REFLECTIONS ON THE MECHANISM OF ACTION OF HORMONES

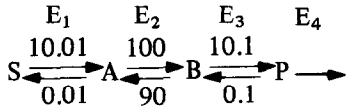
Eric A. NEWSHOLME
Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, England

1. Structure of a metabolic pathway

Biologists are used to the idea that proteins or nucleic acids have a structure, but are surprised when it is suggested that a metabolic pathway has a structure. Pathways have both a thermodynamic and a kinetic structure, which are complementary, but the latter provides an easier-to-understand explanation of how a steady-state flux can be maintained and, further, how it can be changed: one of the earliest papers in which some theoretical principles of metabolic control were discussed in detail was by H. A. Krebs in 1957 [1]. I now submit that, in addition, knowledge of the structure of a pathway is essential in understanding metabolic regulation; failure to obtain and apply such knowledge prior to a detailed study of metabolic regulation, can lead to a wastage of effort. These points have been discussed in two recent articles [2,3]. These theoretical considerations also apply to the question of how hormones influence the flux through metabolic pathways, and the quantitative relationship between the secondary messenger and hormone action; they will be discussed later in this article. First, it is necessary to appreciate the role of the non-equilibrium, near-equilibrium and flux-generating reactions in the structure of a pathway.

1.1. *Non-equilibrium, near-equilibrium and flux-generating reactions*

Three types of reaction occur in metabolic pathways: a single non-equilibrium reaction that approaches saturation with pathway-substrate (the flux-generating step); one or more non-equilibrium reactions which are not saturated with pathway-substrate; and usually more than one near-equilibrium reaction [2]. A reaction is non-equilibrium because the catalytic activity of the enzyme is low in comparison to that of the other enzymes. Hence the concentration of the product(s) is low, so that the rate of the reverse component of the reaction is very much less than the rate of the forward component. A reaction is near-equilibrium if the catalytic activity of the enzyme is high so that the concentration of the product(s) is high and the rate of the reverse component of the reaction is similar to that of the forward component and both are much greater than the overall flux. In the hypothetical example of a simple pathway given below, in which substrate S is converted into end-product P, which is removed by a further reaction E_4, E_1 and E_3 are non-equilibrium, whereas E_2 is near-equilibrium:



The numbers refer to the actual rates (and not the rate constants) in either direction. The roles that these classes of reactions play in a metabolic pathway are given below.

1.1.1. The flux-generating reaction

In order to understand how a steady-state can be established in a metabolic pathway and how the flux through the pathway can be changed from one steady-state to another, it is first necessary to appreciate the significance of the flux-generating reaction. A flux-generating reaction is the first reaction in the pathway that is saturated with pathway-substrate (defined as the substrate which represents the flow of matter through the pathway). In the hypothetical metabolic pathway given above, the enzyme E_1 catalyses the flux-generating step (i.e., it approaches saturation with S). The other reactions in the pathway must be regulated in such a way that they are able to respond to the flux generated at E_1. In the simplest situation, these reactions will be regulated via changes

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

in the concentration of their pathway-substrates (i.e., A,B) which are termed internal effectors. For example, an increase in the activity of enzyme E_1 will increase the flux through the pathway via increases in the concentrations of A and B, which will increase the activities of enzymes E_2 and E_3, respectively. This regulatory relationship is discussed further in section 2. The importance of the concept of the flux generating step should be appreciated when it is considered that many conventional metabolic pathways, at least as described in many textbooks, cannot, by themselves, generate or maintain a steady-state flux. They represent biochemical or truncated pathways which may be of value in understanding the function of a particular pathway in a given cell, but can provide misleading information in relation to control of the flux through pathway. A steady-state flux, and therefore one that can be controlled, can only be achieved if the first step in the pathway approaches saturation with pathway substrate; if it is not, a decrease in the concentration of this substrate will reduce the activity of the enzyme and hence the flux through the pathway. An example may help to explain this important point. In the conventional pathway of glycolysis-from-glucose in muscle, none of the non-equilibrium reactions in this pathway is saturated with pathway substrate. Consequently, utilisation of glucose by muscle could rapidly lead to a reduction in blood glucose concentration which, in turn, would reduce the rate of glycolysis. This is not a trivial point: a marathon runner can use at least 5 g glucose every minute so that, since the total amount of glucose in the extracellular space is only 20 g, the runner would be severely hypoglycaemic in 3–4 min [4]. Glycolysis can only be maintained if glucose is released into the bloodstream at a rate equal to the rate of glucose utilisation. This is achieved either by glucose absorption from the intestine (in the absorptive state) or by glucose release from the liver (in the post-absorptive state).

A flux-generating step is identified by the following criteria: the reaction is non-equilibrium; the K_m of the enzyme catalysing the reaction is considerably lower than the in vivo substrate concentration; the flux through the pathway cannot be modified by changing the in vivo concentration of pathway-substrate of that reaction. Application of these criteria to several accepted metabolic pathways provides some interesting conclusions (see table 1). The supply of glucose to muscle and perhaps to other tissues is achieved by two pathways; one starts with glycogen in the muscle and represents anaerobic glycolysis whereas the other starts with liver glycogen and represents aerobic glycolysis. Biochemical and physiological data on muscle have led to similar conclusions [5–7]. Fatty acid oxidation in muscle starts with triglyceride lipase in the adipose tissue, as does fatty acid oxidation and ketogenesis in the liver [3]. By analogy, the citric acid cycle is really two pathways since it contains two flux-generating steps, these are catalysed by citrate synthase and by 2-oxo-glutarate dehydrogenase. This has considerable implications for the role of the citric acid cycle in muscle especially at rest but this will be discussed elsewhere [8]. Similarly, gluconeogenesis in the liver may be two pathways since pyruvate carboxylase and fructose biphosphatase may both catalyse flux-generating steps. The importance of this sort of information in approaching the problem of metabolic control will be illustrated in sections 2 and 3.

1.1.2. Near-equilibrium reactions

Probably the most important function of a near-equilibrium reaction in metabolic control is to provide a high sensitivity to changes in the concentrations of substrate and/or products of the reaction.

