STUDIES
ON THE MECHANISMS OF PHOSPHORYLASE ACTIVATION
IN SKELETAL MUSCLE

by

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We accept this thesis as conforming to the
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Date September 5th, 1969
ABSTRACT

The effects of epinephrine and electrical stimulation on the activation of glycogen phosphorylase were studied in isolated rat diaphragm and frog sartorius, and rat gastrocnemius in vivo. The resting ratio of phosphorylase, as expressed by the ratio of phosphorylase a to total phosphorylase (-AMP/+AMP ratio) was found to be low of the order of 0.05 in diaphragm and sartorius. In rat gastrocnemius this value was high at 0.26. As measured by the ratio of activity at pH 6.8 to that at pH 8.2 (pH 6.8/8.2 ratio), phosphorylase kinase was essentially in its inactive form under conditions of no stimulation. On treatment with epinephrine, phosphorylase was activated, and there was a significant increase in the activity ratio for phosphorylase kinase, up to 10-fold, indicating that conversion of nonactivated kinase to its activated form had occurred. Epinephrine also produced marked increases, up to 15-fold, in the tissue levels of adenosine 3',5'-monophosphate (cyclic AMP). When muscle was induced to contract by electrical stimulation, phosphorylase was markedly and rapidly activated. In contrast to the effect of epinephrine, electrical stimulation produced no conversion of phosphorylase kinase to its activated form as measured by the pH 6.8/8.2 ratio. This was found for both direct and neural stimulation, at various frequencies, for different times of stimulation, in vitro and in vivo, and in two species. No increase in the tissue levels of cyclic AMP were detected on electrical stimulation. It was concluded that the mechanism of activation of phosphorylase during electrical
stimulation is basically different from that produced by adrenergic amines. The data strongly suggest that during muscle contraction phosphorylase is activated by a mechanism which does not involve conversion of phosphorylase kinase to its activated form.

In further work, the relationship between phosphorylase activation and muscle contraction was studied. It was found that for any given frequency of stimulation, phosphorylase was activated within 2 sec to a particular ratio for that frequency. On further stimulation, the ratio did not increase. When the temperature was lowered, the steady state phosphorylase ratio for a given frequency was lowered, but activation still occurred rapidly. In experiments in which calcium was removed from the medium by using chelating agents, a correlation was demonstrated between phosphorylase activation and contractile tension. From these results it appears that the mechanism of phosphorylase activation is closely coupled to the contractile mechanism. It is proposed that calcium ion, which is important in excitation-contraction coupling and tension development, is responsible for phosphorylase activation. It is further suggested that calcium ion released into the myoplasm may act with nonactivated phosphorylase kinase to catalyse the conversion of phosphorylase \( b \) to phosphorylase \( a \).
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ABBREVIATIONS

Commonly used abbreviations in biochemistry, as well as the system of referencing, have been taken in the most part from The Journal of Biological Chemistry. In particular, the following abbreviations have been used:

phosphorylase  α-1,4-glucan:orthophosphate glucosyltransferase, E.C. 2.4.1.1

phosphorylase kinase  ATP:phosphorylase phosphotransferase, E.C. 2.7.1.37

EDTA  ethylenediaminetetraacetic acid

EGTA  ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetate

Tris  Tris (hydroxymethyl) aminomethane

KAF  kinase activating factor

cyclic AMP  adenosine 3',5'-monophosphate

5'-AMP  adenosine 5'-phosphate

ATP  adenosine 5'-triphosphate
FOR VIVIENNE
1 INTRODUCTION

1.1 General and Historical

Prior to 1956, biochemistry had been mainly concerned with the elucidation of metabolic pathways and the isolation and characterisation of the related enzymes. Mechanisms for the integration and control of metabolism were not known, although mass action effects were considered to play some role. The first clear demonstration of metabolic control at the molecular level was provided by the studies of Umbarger in 1956. In disrupted cells, he showed that isoleucine specifically inhibited threonine dehydrase, the first enzyme unique to the synthesis of isoleucine. This type of end product inhibition was demonstrated also in the biosynthetic pathway for pyrimidines at about the same time by Yates and Pardee. This pioneering work began an era in the study of biological control mechanisms, and it has become clear that other mechanisms, besides end product inhibition, serve to control the activity of enzymes within the cell. Some of these mechanisms are important in the control of glycogenolysis in activating and inactivating phosphorylase, the enzyme which catalyses the breakdown of glycogen. The subject of this thesis is a study of some of the factors which control the activity of glycogen phosphorylase in skeletal muscle.

