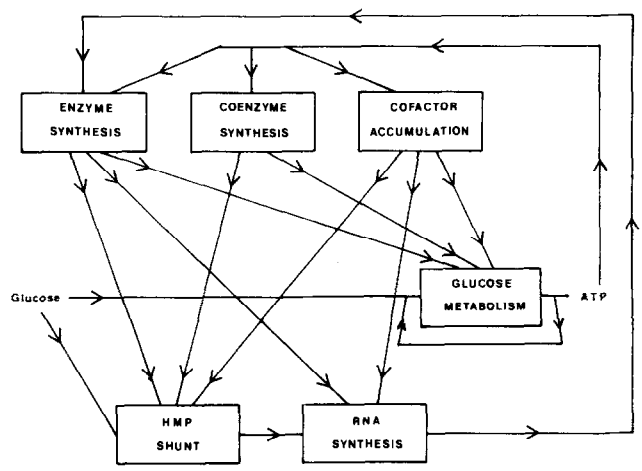


Fig. 2. The action of the system is to produce its own catalytic requirements, which, in turn, promote the action of the system. Each complete loop in the diagram is a positive feedback loop. (Control mechanisms are not shown.)

In addition, various processes combine to make structural materials; structure permits spatial organisation, which has a favourable effect on many reactions by holding active elements in proximity.

Just as the cell's internal needs are met by its own internal action, so its external needs are met with the aid of its external action. Each cell contributes, in its own way, to the various bodily functions; these form a network of loops which deliver to the cells all that they require. This again expresses a circular relationship, illustrated in Fig. 3. The point of interest is that each stage in the diagram tends to permit or promote the next, and so on around each of the loops. Positive feedback is therefore an inherent part of their character.

These loops should not be ignored merely because their normal function is unobtrusive—for example, moderate changes in nutrient supply do not much alter organ performance. Many positive feedback effects are "saturated"

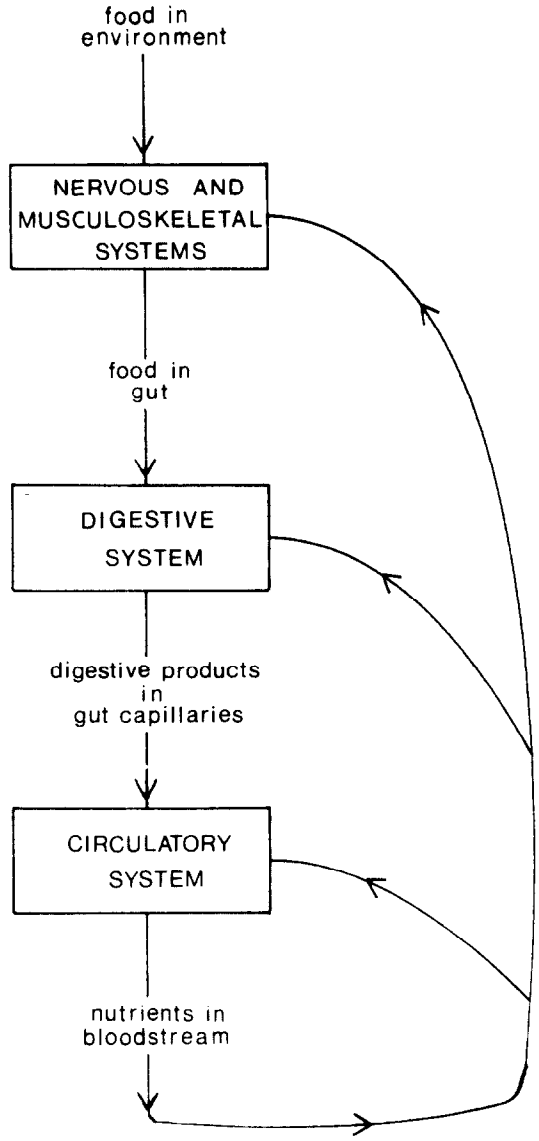


Fig. 3. A small part of the physiological network of positive feedback loops. Food in the environment is transformed, by various physiological systems, into nutrients in the bloodstream; yet the presence of the latter is required for each stage in the transformation.

and roughly constant in the physiological range, and for this reason they largely escape comment. Nevertheless, their status as important variables becomes clear in certain abnormal situations, e.g. in circulatory shock (1).