Table 1
Flux-generating steps for various pathways in muscle

Pathway	Flux-generating step
1. Glycogen to lactate	Muscle glycogen phosphorylase
2. Glucose to pyruvate	Hepatic glycogen phosphorylase
3. Pyruvate to acetyl-CoA	Pyruvate dehydrogenase
4. Acetyl-CoA to 2-oxoglutarate	Citrate synthase
5. 2-Oxoglutarate to oxaloacetate	2-Oxoglutarate dehydrogenase
6. Fatty acid to acetyl-CoA	Adipose tissue triglyceride lipase

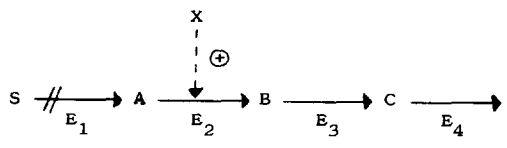
Consequently, they are ideal reactions to transmit the flux generated at the flux-generating reaction [2,3,9,10]. Thus, a small increase in substrate concentration could transmit a large increase in flux generated at the flux-generating step. However, this high sensitivity to substrates and products means that these reactions are much less sensitive to the effects of allosteric regulators, which include the secondary messengers of hormones. Hence, it is unlikely that near-equilibrium reactions will be sites for hormonal control of the flux through metabolic pathways. This is a particularly important point which may be easily overlooked by endocrinologists searching for metabolic effects of hormones, but who have little experience of the theory underlying the control of flux through metabolic processes.

1.1.3. Non-equilibrium reactions not saturated with substrate

Non-equilibrium reactions that are not saturated with substrate play an important role in directing a pathway, particularly towards its end and in the middle of a long pathway. (Flux-generating steps determine direction of a pathway at its beginning.) The major advantage of the non-equilibrium reactions in metabolic control is that the enzymes catalysing such reactions can be controlled by allosteric factors. Hence they provide potential sites for hormonal control via the effects of secondary messengers, provided that this is co-ordinated with control of the flux-generating step. The major disadvantage of the non-equilibrium reaction is a poor sensitivity to changes in concentration of both allosteric effectors and substrates(s). Means for increasing sensitivity of such reactions and particularly the role of hormones in increasing sensitivity is discussed in section 5.

2. Control of the flux through a physiological pathway

In the following hypothetical linear pathway the flux (J) is generated at reaction E_1 (the flux generating step which is denoted by the sign -//->):



This flux is transmitted along the pathway by the response of the subsequent reactions to the metabolic intermediates A, B and C, so that these metabolites link all the component reactions of the pathway and help to produce the overall steady-state flux. The role of the metabolic intermediates is emphasised by consideration of an activation of one of the enzymes; for example, compound X stimulates enzyme E_2 so that, if the concentration of X was increased, the activity of this enzyme would increase. However, this would result in a decrease in the concentration of the substrate A which would lower the activity of enzyme E_2 until it returned to the original activity, so that the overall steady-state flux would not change. The only difference would be a lowered steady-state concentration of A. This shows that, in this example, the concentration of A is determined by the flux (i.e., the activity of the enzyme that catalyses the flux generating step) together with the kinetic properties of enzyme E_2. Metabolic intermediates whose concentrations are determined by the flux (and which, therefore, help to maintain the steady-state flux) are termed internal effectors for that flux. Two points, which are particularly relevant to consideration of the action of hormones, arise from this discussion:

(i) Hormones (or their secondary messengers) would only be expected to change the activity of an enzyme such as E_2 provided that the activity of the enzyme that catalysed the flux generating step was also similarly changed (see section 3).

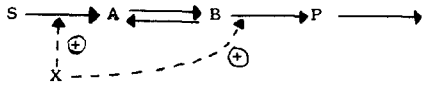
(ii) Many hormones produce their intracellular effects via a change in concentration of a secondary messenger; the concentration of a secondary messenger may be regulated as an intermediate of a pathway which has a structure as indicated above.

The effects of the hormone on the concentration of secondary messengers can be predicted from the structure of the pathway (see section 4).

3. Hormonal action and the flux-generating step

Some hormones modify the flux through metabolic pathways. In general, this effect of the hormone is likely to be mediated through the action of an intracellular secondary messenger (see section 4). An important question is, which reactions in the pathway must the secondary messenger affect? At least the activity of the enzyme that catalyses the flux-generating step must be changed. If the activity of this

enzyme is increased; e.g., the remainder of the reactions in the pathway could respond to the increased flux via increases in the concentrations of pathway-substrates (i.e., internal control). However, it is possible that this control alone may not be sufficient at some non-equilibrium reactions (because of the insensitivity of these reactions). Therefore, it is possible that the hormone, via the secondary messenger, will modify the activity of one or more 'non-equilibrium' enzymes in the pathway in addition to that of the 'flux-generating' enzyme, as follows:



where X is the secondary messenger.

This theoretical approach to the action of hormones can best be explained by reference to examples, such as the hormonal control of fatty acid oxidation in muscle and the effects of insulin on glucose transport and glycogen synthesis in muscle.

3.1. *Flux-generating step for fatty acid oxidation in muscle and effects of hormones*

It is suggested that the flux-generating step for the β-oxidation of fatty acids in muscle (at least as far as the formation of acetyl-CoA) is the lipolytic process in adipose tissue, which is catalysed by triglyceride lipase (together with di- and monoglyceride lipases) (see fig.1). Consequently, in order to control the rate of fatty acid oxidation in muscle, it is necessary to control the activity or triglyceride lipase within the adipose tissue. There are a large number of hormones (e.g., glucagon, adrenaline, noradrenaline, insulin, growth hormone, glucocorticoids) that modify the activity of this enzyme [11]. Indeed, the number of hormones that affect the enzyme is so large that it prompted Steinberg in 1963 [12] to comment that, in consideration of discrimination and selectivity, the number of hormones affecting the lipase was embarrassingly large. In view of the fact that triglyceride within adipose tissue is the major fuel store of the body and that the triglyceride lipase is flux-generating for fatty acid oxidation in muscle and in other tissues, it is not at all surprising that a large number of hormones affect the activity of this enzyme.

3.2. *Flux-generating steps for glucose utilisation in muscle and the effects of insulin*

The flux-generating step for glycolysis-from-glucose in muscle can vary; in the post-absorptive state it is likely to be hepatic phosphorylase, whereas, in the absorptive period, after a carbohydrate-containing meal, glucose absorption from the intestine will be flux-generating (see fig.2). That the hepatic phos-

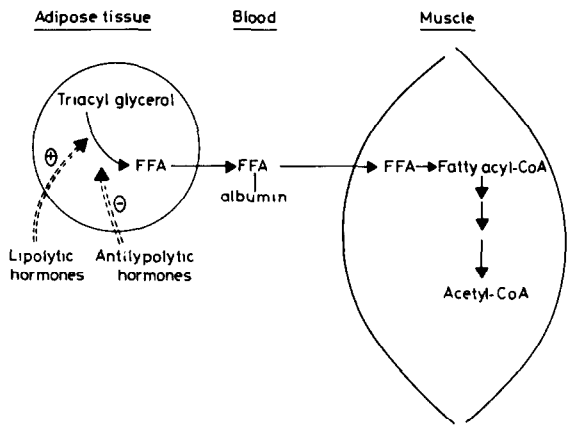


Fig.1. Flux-generating step for fatty acid oxidation by muscle. The flux-generating step is the lipolysis of triacylglycerol to fatty acid (FFA) within the adipose tissue. The triacylglycerol lipase activity is controlled by the balance of effects of lipolytic and antilipolytic hormones. The fatty acids are transported via the bloodstream to the liver in combination with albumin.