Epinephrine, and catecholamines generally, exert marked physiological effects which have been known since the turn of the century when adrenal medullary extracts were found to be active following intravenous administration. Langley noted that such extracts exerted physiological actions which were similar to those produced by
stimulation of sympathetic nerves. These observations led to the hypothesis that chemical substances were released at nerve endings to mediate the effects of neural stimulation. Over the following decades, a heated controversy ensued as to whether transmission at the nerve ending was electrical or humoral. Following the critical experiments of Loewi\textsuperscript{4} beginning in 1921, in which he showed that vagal stimulation of one heart released a substance into the perfusate which modified the rate of a second heart, the humoral theories gradually became accepted. In 1946, Von Euler\textsuperscript{5} showed that norepinephrine, rather than epinephrine, was the chemical transmitter at the sympathetic nerve ending. Although variation is encountered from species to species, epinephrine is the chief amine released from the adrenal medulla.

In addition to their characteristic physiological actions, it had been known for a number of years that the catecholamines could profoundly influence metabolism, particularly the metabolism of carbohydrate and fat. In studying the mechanism by which epinephrine and glucagon stimulated glycogenolysis in the liver, Sutherland and Rall\textsuperscript{6} found that the effect was associated with the increased activity of glycogen phosphorylase. Hepatic phosphorylase was found to exist in an active and an inactive form. Enzymes capable of catalysing the interconversion of the two forms, namely phosphorylase phosphatase and dephosphophosphorylase kinase were isolated and purified. Addition of epinephrine to supernatant fractions of liver homogenate did not stimulate phosphorylase. Hormone treatment of particulate fractions, however, led to the accumulation of a heat-stable factor, which when added to the supernatant containing the enzymes of the
phosphorylase system, led to marked activation of phosphorylase. The heat-stable factor was identified as adenosine 3',5'-monophosphate (cyclic AMP). (For references to early work see Sutherland and Rall.)

Although cyclic AMP was discovered in the liver, this tissue has received little attention in the study of cyclic AMP mediated hormonal actions. Part of the reason for this is that the enzymes are atypical when compared to those of cardiac and skeletal muscle, the liver enzymes being dephosphophosphorylase (inactive) and phosphophosphorylase (active) and dephosphophosphorylase kinase; the inactive form of phosphorylase shows only weak activity in the presence of 5'-AMP. The liver may, however, be useful in studying different mechanisms of phosphorylase activation. The hyperglycaemic response of the liver might have physiological significance with respect to circulating epinephrine released from the adrenal medulla. Although it has been established that transmission at the sympathetic nerve ending is adrenergic, it is not known to what extent the small quantities of amine released are involved in any biochemical changes produced by nerve stimulation. Shimazu and Amakawa have shown that stimulation of the splanchnic (sympathetic) nerve to the liver caused phosphorylase activation, an effect which was not abolished by adrenalectomy. Whether or not phosphorylase activation was mediated by cyclic AMP has not been elucidated. In a further study, these authors compared phosphorylase activation in liver as produced by epinephrine and splanchnic nerve stimulation. Differences were observed in the blocking activity of the beta adrenergic blocking agent, dichloroisoprenaline (DCI), and the authors suggested that
phosphorylase was being activated by two different mechanisms. As discussed later, stimulation of somatic nerves to skeletal muscle, where the transmitter is acetylcholine rather than epinephrine, also results in phosphorylase activation. It will be shown in this thesis that neural or direct stimulation of skeletal muscle causes phosphorylase activation by a non-adrenergic mechanism.

1.2 Cyclic AMP as a Mediator of Hormonal Action

Since the original observations in which cyclic AMP was implicated in mediating the effect of epinephrine in promoting glycogenolysis in liver, the nucleotide has been found in many tissues from four phyla. The concentration of the nucleotide has been found to vary in response to a large number of hormones. Table I is a partial list of those tissues in which cyclic AMP has been located, and the hormones which modify the intracellular levels of the nucleotide. From a consideration of the numerous reactions in which cyclic AMP was involved in the expression of hormonal effects, Sutherland, Oye and Butcher proposed the second messenger theory. According to this hypothesis, a hormone interacts with a component of the cell membrane which catalyzes the intracellular formation of cyclic AMP from ATP. The nucleotide then acts as a second messenger, and the particular response is characteristic of the cell species. Although this theory accounts for phosphorylase activation in different tissues by different hormones (cyclic AMP is the common second messenger), it does not explain how certain hormones act specifically upon particular tissues. Furthermore, it does not account for phosphorylase activation induced by electrical stimulation.
TABLE I

Tissues Containing Cyclic AMP, and Some Hormones Which Change the Concentration of the Nucleotide

The following abbreviations are used in the Table: ACTH, adrenocorticotropic hormone; ICSH, interstitial cell stimulating hormone; LH, luteinising hormone; MSH, melanocyte stimulating hormone; TSH, thyroid stimulating hormone.