All this is not to deny the importance of control effects, such as negative feedback, as described in the literature (2). But it should be more clearly recognised that these controls act upon processes which themselves form a network of positive feedback effects. After all, control mechanisms are only one aspect of a system, and whatever is controlled must in the first place be generated. The living system is remarkable in that it continuously generates itself, an attribute which implies positive feedback.

INSTABILITY

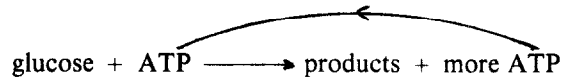
How does the framework of positive feedback affect the organism's behaviour?

It is well known that a system containing positive feedback may be unstable. For example, if A causes an increase in B, while B in turn causes an increase in A, then an initial increase in A may set off a vicious circle in which both A and B increase explosively. Conversely, an initial decrease in A may lead to a rapid collapse in both A and B.

But no real system, perhaps, consists solely of positive feedback. There will also be effects of one sort or another which tend to oppose change, and therefore favour stability. A more complex dynamic profile then results, which may be illustrated by an example taken from metabolism.

Glycolysis

The requirement of ATP as substrate in glycolysis constitutes a positive feedback effect, as may be seen if the glycolytic process is considered as a whole:



Although there are strong control mechanisms (negative feedback effects) within the system, it seems reasonable to ask whether the positive feedback loop has any important destabilising effect on its behaviour.

At a theoretical level, it is possible to approach this problem by drawing the steady-state curves representing ATP production (by glycolysis) and ATP utilisation (by an ATPase, for example), which together constitute an ATP turnover system. The intersections of the curves give the possible steady states of the system, and the properties of each state may then be considered in turn.

For glycolysis, the features which determine the general shape of the curve may be stated. Since no reaction will occur in the absence of ATP, the curve begins at the origin. When adequate ATP is present, glycolysis achieves a high rate of ATP production; and when [ATP] is excessively high, the rate is curtailed by control mechanisms (3). A further feature is the theoretical expectation that the curve should have horizontal slope at the origin, for reasons given in Appendix I.

In contrast, the curve representing ATP utilisation (by an irreversible process requiring a single molecule of ATP as substrate) will approach the origin with non-horizontal slope.

Fig. 4 shows the relative disposition of such curves and suggests that turnover will be inherently unstable. The

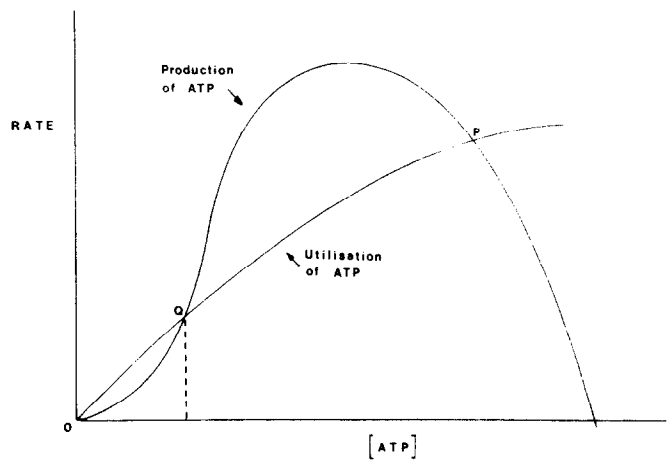


Fig. 4. Theoretical curves. Owing to its complex shape, the ATP production curve has 3 intersections with the ATP utilisation curve, at O, P and Q. This implies unstable behaviour, as discussed in the text.

curves show not one, but three possible steady states, at O, P and Q. For [ATP] higher than at P, the rate of ATP utilisation exceeds that of its production, so that [ATP] will fall to P. For [ATP] between Q and P, production exceeds utilisation, and so [ATP] will rise to P. But for [ATP] between O and Q, utilisation will exceed production, and [ATP] will fall to zero.