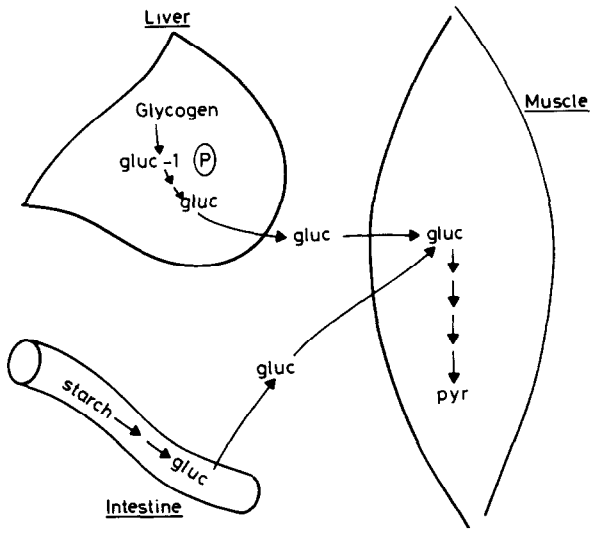


Fig.2. Flux-generating steps for glycolysis in muscle. In the absorptive state, glucose transport across the luminal membrane of the epithelial cell of the intestine is probably flux-generating for glycolysis-from-glucose in the muscle. In the post-absorptive state, hepatic phosphorylase will be flux-generating.

phorylase is flux-generating for glycolysis in muscle is consistent with the large number of hormones that increase the activity of this enzyme under conditions when glycolytic flux in muscle is increased (e.g., sustained exercise). However, it is well established that insulin stimulates transport of glucose into muscle, which is not a flux-generating step. Since insulin reduces the rate of glycogenolysis in the liver [13], it might be expected that it should inhibit rather than activate the transport of glucose across the muscle cell membrane. The activating effect of this hormone can be explained on the basis of the relationship between glucose absorption from the intestine and the stimulation by insulin of glycogen synthesis in muscle. Insulin stimulates the flux-generating reaction for glycogen synthesis in muscle, glycogen synthetase. However, stimulation of this enzyme alone would not lead to an increase in the steady-state rate of glycogen synthesis but would cause a reduction of the concentrations of UDP-glucose and hexose monophosphates in muscle. It could not lead to an increase in the rate of conversion of extracellular glucose to glycogen, since there would be no stimulation of glucose transport into the muscle. Hence insulin, which is secreted in relation to the rate of glucose absorption from the intestine and which stimulates the activity of glycogen synthetase, also stimulates glucose transport. Thus, the flux of glucose from the intestinal lumen to glycogen in the muscle is facilitated by the insulin stimulation of the flux-generating step, glycogen synthetase and the non-flux-generating step, glucose transport (see fig.3).

The major problem with the stimulation of glucose transport by insulin is that, if this effect occurs in the absence of the high rate of absorption of glucose from intestine, it could result in hypoglycaemia. This suggests that high levels of insulin, in the absence of high rate of carbohydrate absorption from the gut, would be dangerous, since it would increase the activity of a non-flux generating step, and result in a decreased concentration of an important metabolic intermediate, namely blood glucose. Thus, the effectiveness of insulin as a murder weapon by medically knowledgeable personnel is explicable on the basis of this theory [14].

3.3. *Experimental approaches to hormone action*

These considerations suggest that hormones will modify the activities of enzymes that catalyse flux generating steps and, in addition, enzymes that catalyse other non-equilibrium reactions in the pathway, to provide a concerted mechanism of control. Consequently, the question as to which reaction(s) in a pathway, a hormone (or its secondary messenger) will be likely to affect, can only be answered when the structure of the pathway is known. The first experimental stage is to identify the near- and non-equilibrium reactions in the pathway. This can be done by established methods [15]. The second stage is to identify the flux-generating step, for which the experimental criteria have been given in section 1 (see also [2]). The demonstration that a hormone can modify a reaction in a pathway via external regulation is carried out by allowing the hormone to modify the flux through the pathway in an intact tissue preparation and measuring the steady-state changes in concentrations of pathway substrates. If the concentration of substrate changes in the opposite direction to the flux, this indicates that external regulation, due to the presence of the hormone, operates at that reaction [2]. The identification of the external regulator (i.e., secondary messenger) is obtained by investigating the properties of the enzyme in vitro. The search for the compound that activated hepatic phosphorylase in response to adrenalin led to the discovery in 1958 of cyclic AMP [16]. The current interest in factors that regulate the rate of interconversion between glycogen synthetase *a* and *b* in muscle is explained by the fact that this might lead to identifi-

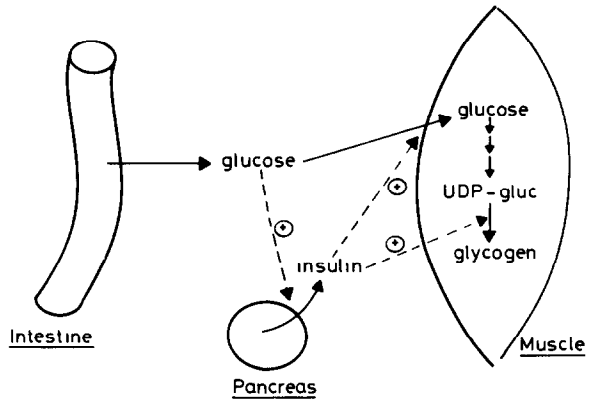


Fig.3. Effects of insulin on glucose uptake and glycogen synthesis in muscle. Insulin stimulates the activities of glycogen synthetase and the glucose-transporting system in muscle. It is suggested that only high concentrations of insulin will have these effects, and that such concentrations will only be apparent after a carbohydrate meal.

cation of the second messenger for insulin [17]. Confirmation that an effector in vitro is in fact the regulator in vivo is different and requires extension of the experiments to the intact system [2,15].