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<tr>
<th>Tissue</th>
<th>Hormone</th>
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<tr>
<td>Adipose Tissue</td>
<td>Catecholamines, ACTH, TSH, LH, Glucagon, Insulin, Prostaglandins</td>
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<tr>
<td>Adrenal Cortex</td>
<td>ACTH</td>
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<tr>
<td>Corpus Luteum</td>
<td>LH</td>
</tr>
<tr>
<td>Ovary</td>
<td>LH</td>
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<tr>
<td>Testis</td>
<td>ICSH</td>
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<tr>
<td>Uterus</td>
<td>Catecholamines</td>
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<tr>
<td>Heart</td>
<td>Catecholamines, Glucagon</td>
</tr>
<tr>
<td>Liver</td>
<td>Catecholamines, Glucagon, Insulin</td>
</tr>
<tr>
<td>Frog Skin</td>
<td>MSH, Catecholamines, Melatonin</td>
</tr>
<tr>
<td>Toad Bladder</td>
<td>Vasopressin</td>
</tr>
<tr>
<td>Kidney</td>
<td>Vasopressin, Prostaglandins</td>
</tr>
<tr>
<td>Kidney and Bone</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>Parotid Gland</td>
<td>Epinephrine</td>
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<tr>
<td>Pancreas</td>
<td>Glucagon, Catecholamines</td>
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For these reasons, attention has been focussed upon the enzyme system responsible for the formation of the cyclic nucleotide, adenyl cyclase. Early studies by Sutherland's group\textsuperscript{11} showed that the enzyme was widely distributed and was present in all mammalian tissues studied with the exception of mature mammalian erythrocytes. It has also been found in birds, reptiles and amphibia, several invertebrates including insects, segmental and flat worms, and in such unicellular organisms as bacteria and slime moulds. In some tissues the enzyme is highly specific; for example, the corpus luteum responds to luteinising hormone alone,\textsuperscript{12} whereas in the isolated fat cell, epinephrine, norepinephrine, glucagon, adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH) and luteinising hormone (LH), all serve to activate the enzyme.\textsuperscript{13} Hormonal specificity may reside in the adenyl cyclase of the particular tissue. Robison, Butcher and Sutherland\textsuperscript{14} have proposed a model for adenyl cyclase based upon the aspartate transcarbamylase/CTP system.\textsuperscript{2} In the model, adenyl cyclase, a particulate enzyme, is part of the cell membrane. The enzyme consists of at least two subunits, a regulatory subunit, which faces the extracellular fluid, and a catalytic subunit, the active centre of which is in contact with the cell interior. A hormone interacts with the regulatory subunit which then modifies the activity of the catalytic unit. The receptor subunit on the cell surface is thought to vary and confer specificity.

1.3 \textbf{Phosphorylase Activation in Cardiac Muscle}

As a result of the pronounced physiological effects of the catecholamines in heart, the phosphorylase system has been studied in
this tissue to elucidate the relationship, if any, between metabolic and contractile events. Hess and Haugaard\textsuperscript{15} demonstrated an increase in phosphorylase a in perfused rat hearts with epinephrine, and Belford and Feinleib\textsuperscript{16} showed that epinephrine increased phosphorylase a activity and contractile force when applied to electrically driven rat atria. Initial dose studies indicated that a close correlation existed between phosphorylase activation and the inotropic effect. Increased energy mobilisation as a result of activation of the enzyme was considered to be responsible for the increase in contractile force. Later studies by Mayer, Cotten and Moran,\textsuperscript{17} using open-chest dog heart preparations, differentiated the two effects by showing that low doses of epinephrine significantly increased ventricular contractile force without altering phosphorylase a levels. Similarly, studies in perfused rat hearts by Drummond, Valadares and Duncan\textsuperscript{18} showed that low doses of epinephrine caused significant increases in contractile force without augmenting phosphorylase a levels. At higher doses, both events occurred, but these results indicated that no direct correlation existed between the two events, and that epinephrine could affect the contractile system without influencing phosphorylase levels. Williamson and Jamieson\textsuperscript{19} corroborated these findings using another technique and concluded\textsuperscript{20} that neither energy nor the intermediates of glycogenolysis were responsible for the inotropic action of epinephrine. The problem was to decide whether a common mechanism caused the two events, but at a different rate, or whether the metabolic events followed as a result of the increased demand for energy. To investigate this problem, temporal studies on the activity of phosphorylase and tissue levels of
cyclic AMP have been performed. Sutherland and Robison\textsuperscript{21} found that
epinephrine administration to perfused rat hearts produced a
transient increase in cyclic AMP levels, followed by an inotropic
response which was at its greatest in 20 sec, in turn followed by
maximum phosphorylase \textsubscript{a} levels in 45 sec. These results suggested
that since cyclic AMP levels increased before either event, it was
possible that the nucleotide was responsible for both the inotropic
response and phosphorylase activation.