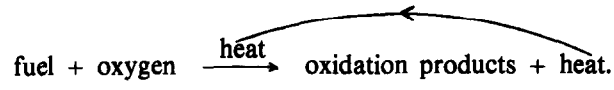
Thus P represents an active state which the system maintains in a stable manner, provided that [ATP] is greater than at Q. Q represents a critical condition below which the system shows no tendency to return to the active state, but instead will exhaust its ATP and stop working.

Appendix II describes evidence that this type of instability can indeed be demonstrated experimentally in a reconstituted glycolytic system.

Positive feedback implies a departure from ideal control. As the system contains both positive and negative feedback loops, it is not surprising that it should show some compromise between a tendency to remain stable and a tendency to become unstable: the outcome, in this case, is that stability is confined to a limited range only. Instability is likely to be a fundamental problem in any system that needs its own product in order to work.

Comparison with Everyday Systems

This sort of behaviour is by no means unfamiliar in everyday life. Consider, for example, the burning of fuel, as in a domestic fireplace. The chemical process of oxidation is subject to a strong positive feedback effect, as follows. The reaction produces a large amount of heat, some of which is retained at the reaction site, where it raises the temperature of the reactants; and higher temperature makes the reaction go faster. Thus heat can be regarded as both a product and a catalyst of the reaction:



When the rate curves for heat production and heat dissipation are plotted against temperature, it turns out that their disposition resembles that of Fig. 4. (A formal treatment for tank reactors may be found in Ref. 4.) Consequently, there is a critical temperature for ignition and quenching of the reaction. That is why coal does not burn spontaneously, but must first be ignited by having its temperature raised above a certain level; and why a momentary gust of wind may put a fire out by lowering the temperature sharply.

Or consider the case of the motor-car engine, in which positive feedback is again important. Motion in the engine causes it to take in fuel, which, on combustion, drives the engine faster. (The motion does not build up excessively, because certain limiting effects come into play at higher speeds.) The curve for engine power production accordingly has a "hump" shape, and has more than one intersection with a curve describing load (5). Curves of this kind explain why the engine does not start spontaneously, but must be cranked up to a critical rate of motion before it will run with its own impetus; and also why the engine will stall if the speed falls below a certain value.

These three systems have in common the feature of positive feedback, which is the basis at once of their self-sustaining quality and of their inherent instability. They differ from the living process in a matter of degree. The simpler systems meet only one of their operative needs (heat, motion, ATP) by means of positive feedback, while other needs (coal, petrol, glucose) are supplied by external agents; whereas in the living process, all of its many needs are met through the intervention of positive feedback. The organism is thereby rendered independent, or autonomous—at the price, however, of its imperfect stability.

DEATH

When blood supply is interrupted, cells may lose their ability to recover, so that they will die even if nutrient is subsequently restored. However, no serious structural damage can be identified until some time after death, and the immediate cause of their "irreversible" loss of function is obscure (6).

A Dynamic Approach

The cell's content of positive feedback suggests a dynamic approach to this problem. This would seem difficult in view of the complexity of Fig. 2, but there are two considerations which appear to simplify matters.

Firstly, our main concern is to identify the earliest change which determines "irreversibility", and not to describe the whole process of subsequent breakdown. Early destabilisation is likely to be mediated by fast-acting positive feedback loops rather than by slow ones, so we can confine our attention to those loops in which every step consists either of intermediary metabolism or transport. Biosynthetic processes are generally much slower, and loops containing these will not be considered.

Examples of fast-acting positive feedback loops are the feedback of ATP as substrate in its own production, as in glycolysis; the active (ATP-requiring) accumulation of ions, such as Mg^{2+}, which in turn are required for the production of ATP; and the active removal of ions, such as Ca^{2+}, which would inhibit ATP production.

A second simplification is merely to note that ATP figures in most positive feedback loops.

Hypothesis

These points suggest the hypothesis that the onset of ischaemic cell death is determined by a critical change of [ATP], causing destabilisation by fast positive feedback. The emphasis here is on the dynamic role of the cell's labile constituents, and not on any damage to the enzymic machinery.