One of the major problems in metabolic control is that it is easy to place the biochemical cart before the horse. Thus, the properties of an enzyme are investigated in vitro and on the basis of such properties, a hypothesis of control is put forward. If the hypothesis has some physiological attractions, it can be readily accepted despite lack of evidence that it has any application to the in vivo condition. Properties of enzymes are usually obtained in a very dilute artificial medium in the cuvette of a given spectrophotometer and immediately extrapolated to the in vivo system. The phenomenon of 'exciting' properties being discovered in such a way has become known as a 'storm in a cuvette'. Perhaps a better description of this situation was provided by Charles L. Dodgson (alias Lewis Carroll) in 1871 [18] as follows:

> *"As she said this, she looked up, and there was the Cat again, sitting on a branch of a tree.*
>
> *'Did you say pig, or fig?' said the Cat.*
>
> *'I said pig,' replied Alice; 'and I wish you wouldn't keep appearing and vanishing so suddenly: you make one quite giddy.'*
>
> *'All right,' said the Cat; and this time it vanished quite slowly, beginning with the end of the tail, and ending with the grin, which remained some time after the rest of it had gone.*
>
> *'Well! I've often seen a cat without a grin', thought Alice; 'but a grin without a cat! It's the most curious thing I ever saw in all my life!' "*

Biochemists are almost always interested in the grin rather than the cat which Alice realised as being 'most curious'. The 'grin', of course, can be studied as a phenomenon, the difficulty arises when such studies are extrapolated to the 'cat' without rigorous testing of such extrapolations.

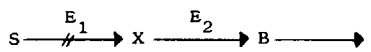
Knowledge of the structure of the pathway can provide some guidance as to which reactions will be wise to study, and the examples given in this section indicate the scientific success that can be achieved from selection of the correct reaction to study. One example from the recent literature illustrates the danger. The mitochondrial adenine nucleotide translocase catalyses the transport of ADP into the mitochondria and ATP out of the mitochondria [19]. This is an extremely important reaction in energy transfer in the cell, but the available evidence suggests that it is not flux-generating for energy transfer and that it is likely to be a near-equilibrium reaction. Consequently, it is unlikely to be a site for allosteric or hormonal regulation. Nonetheless, it has been suggested on the basis of experiments with isolated mitochondria, that the physiological effects of thyroxine on energy metabolism may be explained via its effect on the translocase [20]. The structure of the energy transfer pathway in muscle would suggest that this is unlikely to be a hormone sensitive reaction and any further in vitro studies with thyroxine on this process would provide information only on the 'grin'.

4. Secondary messengers and hormone action

The concept of the secondary messenger was proposed by Sutherland and colleagues in 1965 [21] to account for the role of cyclic AMP in mediating the intracellular response to a hormone. In this elegant scheme, the hormone is seen as the primary messenger which, after release from the endocrine gland and transport in the bloodstream, modifies metabolism within the target tissue(s) by changing the concentration of the secondary messenger. Since 1965, a considerable amount of work has been directed towards understanding both the molecular events by which a hormone interacts with a tissue and causes a change in the intracellular concentration of a secondary messenger, such as cyclic AMP, and how the latter produces the physiological response in the target tissue. It is generally accepted that some hormones bind to a specific receptor on the surface of the cell, that this binding enhances the activity of a specific enzyme such as adenylate cyclase and that this leads to an increase in the steady-state concentration of the secondary messenger in the tissue. Since, the change in the secondary messenger concentration is important in producing the response to the hormone, the theoretical principles underlying the concentration changes are of considerable importance. It has not, in general, been realised that the secondary messenger system is formally analogous to a metabolic pathway and must comprise a structure as described in section 1. Maintenance of steady-state concentrations and the ability to change this concentration demand an organised structure. The earlier discussion concerned the origin and control of flux, but since flux and concentrations of intermediates are intimately related, changes in flux will produce defined changes

in the concentration of an intermediate involved in the transmission of that flux. Thus, the concentration of a secondary messenger can be predicted if the flux and the properties of the enzymes that utilise the messenger are known.

A simple model for a pathway which produces and utilises a secondary messenger X is described as follows:



The term 'utilises' is used in the context of secondary or local messengers to mean conversion of the messenger to a less active or non-active metabolite of the messenger.

In this model, E_1 is the flux generating reaction and E_2 is a non-equilibrium reaction. It is important to appreciate that the reaction catalysed by E_1 must be flux generating (or pseudo-flux generating; see [2]) if changes in the activity of this enzyme are to modify the steady-state concentration of the secondary messenger, X. If this reaction were not flux generating, a change in the activity of the enzyme would not change the flux through this pathway, so that there would be no change in the steady-state concentration of X. On the other hand, changes in the activity of enzyme E_2 should always cause a change in the steady-state concentration of X, although there would be no change in the flux through the pathway.

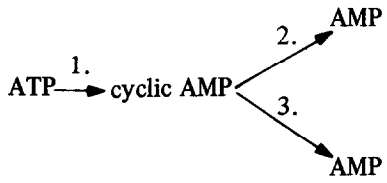
Once a secondary messenger system is identified, it is tacitly assumed that changes in the enzyme that produces the messenger must always be capable of changing the concentration of the messenger. The danger is that the enzyme will be studied in vitro and any properties that are discovered in this way could immediately be assumed to apply to the situation in vivo. Such properties will only be relevant to changes in the concentration of secondary messenger if the 'secondary messenger-producing enzyme' catalyses a flux generating reaction. These considerations lead to the important question, does adenyl cyclase catalyse a flux-generating step? If it does not, it is possible that all the properties concerning hormone activation or receptor interaction may be of little value for understanding how the changes in the steady-state concentration of cyclic AMP are brought about. It has indeed been shown that adenyl cyclase catalyses a flux-generating step [22].

The structure of the 'secondary messenger pathway' is useful not only from indicating which enzymes should be studied in detail in vitro but knowledge of the properties of these enzymes enable the concentration of the secondary messenger to be calculated. Thus, the system can be described mathematically and the concentration of X is given by the following equation:

$$[X] = \frac{V_{E_1} \cdot K_{m_{E_2}}}{(V_{E_2} - V_{E_1})}$$

where V_{E_1} and V_{E_2} are the maximal activities of enzymes E_1 and E_2, respectively, and $K_{m_{E_2}}$ is the K_m of E_2 for X (see appendix A for derivation of equation). The usefulness of the model is that from a knowledge of the quantitative effect(s) of the hormone on enzymes E_1 (assuming it catalyses a flux-generating reaction) and/or E_2, precise changes in the concentration of the secondary messenger are predictable. It is assumed that the flux through the pathway is equal to the maximal activity of enzyme E_1, and that the activity is changed by the effect of the hormones.

Although this model provides a useful introduction it is an oversimplification. Thus, for many secondary messenger systems the utilisation process comprises at least two reactions, which have different properties. This complication must be included in the model. For the cyclic AMP system, there are at least two phosphodiesterase enzymes whose properties are different. The following reaction sequence describes the model for cyclic AMP:



The activities of adenylate cyclase and high- and low-K_m phosphodiesterase are represented by 1, 2 and 3 respectively. The following assumptions are implicit in the model:

(i) Adenylate cyclase is the flux-generating step and its maximal activity under any given condition represents the flux through the cyclic AMP pathway;

(ii) All three enzymes catalyse non-equilibrium reactions;

(iii) Both phosphodiesterase enzymes obey Michaelis–Menten kinetics.