If cyclic AMP was directly responsible for the inotropic
effect, it should be possible to elicit the same response by infusion
of the nucleotide into the heart. Such attempts have been largely
unsuccessful because the nucleotide does not traverse biological
membranes. However, Levine and Vogel\textsuperscript{22} have reported both an increase
in heart rate and cardiac output in dogs following intravenous injec­
tion of high non-physiological doses (4 to 8 mg/kg) of cyclic AMP.
Whether the effects can be attributed to the low doses of the nucleo­tide actually entering the cells, or indirect effects such as the
fall in blood pressure which occurred, cannot be adequately assessed.

In further studies of the role of cyclic AMP in the inotropic
response, Cheung and Williamson\textsuperscript{23} showed that continuous infusion of
epinephrine caused the levels of the nucleotide to increase and then
fall to a new but elevated level, while the contractile force reached
a plateau. These experiments showed that although cyclic AMP may be
involved in initiating inotropism, it was not responsible for main­
taining the effect. These authors\textsuperscript{23} suggested that the nucleotide
was involved in some indirect way and might serve to increase the
availability of ionised calcium in the muscle. Although there is no
evidence for this hypothesis, the concept that calcium may be important in phosphorylase activation, particularly in skeletal muscle during contraction, will be discussed later.

To establish the epinephrine induced sequence of phosphorylase activation in cardiac muscle, the effects of the amine on phosphorylase and phosphorylase kinase activities and cyclic AMP levels have been studied. Krebs, Graves and Fischer\textsuperscript{24} had shown that phosphorylase kinase can be extracted from skeletal muscle in a form which is essentially inactive at pH 7 and below, but at higher pH the activity increases to a maximum at pH 8.5. The enzyme is converted to an activated form by incubating with ATP and magnesium ion, a reaction which is facilitated by cyclic AMP. The activated form of phosphorylase kinase is active at both physiological pH and pH 8.2. A measure of the activation of this enzyme is obtained from the ratio of activity at these two pH values. Hammermeister, Yunis and Krebs\textsuperscript{25} using perfused rabbit hearts found that epinephrine increased phosphorylase and cyclic AMP levels; the resting pH 6.8/8.2 ratio for phosphorylase kinase was high in unstimulated hearts, and did not increase with epinephrine. Drummond and Duncan\textsuperscript{26} using refined assay controls were able to show that phosphorylase kinase was essentially in the nonactivated form in perfused rat hearts, and that the ratio was rapidly increased by epinephrine.

The studies which have been described above have established that epinephrine induced phosphorylase activation in cardiac muscle is associated with increased intracellular levels of cyclic AMP; this is followed by conversion of nonactivated phosphorylase kinase to its
activated form, and the subsequent conversion of phosphorylase $b$ to phosphorylase $a$. There is still no clear indication as to how epinephrine produces the inotropic effect or the extent to which cyclic AMP is involved in the contractile event, if at all. Whether or not phosphorylase activation can be achieved in cardiac muscle by mechanisms which do not involve activation of phosphorylase kinase or increased cyclic AMP levels is not known. Phosphorylase activation occurs during anoxia, and there is conflicting evidence for the involvement of adrenergic mechanisms in this effect. Mayer, Williams and Smith\textsuperscript{27} showed that pretreatment with reserpine had no effect, but the beta blocking drug, pronethalol, partially blocked the increase in phosphorylase $a$ activity in the anoxic rat heart. Elevation of calcium\textsuperscript{28} and potassium depolarization\textsuperscript{29} have also been reported to increase phosphorylase $a$ levels in perfused rat hearts. Although mediation of this effect through release of catecholamines appears unlikely, the mechanisms responsible have not been elucidated.

1.4 Phosphorylase Activation in Smooth Muscle

Depending on the location, species and state of the tissue, epinephrine exerts a variety of actions on smooth muscle, and attempts have been made to link the biochemical and physiological events as in cardiac muscle. In uterine muscle, Diamond and Brody\textsuperscript{30} showed that epinephrine increased the activity of phosphorylase $a$ while depressing motility. A correlation between the two events was shown in dose studies and with beta adrenergic blocking drugs. A series of smooth muscle stimulants, including acetylcholine, serotonin and barium
chloride, were found to increase the motility of rat uterus, and once again phosphorylase was activated. Thus, according to these observations, phosphorylase can be activated in smooth muscle under conditions of increased and decreased motility, and it is difficult to explain how the same mechanism would be involved in both cases. The enzymes responsible for one mechanism of phosphorylase activation in smooth muscle have been studied by Mohme-Lundholm. A phosphorylase kinase has been shown to be present and increased levels of cyclic AMP have been reported to occur in response to epinephrine in guinea pig taenia coli. Phosphorylase activation in smooth muscle associated with decreased motility appears to be qualitatively similar to the adrenergic mechanism in cardiac and skeletal muscle. It is not known whether cyclic AMP has any role in relaxation. It is not known how phosphorylase activation occurs under conditions of increased motility, but it is probably not associated with increased levels of cyclic AMP, and may be related to ionised calcium levels.