Existing Evidence

How does this hypothesis stand in relation to existing experimental evidence? When cells are deprived of substrate, ATP content falls after a time and may reach drastically low levels, as was observed in *Escherichia coli* (7), dog liver (8), rat heart (9) and mouse brain (10). In the first three of the above studies, the degree of recovery after restoration of substrate was also investigated, and the findings (though expressed in a variety of ways) indicate that substantial or complete loss of viability occured when ATP content had fallen to about 0.5mM or less. In this

range it is plausible that glycolysis is limited by scarcity of ATP as substrate, because reported Km values for ATP of the enzymes hexokinase (11), glucokinase (12) and phosphofructokinase (13) are of similar magnitude (0.13, 0.5 and 0.1 mM respectively). The condition of the cells in these experiments would then correspond to the rising limb of the production curve of Fig. 4, the region in which a critical intersection could occur.

Large ionic shifts have also been observed in ischaemic brain (14) and in ischaemic heart (15). A mechanism of myocardial cell death has been suggested which involves Ca^{2+} shift (16). Severe ischaemia leads to net influx of Ca^{2+}, possibly owing to depletion of the ATP required for active Ca^{2+} removal. Increased intracellular $[Ca^{2+}]$ causes uncoupling of oxidative phosphorylation (17), thereby leading to further depletion of ATP. This amounts to a positive feedback effect, and might well be associated with critical values of [ATP] and $[Ca^{2+}]$, thus explaining the irreversible loss of oxidative phosphorylation—though it would not, in itself, explain why adequate ATP fails to be regenerated via glycolysis.

Thus ischaemic death is indeed associated with important changes in intracellular ATP and ionic content. It remains to decide whether any of these constituents has a critical role.

Further Testing

The best test of such a hypothesis would be its use in an attempt to resuscitate dead cells. Using cells which have been starved until viability is lost, a systematic attempt should be made to correct the intracellular content of ions, intermediary metabolites, and, perhaps most importantly, ATP. Intracellular [ATP] could be raised, either directly by micro-injection or by providing ATP in the extracellular environment, or indirectly by making use of certain metabolic pathways which can yield ATP without requiring any pre-existing ATP.

This has already been achieved in the case of the human red blood cell (18). Red cells become severely depleted of ATP after cold storage for several weeks, and their ability to survive in a patient's circulation after transfusion is then impaired. When such ATP-depleted red cells are briefly incubated with nucleosides before transfusion, their ATP content is restored, and their post-transfusion survival is improved.

A similar approach should be pursued with other cell types, not excluding those of the heart or the brain. There seems to be a belief that such relatively simple measures would be of no avail unless some more fundamental derangement could be identified and treated. But if the labile constituents are seen as elements in a positive feedback network, it is plausible that their disturbance may be adequate to cause destabilisation, and that their correction may restore vitality after ischaemic death.

APPENDIX I — THEORETICAL

Because two molecules of ATP are required as substrate in glycolysis, the rate curve has zero gradient at the origin, as indicated below.

As a first model of the glycolytic pathway, consider a process having the overall stoicheiometric form

$$G + 2ATP \rightleftharpoons 2L + 4ATP$$

where G and L represent glucose, and lactic acid respectively. The system is taken to be in the steady state and [G] and [L] are assumed to be constant.

As [ATP] approaches zero, the mass action ratio $[L]^2 [ATP]^4 / [G] [ATP]^2$ approaches zero. The mass action ratio will therefore have a smaller value than the equilibrium constant. It follows that the process will then proceed from left to right

$$\text{i.e. } R > 0 \text{ for } [ATP] \to 0. \qquad (1)$$

where R is the rate in the steady state and the forward direction is positive.

Choose an intermediate I so that the process can be divided into two segments having the stoicheiometric form

$$G + 2ATP \rightleftharpoons I \qquad (a)$$

$$I \rightleftharpoons 2L + 4ATP \qquad (b)$$

These reactions proceed from left to right, since the overall reaction proceeds from left to right. Hence the mass action ratios will be smaller than the corresponding equilibrium constants. If K_a is the equilibrium constant for (a),

$$[I] < K_a[G][ATP]^2 \qquad (2)$$

Consider the first kinetic step in process (b):

$$I \overset{k}{\rightleftharpoons} \ldots \qquad (c)$$

where k is the rate constant for the forward step and Δ is the rate of the reverse reaction in (c).