Consequently, the model is described mathematically as follows:

$$V_1 = \frac{V_2 S}{K_{m_2} + S} + \frac{V_3 S}{K_{m_3} + S}$$

where S is the concentration of cyclic AMP and subscripts 1, 2 and 3 refer to the enzymes as indicated above. Consequently, the concentration, S, can be calculated from the equation:

$$S = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

(see appendix B for derivation of equation)

where $a = (V_2 + V_3 - V_1)$

$$b = (V_2 K_{m_3} + V_3 K_{m_2} - V_1 K_{m_3} - V_1 K_{m_2})$$

$$c = (V_1 \cdot K_{m_2} \cdot K_{m_3})$$

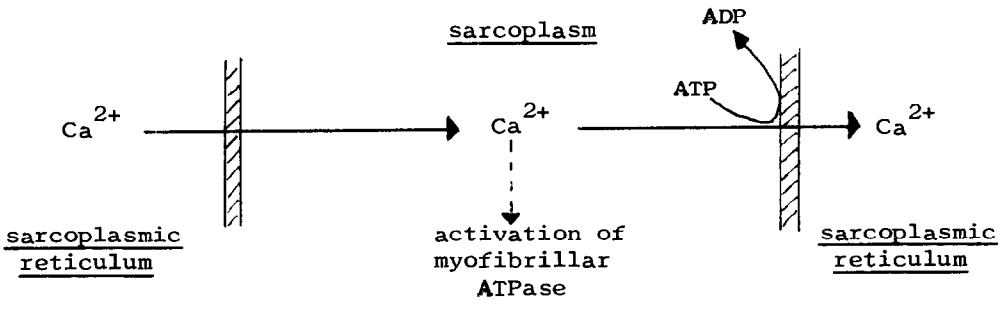
Thus, the basal concentration of cyclic AMP in a tissue can be calculated from the above equation from values of the maximal activities for the three enzymes and values of K_m for the two phosphodiesterase enzymes. In addition, the increase in the concentration of cyclic AMP produced by the action of a hormone can also be quantitatively predicted if the effects of the hormone on the cyclase and phosphodiesterase enzymes are known. The relevant information would be included in the mathematical model given above.

The basal and hormone-stimulated activities of adenylate cyclase and the maximal activities and K_m values of the two phosphodiesterases have been measured in many different tissues so that the concentration of cyclic AMP can be calculated according to the model [22]. Where possible, the calculated concentrations of cyclic AMP have been compared to the measured concentrations reported in the literature [22]. In general, there is reasonable agreement between calculated and reported concentrations although, in some tissues, there are marked differences for both the basal and hormone-stimulated concentrations (i.e., gastrocnemius muscles of both the mouse and rat and heart ventricle of the frog). In these latter cases, the calculated concentration is almost an order of magnitude lower than the measured concentration. However, if only the extent of stimulation by the hormone is considered (i.e., stimulated concentration/basal concentration) there is remarkable agreement. This suggests that the actual measurements of cyclic AMP may be in error and that the model has some validity [22].

Most discussions of secondary messengers in the literature centre on the cyclic nucleotides and more recently on Ca^{2+}. Consideration of the secondary messenger as an intermediate in a physiological metabolic pathway enables other messenger systems, which have not usually been considered to have any metabolic similarity to the cyclic nucleotide systems, to be included in a secondary messenger classification. In addition to Ca^{2+}, neurotransmitters and adenosine can be included.

4.1. *Calcium ions as secondary messengers*

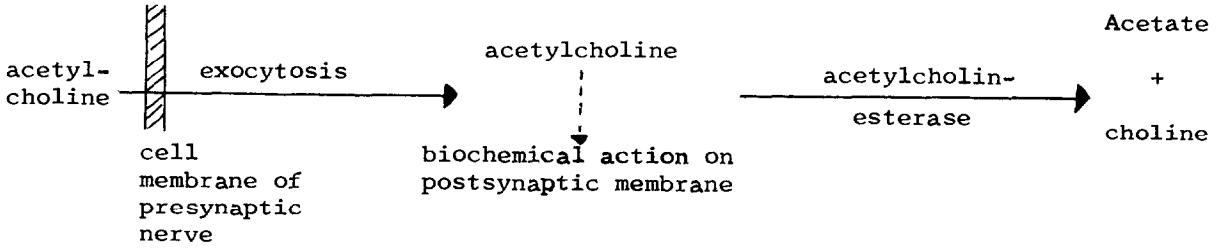
In resting muscle, most of the Ca^{2+} in the fibre is contained within the sarcoplasmic reticulum and the mitochondria [23]. The action potential causes a release of Ca^{2+} from the reticulum resulting in an increase in the sarcoplasmic concentration of Ca^{2+}, which causes activation of the myofibrillar ATPase (and hence contraction). The sarcoplasmic Ca^{2+} concentration is lowered by re-uptake into the sarcoplasmic reticulum and the mitochondria by a process that requires the utilisation of ATP [23]. Consequently this represents another messenger-model system:



It is suggested that the Ca^{2+}-release process is flux- or pseudo-flux-generating and that Ca^{2+} uptake by the reticulum is first order, for which there is some evidence [23,24]. This messenger system could be quantitatively analysed by the model similar to that for cyclic AMP described above.

4.2. *Neurotransmitters as secondary messengers*

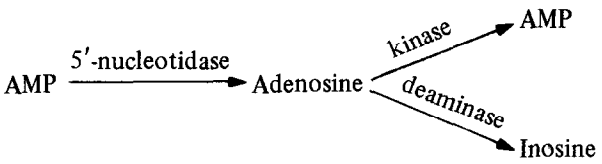
Most if not all neurotransmitters are concentrated in vesicles in the presynaptic nerve terminals. The process of exocytosis causes release of the contents of these vesicles into the synaptic cleft. The neurotransmitter is 'utilised' either by re-absorption into the pre-synaptic nerve terminal and/or by enzymatic destruction in the cleft. Acetylcholine is used as an example to illustrate how the messenger-model can be applied to neurotransmitters:



It is suggested that the exocytotic process is either flux-generating or pseudo-flux-generating. The utilisation of acetylcholine, on the other hand, is probably first order. Thus, the K_m value of brain acetylcholinesterase for acetylcholine is about 100 μM [25] but it is likely that the concentration of acetylcholine in the synaptic cleft is much less than this, since as little as 0.0001 μM acetylcholine excites or inhibits many organs and tissues, and 1 μM acetylcholine is sufficient for a maximal response [26]. If the flux through the system and the properties of the utilisation system were known, it would be possible to analyse the system quantitatively.