1.5 Phosphorylase Activation in Skeletal Muscle

From experiments on frog and rat skeletal muscle, Cori in 1956 showed that the rate of glycogen breakdown was increased several hundred-fold during tetanic stimulation. Resting levels of phosphorylase were high, but Cori suggested even at that time that these values were the result of inadequate methodology and predicted that only 1% of the phosphorylase need be in the active form to account for the rate of glycogen breakdown in resting muscle. Electrical stimulation produced a rapid increase in phosphorylase and Cori
concluded that a correlation may exist between the rate and amount of work performed and the degree to which phosphorylase became activated during the contraction process. Using the excised anterior tibial muscle of the mouse, Rulon, Schottelius and Schottelius\textsuperscript{35} found that phosphorylase levels increased rapidly with the onset of electrical stimulation, the degree of activation increasing with the time of stimulation. They concluded that phosphorylase was activated sufficiently rapidly to bring about increased rates of glycogenolysis relatively early during tetanic stimulation.

To study the relationship between phosphorylase activation and the contractile mechanism, Danforth, Helmreich and Cori\textsuperscript{36} examined the effects of electrical stimulation and epinephrine on phosphorylase activation in the sartorius muscle of the frog. Because of the high resting levels of phosphorylase in the two previous studies,\textsuperscript{34,35} they paid particular attention to preparative procedures. By freezing the tissue at \(-160^\circ\) and homogenising in a glycerol-containing buffer at \(-30^\circ\) to prevent activation of phosphorylase by phosphorylase kinase during the extraction procedure, they obtained much lower resting levels of phosphorylase. They were then able to demonstrate more clearly the changes produced on electrical and adrenergic stimulation. During electrically induced contractions phosphorylase was activated extremely rapidly with a half time of 0.7 sec. On incubation with epinephrine at 30°, phosphorylase was activated, but much more slowly with a half time of 370 sec. From a kinetic analysis of the rate of activation and inactivation of phosphorylase during electrical stimulation, these authors concluded that activation was due to changes in
the activity of phosphorylase kinase, although no measurements of the latter were actually performed. They considered that phosphorylase activation by the two types of stimulation were occurring by different mechanisms at the phosphorylase kinase level. The only real evidence that the two mechanisms might be different was provided by the observation that dichloroisoprenaline inhibited the effect of epinephrine, but had no effect on the increase in phosphorylase activity produced during muscle work.

The first experimental evidence that the mechanisms for phosphorylase activation in skeletal muscle produced by epinephrine and electrical stimulation might be different was provided by the studies of Posner, Stern and Krebs. They compared the effects of the two types of stimulation in rat gastrocnemius and frog sartorius in vivo. In both rat and frog, intracardial injection of epinephrine increased the level of phosphorylase activity, the pH 6.8/8.2 activity ratio for phosphorylase kinase, and the tissue concentration of cyclic AMP. Electrical stimulation produced a rapid and marked increase in phosphorylase activity in frog muscle and an increase in the pH 6.8/8.2 ratio for phosphorylase kinase. In the rat, stimulation of the posterior tibial nerve produced some increase in the activity of both enzymes, but the results were variable. In both tissues, the levels of cyclic AMP remained unchanged on electrical stimulation. The cyclic nucleotide appeared to be involved in the mediation of the effects of epinephrine, but not those of electrical stimulation. Both types of stimuli increased the pH 6.8/8.2 ratio for phosphorylase kinase. It appeared, therefore, that during contraction, phosphorylase kinase was being converted to its activated form by a mechanism not
Involving cyclic AMP.

Other mechanisms have been demonstrated which increase glyco-
genolysis without the conversion of phosphorylase \( b \) to phosphorylase \( a \). Phosphorylase \( b \) is normally inactive except in the presence of 5'-AMP which acts as a positive effector with a \( K_m \) of \( 5 \times 10^{-5} \) M. Phosphorylase \( b \) was considered to be catalytically inactive in resting tissue, but it then became apparent that tissue concentrations of 5'-AMP were sufficiently high to keep the enzyme active at all times. Studies by Parmeggiani and Morgan\(^38\) showed, however, that ATP was an inhibitor of the action of 5'-AMP on phosphorylase \( b \). Under resting conditions the enzyme would be essentially inactive but critically dependent upon the relative levels of the two nucleotides. In simulated studies of the phosphorylases under aerobic and anaerobic conditions, these authors\(^39\) concluded that physiological regulation of glycogenolysis occurred by two completely different mechanisms: the conversion of phosphorylase \( b \) to \( a \), and the regulation of the activity of phosphorylase \( b \) by changes in the relative concentration of the two effectors.