$$\therefore R = k\,[I] - \Delta$$

$$\therefore R < k\,[I]\,.$$

Combining with (2),

$$R < k\,K_a[G][ATP]^2$$

$$\therefore R < K[ATP]^2 \qquad \text{where K is constant;}$$

and combining with (1),

$$0 < R < K[ATP]^2 \qquad \text{for } [ATP] \to 0.$$

Fig. 5 illustrates the constraints thus placed upon the curve representing R as a function of [ATP]. The curve

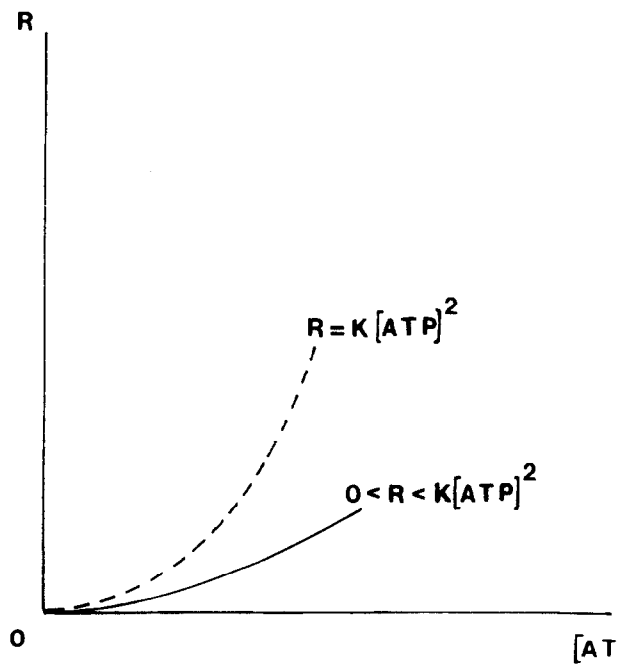


Fig. 5. The ATP production curve, for which $0 < R < K[ATP]^2$, has zero gradient at the origin. The broken line shows $R = K[ATP]^2$.

lies above the horizontal axis, but below the parabola, shown as a broken line, at which $R = K[ATP]^2$. Both of these limiting lines have zero gradient at the origin: therefore the curve representing R as a function of [ATP] has zero gradient at the origin.

This conclusion is independent of most of the kinetic details of the glycolytic pathway, because the mass action ratio, in the same way as the thermodynamic free energy change, provides a general criterion of the occurrence of chemical change, without reference to its kinetics. It is therefore not necessary to discuss any control mechanisms explicitly.

Similar considerations will apply to a model in which ADP and inorganic phosphate enter into the reaction; in which the adenylate kinase reaction (ATP + AMP $\rightleftharpoons$ 2ADP) takes place; in which glycolysis is linked to oxidative phosphorylation via pyruvate oxidation; and in which glucose and lactate concentrations are not necessarily constant, but may be subject to active or passive transport processes.

APPENDIX II — EXPERIMENTAL

Some experiments were carried out to demonstrate the phenomenon of dynamic instability in an ATP turnover system. Because it is not easy to manipulate ATP content in intact cells, these observations were made with a reconstituted enzyme system in vitro, the individual enzymes having been purchased in purified form (Sigma Chemical Co., London).

Ability to Consume Glucose

The system comprised ATP production via glycolysis (glucose to lactic acid) and an ATP-utilising reaction.

The enzymes of the glycolytic pathway were used in proportions roughly comparable to those found in various mammalian tissues (2). Nevertheless, it is emphasised that the composition and conditions of the system were largely arbitrary, having been chosen merely to provide a working model to test the expectation of instability. Precise simulation of any particular cell was not intended.

In order to avoid certain problems arising from the insoluble nature of commercially-available ATPase, ATP utilisation was effected by means of glucose-6-phosphate dehydrogenase, which "wastes" the ATP molecule used in forming glucose-6-phosphate. The system also contained glutathione reductase as a means of regenerating $NADP^+$. As this mechanism of ATP utilisation is unimolecular with respect to ATP, its behaviour should be essentially similar to that considered for ATPase.

The activity of the system was started at time zero by adding various amounts of ATP, and its ability to metabolise glucose was observed. Fig. 6 shows that with large amounts of ATP (0.3 mM or more), the system consumed glucose freely, but with smaller amounts of ATP (0.15 mM or less), glucose consumption failed to proceed.