4.3. *Adenosine as a secondary messenger*

Adenosine is considered to be produced from AMP by the action of 5′-nucleotidase and it is utilised by the action of adenosine deaminase and adenosine kinase. Adenosine is considered to have a number of regulatory roles in both biochemical and physiological processes [27]. Consequently, the control of the concentration of this messenger may be described by the following model system:



The properties of these enzymes from rat heart have been investigated in detail and a model system, based on these properties, has been proposed in [28].

5. Hormonal action and sensitivity in metabolic control

The above discussion has focused on one important aspect of metabolic control, the identification of reactions that should be affected by hormones or their secondary messengers. This approach enables properties of enzymes to be investigated in vitro with some confidence that the properties will be of relevance in vivo. However, this implies that if hormones affect, directly or indirectly, a flux-generating step, they will cause a change in flux through that pathway. This is not necessarily the case. It is suggested that some hormones may cause changes in the control mechanism so that the response to other hormones or other regulators is markedly increased. In order to understand how this could be brought about, it is necessary to appreciate the problems of sensitivity in metabolic control.

4.4. *Sensitivity in metabolic control*

Sensitivity can be defined as a function that measures the magnitude of a response to given stimulus (e.g., the change in flux through a metabolic pathway in response to a change in concentration of a secondary messenger, i.e., the ratio of the relative (percentage) change in the response to the relative change in stimulus). If the concentration of a messenger (x) changes by Δx, the relative change is $\Delta[x]/[x]$; if

the change in $[x]$ modifies the flux through a metabolic pathway from J to ΔJ, the sensitivity of J to the change in $[x]$ is given by the ratio $(\Delta J/J)/(\Delta[x]/[x])$. The sensitivity as the mathematical limit of this ratio is denoted by the symbol s_x^J, so that:

$$s_x^J = (dJ/J)/(d[x]/[x])$$

$$= d \ln J/d \ln [x]$$

However, the interpretation of what is meant by sensitivity is probably easier to understand if this equation is integrated to provide a power equation [10]:

$$J = \lambda [x]^{s_x^J}$$

where λ is a constant of integration whose value is not required for the present analysis. Although there are limitations in the use of this equation for calculation of sensitivity [10], it is useful in providing a means for comparing the effectiveness of various mechanisms for increasing sensitivity. Although there are a large number of different mechanisms for specific control of different enzymes, when the problem of improving sensitivity of non-equilibrium reactions to changes in concentrations of regulators is considered, it can be seen that the mechanisms can be separated into a very small number of classes. Hormones play a role in a least two of these classes.

4.4.1. Requirement for sensitivity in metabolic control

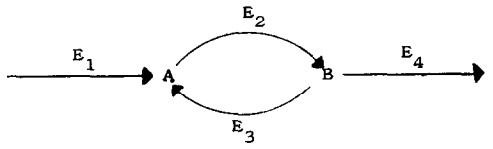
If an enzyme responds in a hyperbolic manner to a change in concentration of a messenger, the value of the sensitivity in the above power equation will vary between 1 and 0. Consequently, if the flux through the reaction is required to increase 100-fold, the smallest possible change in messenger to produce this effect would be greater than 100-fold.

In many cases, the flux through a metabolic pathway can change by greater than 100-fold. Indeed, in order to provide the energy for sprinting, the rate of glycolysis-from-glycogen increases by at least 1000-fold in the leg muscles of man [29,30]. If the response of the non-equilibrium reactions of glycolysis to the regulators were hyperbolic, this would require a greater than 1000-fold increase in the concentrations of the regulator(s). Does this occur? Two non-equilibrium reactions in this pathway are those catalysed by glycogen phosphorylase and phosphofructokinase. The activity of the latter enzyme is regulated by changes in the concentrations of ATP, AMP, NH_4^+, creatine phosphate, fructose bisphosphate, phosphate and citrate [31]. The concentrations of most of these regulators have been measured in the leg muscles of man at rest and during sprinting, and they do not change by more than 2- or 3-fold [32]. Consequently, a marked improvement in sensitivity is required over that provided by the interaction of an enzyme, which catalyses a non-equilibrium reaction, with even a multiplicity of regulators in a hyperbolic manner. It is considered that this improvement is achieved by co-operativity, substrate cycles and interconversion cycles; these represent a logical series of biochemical mechanisms for improving sensitivity in metabolic control [9,30]. Since some hormones appear to play a role in improving sensitivity by means of substrate and interconversion cycles these will be discussed below.

4.5. *Mechanisms for improving sensitivity in metabolic control*

4.5.1. Substrate cycles

It is possible for a reaction, which is non-equilibrium in the forward direction of a pathway (i.e., A→B; see below) to be opposed by a reaction that is non-equilibrium in the reverse direction of the pathway (i.e., B→ A). (Both reactions must, of course, be chemically distinct.) These two separate reactions are catalysed by enzymes E_2 and E_3 in the following hypothetical scheme:



If the two enzymes are simultaneously catalytically active, a substrate cycle, in which A is converted to B and the latter is converted back to A, is established.

The precise quantitative role of substrate cycles in improving sensitivity has been developed by Newsholme and Crabtree [9]: the intrinsic sensitivity is given by (1 + cycling rate/flux). In other words, sensitivity will be high only when the rate of cycling is high and the flux is low. However, high rates of cycling for prolonged periods would cause a large amount of chemical energy to be converted into heat

with the inherent danger of hyperthermia [9]. Hence, it is suggested that cycling rates will be increased only when they are required to provide an improvement of sensitivity. The mechanism for increasing the rate of cycling, prior to the requirement for increased flux, is considered to be hormonal. In this way, hormones will not be involved nor will they interfere in the basic control mechanism, but they will improve sensitivity when it is required. Thus, I consider that an important effect of some hormones is to increase the rates of substrate cycling so that the sensitivity of the control system to changes in metabolic (or other) regulators is markedly increased. This aspect of hormone action appears to have been neglected by most endocrinologists, so that we have a dearth of information as to which hormones could affect which cycles. A speculative example is given below.