Karpatkin, Helmreich and Cori\(^40\) examined the effects of electrical stimulation and epinephrine on glycogen breakdown in skeletal muscle by measuring the concentrations of glucose 6-phosphate and lactate. Electrical stimulation was found to increase the levels of both intermediates, whereas epinephrine increased only glucose 6-phosphate levels. Although these observations do not in themselves provide evidence for different mechanisms of phosphorylase activation, the two types of stimulation clearly differ physiologically. The authors concluded that electrical stimulation increased the activities of both phosphorylase and phosphofructokinase (a key enzyme of
glycolysis), whereas epinephrine increased only phosphorylase activity. The physiological relationship between the two types of stimulation in increasing the levels of phosphorylase a is not clear. Danforth and Helmreich reported that when repetitive stimuli were applied to frog sartorius, there was a lag period before levels of phosphorylase a increased. Low doses of epinephrine which increased phosphorylase a levels slightly were found to abolish the lag period.

There is evidence as indicated above that glycogenolysis in skeletal muscle can be effected by a number of mechanisms. In particular, increased levels of phosphorylase a can be produced by epinephrine and during electrically induced contraction. The subject of this thesis is to further investigate the mechanisms by which phosphorylase becomes activated in skeletal muscle. The effects of electrical stimulation and epinephrine have been studied on phosphorylase, phosphorylase kinase and cyclic AMP levels in isolated rat diaphragm, isolated frog sartorius and rat gastrocnemius in vivo.
2.1 Materials

2.1.1 Purification of Glucose 1-Phosphate

Glucose 1-phosphate was purified in the following manner. The sodium or potassium salt, 10 g, was dissolved in 40 ml of water, and to this solution was added 3 ml of magnesia mixture. The alkaline solution was allowed to remain at 2° overnight. The precipitate of magnesium ammonium phosphate was removed by centrifugation. About 40 g of washed Amberlite IR-120 (H\(^+\) - form) was added to the ice-cold solution until the pH dropped to 2.5 to 3.0, and no longer gave a positive test for magnesium ion with ammoniacal phosphate solution. The ion exchange resin was removed by filtration and the cold solution adjusted to pH 8.0 with 5 N KOH. One volume of cold absolute ethanol was added to effect crystallisation. After several hours at 4°, the crystals were filtered off, and the material re-crystallised from 50% ethanol. The crystals were finally washed in absolute ethanol and dried in vacuo.

2.1.2 Purification of Glycogen

Commercial glycogen, 25 g, was dissolved in water. Ten g of purified charcoal (Norit A), which had been washed four times to remove fines, was added, and the mixture stirred for 10 min. The solution was filtered through a charcoal filter bed on a large Buchner funnel with the aid of suction. The filtrate was adjusted to pH 8.0 with 1 N KOH, and 10 g of washed Dowex 1-X4 (Cl\(^-\)-form) added, and stirred for 10 min. The ion exchange resin was removed by filtration and the filtrate was brought to 50% ethanol with
stirring and left in ice for 1 hour. The precipitated glycogen was removed by centrifugation, resuspended in 300 ml of 95% ethanol, stirred for 15 min and centrifuged. The material was dried in an evacuated desiccator over potassium hydroxide pellets.