The poor activity of the samples with low initial [ATP] cannot be attributed to a defect in setting up the glycolytic apparatus. Except for [ATP], their initial composition was identical with that of the active samples. Nor does it appear that any serious deterioration occurred later. At the end of

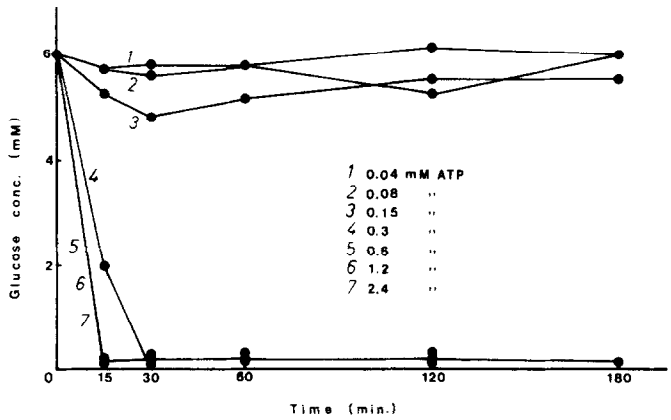


Fig. 6. Metabolism appears to fail at low [ATP]. An enzyme mixture was freed of excess $(NH_4)_2SO_4$ by dialysis; phosphofructokinase, however, was used without dialysis. Coenzymes and cofactors were added to buffered saline and pH adjusted to 8.0 before adding to the enzyme mixture. The composition of the final reaction medium, calculated on the basis of the maker's product specifications, was as follows. Enzyme concentrations were (μkatal/ml): hexokinase (yeast) 0.03, glucose-phosphate isomerase (y.) 0.17, 6-phosphofructokinase (rabbit muscle) 0.08, fructose-bisphosphate aldolase (r.m.) 0.10, phosphotriose isomerase (r.m.) 0.97, glyceraldehyde-phosphate dehydrogenase (y.) 0.67, phosphoglycerate kinase (y.) 0.37, phosphoglyceromutase (r.m.) 0.17, enolase (y.) 0.20, pyruvate kinase (r.m.) 0.53, lactate dehydrogenase (beef heart) 0.37, adenylate kinase (r.m.) 0.33, glucose-6-phosphate dehydrogenase (y.) 0.008, gluthathione reductase (y.) 0.008. Other factors were (mM): NAD^+ 1.2, $NADP^+$ 0.9, 2-3 diphosphoglycerate 3, AMP 2.2, oxidised glutathione 14, glucose 6. Ionic constituents were (mM): Na^+ 110, K^+ 8, Mg^{2+} 6, NH_4^+ 22, tris 10, Cl^- 120, inorganic phosphate 9, SO_4^{2-} 11. Temperature was 37°C and pH was 8.0. The mixture was divided into 7 portions and the indicated amounts of ATP were added in a small volume to start the reaction. Glucose was estimated using a glucose oxidase/peroxidase method (Sigma kit 510).

the 3-hour period, the inactive samples were further tested by adding 2.4 mM ATP—half an hour later, glucose had entirely disappeared from these, indicating that the system was still functionally competent, and needed only an adequate amount of ATP to trigger it off.

This perhaps leaves the possibility that enzyme function might have been held back by deficiency of the adenine nucleotide pool rather than of ATP itself, a situation which might arise if AMP were degraded (by contaminants) during the experimental period. A further experiment rules this out. The enzyme system was reconstituted with the omission of glucose, and the AMP content, as measured by a NADH-linked method (Boehringer) did not change significantly over 3 hours.

If the system's failure at low [ATP] is not due to defective constitution, it presumably has a kinetic basis. But the kinetics of glycolysis do not alone explain this effect. When the system of Fig. 6 was reconstituted with the omission of glucose-6-phosphate dehydrogenase, so that the glycolytic pathway was acting unopposed, "failure" did not occur: glucose was entirely consumed within 1 hour, for all starting ATP levels ranging from 0.08 mM to 2.4 mM (though for initial [ATP] 0.04 mM, glucose disappearance took more than 1 hour, but was completed within 3 hours). Thus the explanation of this phenomenon lies in the balance between the two pathways, and not in either alone.