4.5.2. The fructose 6-phosphate–fructose 1,6-bisphosphate cycle in the control of glycolytic flux and the actions of hormones

The presence of fructose bisphosphatases in a wide variety of muscles from many different animals across the animal kingdom led Newsholme and Crabtree [9,33,34] to propose that this enzyme plays a role in the regulation of glycolysis via the operation of a substrate cycle between fructose 6-phosphate and fructose 1,6-bisphosphate. The above discussion leads to the suggestion that the role of this cycle in muscle of human subjects is to increase the sensitivity of metabolic control at the level of fructose 6-phosphate phosphorylation to changes in the concentrations of the regulators of phosphofructokinase. Because of the problem of hyperthermia, which results from high rates of cycling, it is suggested that when the subject is in the basal metabolic state, the rate of cycling will be very low. However, when exercise is anticipated, the rate of cycling will be raised so that the sensitivity of the metabolic control system is increased. Thus, it is predicted that, in the sprinter on his blocks, the blood levels of the stress hormones, adrenaline, noradrenaline, glucagon and glucocorticoids, will be raised to increase markedly the rate of cycling. This will provide a high, cycling rate:flux, ratio for the phosphorylation of fructose 6-phosphate while the sprinter is on his blocks waiting for the gun. The reaction will now be very sensitive to changes in the concentrations of the regulators of phosphofructokinase. Once the sprint is under way, the rate of ATP hydrolysis will increase markedly and this will lead to decreases in the concentrations of creatine phosphate, ATP and citrate and increases in the concentrations of AMP, phosphate, and NH_4^+ which will increase the activity of phosphofructokinase towards its maximum. This will ensure that the rate of energy production via anaerobic glycolysis increases precisely to the rate required to satisfy the demand for ATP utilisation by the contractile process. It is suggested that one reason for the high performance of elite sprinters is the fact that metabolic control mechanisms are so well developed that they provide maximum sensitivity when required in the control of the energy-producing pathways in muscle [29]. Consequently, the rate of glycolysis can be increased about 1000-fold in a very short time with a minimal disturbance in the concentrations of glycolytic intermediates and especially the [ATP]/[ADP].

4.5.3. Interconversion cycles

Newsholme and Crabtree [33] pointed out that the mechanism of regulation of an enzyme via the conversion of an inactive form into an active form, and vice versa, has similarities to a substrate cycle and, indeed, that this interconversion cycle represented a logical extension of the substrate of the substrate cycle. It has been emphasised that, in some situations, the substrate cycle mechanism of control could produce heat at too rapid a rate to provide a satisfactory control mechanism. Although the cycling between active and inactive forms of enzyme (e.g., between phosphorylase *a* and phosphorylase *b* or pyruvate dehydrogenase *a* and pyruvate dehydrogenase *b*) is similar to that of a substrate cycle, the concentration of the substrate in the cycle (i.e., the enzymes) will, in general, be very much lower (by at least one order of magnitude) than those of the metabolic intermediates in a substrate cycle. Hence, the rate of heat generation will be considerably less. However, although the mechanism for the improvement in sensitivity in the interconversion cycle is similar to that of the substrate cycle, the precise quantitative role of the interconversion cycle in increasing sensitivity has not yet been defined. A qualitative description of the sensitivity has been provided in [9,33]. It is suggested above that the role of some hormones may be to increase the sensitivity of metabolic control systems when required by increasing the rate of substrate cycling. Similarly, hormones could increase the rate of cycling in an interconversion cycle, without markedly changing the proportion of the

active form of the enzyme. However, once the cycling rate has been increased, the effect of a regulator on the activity of one or both of the interconverting enzymes would be to increase the rate of activation (or inhibition) of the target metabolic enzyme. It is suggested that this may be the role of adrenaline in increasing the activity of phosphorylase *b* kinase by phosphorylation. Adrenaline increases the concentration of cyclic AMP in muscle which increases the activity of protein kinase. The latter phosphorylates the β-subunit of phosphorylase *b* kinase, which increases the activity of the enzyme. However, it also phosphorylates the α-subunit which favours the dephosphorylation of the β-subunit by the phosphorylase *b* kinase phosphatase [35]. In this way, the rates of both phosphorylation and dephosphorylation of phosphorylase *b* kinase are increased; i.e., the rate of interconversion cycling is increased. In the case of skeletal muscle, Ca^{2+} are known to play an important role in the control of phosphorylase *b* kinase activity and hence in that of phosphorylase activity. If adrenaline is able to increase the rate of interconversion cycling between active and inactive forms of phosphorylase *b* kinase, the effect of Ca^{2+} on the rate of activation of phosphorylase (i.e., conversion of phosphorylase *b* into *a*) could be markedly increased.

6. Conclusions

The role of substrate cycling in metabolic regulation has been criticised on the grounds that cycling rates that have been measured so far are, in general, rather low [36]. This, however, does not take into account that even low rates of cycling could provide an improvement in sensitivity especially if cycling and co-operativity were combined. There is no doubt that a large number of substrate cycles are present in metabolic pathways: these include the triglyceride–fatty acid cycles, the protein–amino acid cycle, the glycogen–glucose 1-phosphate cycle, the glucose–glucose 6-phosphate cycle, the fructose 6-phosphate–fructose 1,6-bisphosphate cycle and the pyruvate–phosphoenolpyruvate cycle. Although the rates of cycling in the cycles that have so far been measured is low, the question arises whether the rates have been measured under conditions when hormone stimulation of the cycles is occurring. If any of the speculations given above are valid, hormonal stimulation of cycling rates could be a major form of metabolic control which would be operative for relatively short times. However, during those periods the cycling rates should be high and sensitivity to metabolic regulators should also be high. This represents a novel concept in the consideration of hormone action and, if it has any validity, could be of value in understanding diseases in which there is a reduction in responsiveness to biochemical and physiological stimuli. Although it may be a novel concept in its application to hormone action, the general importance of substrate cycling was realised by Charles Dodgson in the last century [18]:

> *" 'Now! Now!' cried the Queen. 'Faster! Faster!' And they went so fast that at last they seemed to skim through the air, hardly touching the ground with their feet, till suddenly, just as Alice was getting quite exhausted, they stopped, and she found herself sitting on the ground, breathless and giddy.*
>
> *The Queen propped her against a tree, and said kindly, 'you may rest a little now'.*
>
> *Alice looked round her in great surprise. 'Why, I do believe we've been under this tree all the time! Everything's just as it was!'*
>
> *'Of course it is,' said the Queen: 'what would you have it?'*
>
> *'Well, in* our *country,' said Alice, still panting a little, 'you'd generally get to somewhere else–if you ran very fast for a long time, as we've been doing.'*
>
> *'A slow sort of country!' said the Queen. 'Now* here, *you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!' "*

Acknowledgements

The theoretical approaches to metabolic control that have been put forward in this paper have been developed in collaboration with Francis Rolleston, Carole Start and, particularly, Bernard Crabtree.

References

[1] Krebs, H. A. (1957) Endeavour 16, 125.
[2] Newsholme, E. A. and Crabtree, B. (1979) J. Mol. Cell. Cardiol. 11, 839–856.
[3] Newsholme, E. A. and Crabtree, B. (1980) in: The Short-Term Regulation of Metabolism in the Liver (Hue, L. and Van de Werve, G. eds) Academic Press, London.
[4] Newsholme, E. A. (1980) Medisport 2, 28–32.
[5] Crabtree, B. and Newsholme, E. A. (1972) Biochem. J. 126, 49–58.
[6] Newsholme, E. A., Zammit, V. A. and Crabtree, B. (1978) Biochem. Soc. Trans. 6, 512–520.