2.1.3 Preparation of Phosphorylase b

Phosphorylase b was prepared essentially as described by Fischer and Krebs. A rabbit was killed by injection into the ear vein of a lethal dose of pentobarbitone (300 mg dissolved in 2 ml of water). The animal was bled from the neck, skinned, and after 5 min the skeletal muscle was removed from the hind limbs and back. The muscle was weighed, passed through a meat grinder and homogenised with an equal volume of water in a Waring blender. The extract was stirred by hand for 10 min, and filtered through two layers of cheese cloth into a large flask chilled in ice. The homogenisation and extraction were repeated once with an equal volume of water, and again with a half volume of water. The pooled extract at 4° was adjusted to pH 5.1 with 1 N acetic acid and centrifuged at 10,000 x g for 10 min at 4°. The supernatant was filtered through a #5 filter paper on a large Buchner funnel, and the cold filtrate adjusted to pH 6.8 by adding solid potassium bicarbonate. Saturated ammonium sulphate solution was added slowly to the filtrate to give 40% saturation (700 ml of saturated ammonium sulphate solution per litre of filtrate), and left overnight in the cold room. The supernatant was syphoned off, and the sedimented protein was centrifuged at 10,000 x g for 15 min at 4°. The residue was dissolved in 20 ml of water and dialysed against 1 mM Tris-HCl buffer pH 7.5 in the cold room for 6 hours. The
following solutions were added to the dialysed protein: (1) freshly neutralised 0.3 M cysteine to give a final concentration of 0.03 M, (2) neutral 0.1 M EDTA to give a final molarity of 0.5 mM, and (3) 2 M unbuffered Tris to adjust the pH to 8.8. The mixture was incubated for 1 hour at 37°, cooled, adjusted to pH 7.0 with 1 N acetic acid, and centrifuged. Phosphorylase was crystallised by adding to the supernatant 1/100 volume of 0.1 M 5'-AMP and 1/200 volume of 2 M magnesium acetate. The solution was cooled and left overnight. The crystals were collected by centrifugation and dissolved in a minimum quantity of neutral 0.03 M cysteine (about 20 ml) by warming to 30°. Recrystallisation was effected by adjusting the solution to 1 mM 5'-AMP and 10 mM magnesium acetate as above. After three more crystallisations (the last using 1/10 the concentration of 5'-AMP) the nucleotide was removed by stirring the solution with 1 g of Dowex 1-X4 (Cl^- form). The enzyme was assayed in the presence and absence of 5'-AMP, and the final solution contained not less than 100,000 Cori units/ml.43 Phosphorylase was stored in the refrigerator at 4° under toluene.

2.1.4 Preparation of Nonactivated Phosphorylase Kinase

Nonactivated phosphosylase kinase was prepared from rabbit muscle according to the method of Krebs et al.44 A large rabbit was injected via the ear vein with a lethal dose of pentobarbitone; the animal was bled from the neck, skinned and the muscle removed and placed on ice. All subsequent procedures were performed in the cold room or at 4°. The muscle was passed through a meat grinder, then homogenised immediately in 2.5 volumes of 4 mM EDTA in a Waring blender.
for 1 min at full speed. The extract was centrifuged at 4000 x g for 40 min, and the supernatant decanted through glass wool to remove lipid. The filtrate was adjusted to pH 6.1 with 1 N acetic acid, left for 10 min, then centrifuged at 4000 x g for 30 min. The supernatant was retained for the preparation of protein kinase, which is described in section 2.1.5. The residue was dissolved in a minimal volume of 0.1 M β-glycerophosphate (sodium β-glycerophosphate), 4 mM EDTA pH 8.2, and then 50 mM β-glycerophosphate and 2 mM EDTA pH 7.0 was added to give a final volume of 165 ml per 1000 g of tissue used. The residue was homogenised briefly in a Potter-Elvehjem homogeniser to effect complete dispersal of the material. The final pH was between 6.8 and 7.2. The suspension was centrifuged in a Spinco Model L preparative ultracentrifuge for 90 min at 78,000 x g. The clear supernatant (30s fraction) was assayed and stored at -18°. One unit of phosphorylase kinase activity was defined as that amount of enzyme which produced 100 units of phosphorylase a in 5 min under the conditions of the assay.

2.1.5 Preparation of Protein Kinase

Protein kinase was prepared as described by Walsh, Perkins and Krebs, from rabbit skeletal muscle to the 78,000 x g supernatant stage. The pH 6.1 supernatant obtained in the preparation of phosphorylase kinase (section 2.1.4) was adjusted to pH 5.5 with 1 N acetic acid, left in ice for 10 min, and centrifuged at 4000 x g for 30 min. The supernatant was adjusted to pH 6.8 with 1 M potassium phosphate buffer pH 7.2, and fractionated by the addition of 32.5 g of ammonium sulphate per 100 ml of solution. The sediment was collected by centrifugation at 4000 x g for 30 min and dissolved in
5 mM potassium phosphate buffer pH 7.0, containing 2 mM EDTA using 60 ml per kg of muscle used. The solution was dialysed extensively against the same buffer, and centrifuged in the Spinco Model L preparative ultracentrifuge for 60 min at 78,000 x g. The supernatant was stored at -18°.