Ability to Regenerate ATP

In a similar enzyme system, the quantity [ATP] + $\frac{1}{2}$ [ADP] was measured at intervals of time (Fig. 7). With high starting [ATP], this quantity reached high values,

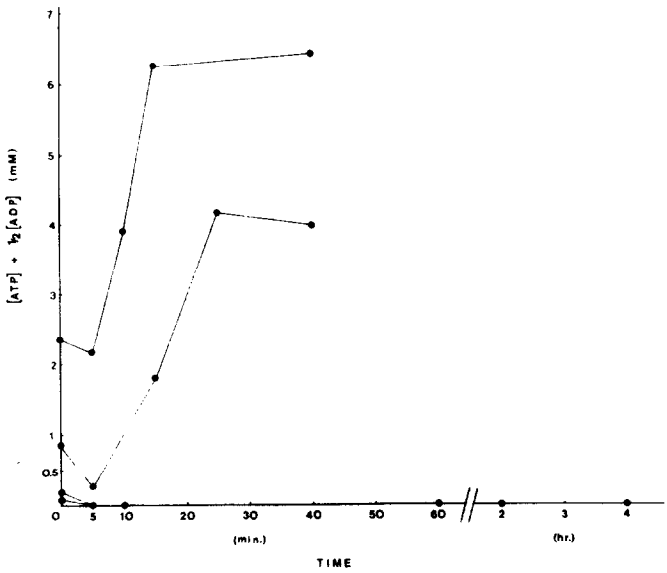


Fig. 7. Regeneration of high-energy phosphate appears unsuccessful when initial [ATP] is low. The composition of the system was similar to that of fig. 6, but with glucose-6-phosphate dehydrogenase 0.02 μkatal/ml. The reaction was started by adding an initial amount of ATP, and samples were taken at intervals of time for immediate assay in a system containing hexokinase, glucose-6-phosphate dehydrogenase, $NADP^+$, adenylate kinase and glucose. The spectrophotometric absorbance change at 340nm was recorded, and the results expressed as [ATP] [ADP].

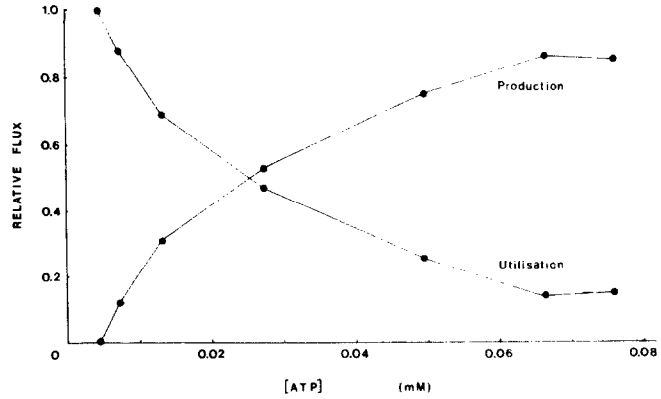


Fig. 8. For various initial amounts of ATP, the graph shows the proportion of ATP which passed down the "production" pathway, and the proportion of ATP which passed down the "utilisation" pathway, and shows a crossover in their relative dominance. The medium contained NaCl 135mM, triethanolamine Cl buffer 30mM at pH 8.1, Mg Cl_2 1mM, hexokinase 0.006 μkatal/ml, glucose-6-phosphate dehydrogenase 0.0012 μkatal/ml, and $NADP^+$ 0.16mM. An initial amount of ATP was added, and the mixture divided into two 4-ml aliquots. In one of these, reaction was started by adding glucose 4mM and the spectrophotometric absorbance change at 340 nm gave a measure of initial ATP content. The other aliquot was used as the test system. After addition of phosphoglucose isomerase 0.07 μkatal/ml and phosphofructokinase 0.035 μkatal/ml, the reaction was started by adding glucose 4 mM. The absorbance change at 340 nm then showed the amount of ATP which gave rise to flux through the glucose-6-phosphate dehydrogenase reaction ("utilisation" pathway). The difference between this and the total ATP was taken as the amount which entered into the glycolytic pathway.

indicating that glycolysis was successful. With low starting [ATP], the assay showed that [ATP] and [ADP] fell to negligible levels, and failed to rise even after 4 hours.