[7] Newsholme, E. A., Crabtree, B. and Zammit, V. A. (1980) Ciba Found. Symp. 73, 245–258.
[8] Newsholme, E. A. and Crabtree, B. (1980) Trends Biochem. Sci. in preparation.
[9] Newsholme, E. A. and Crabtree, B. (1976) Biochem. Soc. Symp. 41, 61–110.
[10] Crabtree, B. and Newsholme, E. A. (1978) Eur. J. Biochem. 89, 19–22.
[11] Hales, E. N., Luzio, J. P. and Siddle, K. (1978) Biochem. Soc. Symp. 43, 97–135.
[12] Steinberg, P. (1963) in: Control of Lipid Metabolism (Grant, J. K. ed) pp. 111–138, Academic Press, London, New York.
[13] Van de Werve, G., Hue, L. and Hers, H. G. (1977) Biochem. J. 162, 135–142.
[14] Birkinshaw, V. P., Gurd, M. R., Randall, S. S., Curry, A. S., Price, D. E. and Wright, P. H. (1958) Brit. Med. J. 2, 463–468.
[15] Newsholme, E. A. and Start, C. (1973) in: Regulation in Metabolism, Wiley, London, New York.
[16] Sutherland, E. W. (1962) Harvey Lect. 57, 17–33.
[17] Nimmo, H. G. and Cohen, P. (1977) Adv. Cyclic Nucl. Res. 8, 145, 266.
[18] Carroll, L. (1871) Alice's Adventures in Wonderland, J. M. Dent and Sons Ltd., London.
[19] Klingenberg, M. (1976) in: Enzymes of Biological Membranes (Martonis, A. ed) vol. 3, pp. 383–438, Plenum, New York.
[20] Hoch, F. L. (1977) Arch. Biochem. Biophys. 178, 535–545.
[21] Sutherland, E. W., Øye, I. and Butcher, R. W. (1965) Rec. Prog. Horm. Res. 21, 623–642.
[22] Arch, J. R. S. and Newsholme, E. A. (1976) Biochem. J. 158, 603–622.
[23] Hasselbach, W. (1964) Progr. Biophys. Mol. Biol. 14, 167–222.
[24] Weber, A. (1966) Top. Biocenerget. 1, 203–254.
[25] Chan, S. L., Shirachi, P. Y. and Trevor, A. J. (1972) J. Neurochem. 19, 437–447.
[26] Michelson, M. J. and Danilov, A. F. (1971) in: Fundamentals of Biochemical Pharmacology (Bacq, Z. M., Capek, R., Paoletti, R. and Renson, J. eds) pp 221–253, Pergamon, Oxford, New York.
[27] Arch, J. R. S. and Newsholme, E. A. (1978) Essays Biochem. 14, 82–124.
[28] Fisher, D. N. (1980) PhD Thesis, Oxford University.
[29] Newsholme, E. A. (1978) Biochem. Soc. Symp. 43, 183–205.
[30] Newsholme, E. A. (1980) Medisport 2, 155–157.
[31] Sugden, P. H. and Newsholme, E. A. (1975) Biochem. J. 150, 113–122.
[32] Vaage, O., Newsholme, E. A., Grønnerød, O. and Hermansen, L. (1978) Acta Physiol. Scand. 102, 11A–12A.
[33] Newsholme, E. A. and Crabtree, B. (1973) Symp. Soc. Exp. Biol. 26, 429–460.
[34] Crabtree, B. and Newsholme, E. A. (1975) in: Insect Muscle (Usherwood, P. N. R. ed) pp. 405–494. Academic Press, London, New York.
[35] Cohen, P., Nimmo, H. G. and Proud, C. G. (1978) Biochem. Soc. Symp. 43, 69–95.
[36] Katz, J. and Rognstad, R. (1978) Trends Biochemical Sci. 3, 171–174.

Appendix A

The reaction sequence is:

$$A \xrightarrow{E_1} X \xrightarrow{E_2} B$$

Both reactions are non-equilibrium and are assumed to be enzyme catalysed; reaction A to X is zero order (i.e., substrate is present in excess) and the enzyme that catalyses the X to B reaction obeys Michaelis–Menten kinetics. The equation governing this situation is:

$$X = \frac{V_{\max_{E_1}} \cdot K_{m_{E_2}}}{(V_{\max_{E_2}} - V_{\max_{E_1}})}$$

Let $V_{\max_{E_1}}$ be V_1, $V_{\max_{E_2}}$ be V_2 and $K_{m_{E_2}}$ be K. Then

The equation is derived as follows:

$$\frac{dX}{dt} = V_1 - V_2$$

where V_2 represents the actual activity of enzyme E_2. However:

$$V_2 = \frac{V_2 . X}{(K + X)}$$

Then

$$\frac{dX}{dt} = V_1 - \frac{V_2 \cdot X}{(K + X)}$$

When the sequence of reactions A → X → B is in a steady-state condition:

$$\frac{dX}{dt} = 0 \qquad \therefore V_1 - \frac{V_2 X}{(K + X)} = 0$$

$$(V_1 K + V_1 X) - V_2 X = 0$$

$$V_1 K + X(V_1 - V_2) = 0$$

$$\therefore X = \frac{V_1 K}{(V_2 - V_1)}$$

Appendix B

$$V_1 = \frac{V_2 \cdot S}{K_2 + S} + \frac{V_3 \cdot S}{K_3 + S} \qquad \text{(see text)}$$

This equation is solved for S as follows:

$$V_1(K_2 + S)(K_3 + S) = V_2S(K_3 + S) + V_3S(K_2 + S)$$

$$V_1[K_2K_3 + (K_3 + K_2)S + S^2] = [V_2K_3 + V_3K_2]S + [V_2 + V_3]S^2$$

$$[V_2 + V_3 - V_1]S^2 + [V_2K_3 + V_3K_2 - V_1K_3 - V_1K_2]S - V_1K_2K_3 = 0$$

This is equivalent to:

$$aS^2 + bS + c = 0$$

$$S = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

so that:

$$S = \frac{-[V_2K_3 + V_3K_2 - V_1K_3 - V_1K_2]}{2(V_2 + V_3 - V_1)} \pm$$

$$\frac{\sqrt{(V_2K_3 + V_3K_2 - V_1K_3 - V_1K_2)^2 - 4(V_2 + V_3 - V_1)(V_1K_2K_3)}}{2(V_2 + V_3 - V_1)}$$