An early fall in [ATP] + ½ [ADP] is seen in all curves, presumably because some time is required for glycolytic intermediates to build up, so that ATP utilisation is at first unopposed. Subsequently, successful regeneration is achieved in the cases of high initial [ATP], but apparently not at all for low initial [ATP].

A Simplified System

Fig. 8 describes an experiment which further illustrates the mechanism of this effect. In this simplified system, glycolysis is represented by only the first few enzyme steps—glucose to fructose diphosphate. Since these are the steps which are thought mainly to control glycolytic flux, the activity of this short segment provides an indication of how the whole glycolytic pathway would behave.

The system contains this "abbreviated' glycolytic pathway together with the glucose-6-phosphate dehydrogenase reaction. When ATP is added to the system, some of the ATP is used in the sequence leading to fructose diphosphate (glycolysis), and the remainder is used in the sequence leading to 6-phosphogluconate ("utilisation"). These amounts were estimated, to show how the two pathways compete with each other for the available ATP.

The results show that for higher initial [ATP], the flux along the "ATP-production" pathway is greater than the flux along the "ATP-utilisation" pathway, while the reverse applies for low initial [ATP].

These experiments indicate that the ATP turnover system does not operate successfully if [ATP] is less than some relatively low critical value.

Acknowledgements

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REFERENCES

1. Guyton AC, Jones CE, Coleman TG. *Circulatory Physiology: Cardiac Output and its Regulation.* 372—393, Saunders, London, 1973.
2. Newsholme EA, Start C. *Regulation in Metabolism.* Wiley, London, 1973.
3. Atkinson DE. Regulation of enzyme function. Annu Rev Microbiol. **23,** 47-68, 1969
4. Aris R. *Introduction to the Analysis of Chemical Reactors.* 173-184, Prentice-Hall, New Jersey, 1965.
5. Newton K, Steeds W, Garrett TK. *The Motor Vehicle.* 452, Iliffe, London, 1972.
6. Robbins SL. *Textbook of Pathology.* 5-16, Saunders, London, 1962.
7. Chapman AG, Fall L, Atkinson DE. Adenylate energy charge in Escherichia coli during growth and starvation. J Bacteriol. **108,** 1072-1086, 1971
8. Farkouh EF, Daniel AM, Beaudoin J-G, MacLean LD. Predictive value of liver biochemistry in acute hepatic ischemia. Surg Gynec Obstet. **132,** 832-838, 1971
9. Hearse DJ, Chain EB. The role of glucose in the survival and "recovery" of the anoxic isolated perfused rat heart. Biochem J **128,** 1125-1133, 1972
10. Lowry OH, Passonneau JV, Hasselberger FX, Schulz DW. Effect of ischaemia on known substrates and cofactors of the glycolytic pathway in brain. J Biol Chem. **239,** 18-30, 1964
11. Crane RK. *The Enzymes.* 2nd ed *Vol 6,* 47-66, PD Boyer, H Lardy, K Myrback, eds. Academic Press, London, 1962.
12. Walker DG, Parry MJ. *Methods in Enzymology. Vol IX,* 381-388, SP Colowick, NO Kaplan, eds. Academic Press, London, 1966.
13. Lowry OH, Passonneau JV. Kinetic evidence for multiple binding sites on phosphofructokinase. J Biol Chem. **241,** 2268-2279, 1966
14. Maker HS, Lehrer GM. *Handbook of Neurochemistry. Vol VI* 267-310, A Lajtha, ed. Plenum, London, 1971.
15. Shen AC, Jennings RB. Myocardial calcium and magnesium in acute ischemic injury. Am J Path. **67,** 417-440, 1972
16. Wrogemann K, Pena SDJ. Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle diseases. Lancet **1,** 672-674, 1976
17. Lehninger AL. *The Mitochondrion.* 162-164, Benjamin, New York, 1965.
18. Bartlett GR. *The Human Red Cell in Vitro.* 5-29, TJ Greenwalt, GA Jamieson, eds. Grune and Stratton, London, 1974.