2.2 Preparation of Tissues

2.2.1 Rat Diaphragm

Adult female Wistar rats weighing about 200 g were killed by a blow on the head and bled from the neck. The diaphragm, attached to the rib cage, was removed as quickly as possible and placed in ice-cold Tyrode's solution having the following composition (mM): NaCl, 137; KCl, 2.68; CaCl₂, 1.36; NaHCO₃, 11.9; MgCl₂, 0.49; NaH₂PO₄, 0.36; glucose, 11.1; and gassed with 95% oxygen and 5% carbon dioxide. For some experiments, the phrenic nerve was dissected out on each side. The diaphragm was divided into two, trimmed of any fat and extraneous tissue, mounted on a perspex frame by means of three pins, and arranged in a tissue bath containing 100 ml of Tyrode's solution at 37°. A thread from the tissue was attached to a Grass FT03 force displacement transducer and recordings were made on a Grass polygraph. A tension of 5 g was applied to the muscle and a few single shocks were delivered to ensure that the preparation was responsive. A 15 min equilibration period preceded each experimental procedure. At the appropriate instant, the bath was dropped and the tissue frozen with a pair of tongs prechilled in liquid nitrogen while the stimulus was still being applied. The preparation was plunged into liquid nitrogen and any tissue outside the tongs was
removed. The sample was stored in liquid nitrogen until assay.

For direct electrical stimulation of the muscle, 5 volt pulses of 2 msec duration were provided by a Grass stimulator. When stimulating the diaphragm via the phrenic nerve, 4 volt pulses of 0.02 msec duration were used. Diaphragms to be treated with epinephrine were prepared in the same way, but 1 min before addition of the drug (final concentration 10 µg/ml), freshly prepared ascorbic acid was added to the bath to give a final concentration of 1 mg/ml. In those experiments in which the development of tension during tetanus was of interest, an initial pre-tetanus was delivered. After treatment with EDTA or EGTA, a similar tetanus was applied. The tension developed was calculated in grams and the final tension expressed as a percentage of the initial tension. Controls were treated in an identical manner to experimental tissues except that no stimuli were applied.

2.2.2 Frog Sartorius

Various populations of Leopard frogs (Rana pipiens) were used in the experiments. A frog was pithed, the sartorii dissected out, and mounted in a 50 ml tissue bath at 20° in Krebs-Ringer's bicarbonate solution adapted for frog muscle and having the following composition (mM): NaCl, 83; NaHCO₃, 25; KCl, 2; KH₂PO₄, 1.2; CaCl₂, 2.5; MgCl₂, 3.0. The solution was gassed with 95% nitrogen and 5% carbon dioxide. The recording arrangement was similar to that used for diaphragm. The preparation was allowed to equilibrate for 15 min, bearing 0.5 g of tension. Fifteen volt pulses of 2 msec duration were applied directly through a pair of fine wire electrodes placed against
the muscle. For epinephrine treated tissues, ascorbic acid (to give a final concentration of 0.1 mg/ml) was added 1 min before the epinephrine, 5 μg/ml final concentration. At the required time the muscle was frozen rapidly as described for diaphragm. Appropriate controls were treated in a similar manner but with no stimulation.

2.2.3 Rat Gastrocnemius

Rats were anaesthetised by intraperitoneal injection of pentobarbitone, 60 mg/kg. The skin was detached at the ankle and pulled back to expose the gastrocnemius. A firm thread was attached to the Achilles tendon, the distal end of the tendon cut, the thread attached to a transducer, and 10 g of tension applied to the muscle. Fine wire electrodes were inserted into the gastrocnemius and during the 20 min equilibration period, a lamp was placed above the muscle to prevent cooling. The muscle was stimulated for 5 sec with 15 volt pulses of 2 msec duration. At the appropriate instant while the stimulus was being applied, the entire leg below the knee was crushed between large aluminum tongs chilled in liquid nitrogen. The leg was rapidly severed from the animal and plunged into liquid nitrogen. The gastrocnemius was dissected from the frozen tissue and stored in liquid nitrogen. In other animals, epinephrine was injected intracardially with a dose of 5 μg/kg, and after 40 sec, the prepared leg was frozen as above. The success of the injection was judged by an increase in heart rate. No tension was applied to the epinephrine treated muscles, because of the manipulation required to inject intracardially. Tissues from control animals were prepared in a similar manner; only one gastrocnemius from each animal was used as a control or stimulated
muscle. In some experiments the leg of control animals was frozen without removing the skin. This was done to determine whether the rather high resting levels of phosphorylase a (see Results, section 3.4) were due to trauma imposed by surgery.

2.3 **Homogenisation and Preparation of Extracts**

The stored samples were weighed and ground to a fine powder under liquid nitrogen. Diaphragm and gastrocnemius were pulverised in a suitably constructed stainless steel mortar and transferred to a prechilled Potter-Elvehjem homogeniser tube. Frog sartorii were conveniently powdered in a small homogeniser tube (immersed in liquid nitrogen), with the aid of a glass rod. The powdered material was thoroughly homogenised in 10 volumes (5 volumes for diaphragm) of a buffer containing 40 mM β-glycerophosphate, 5 mM EDTA, 20 mM sodium fluoride, and 30 mM cysteine pH 6.8, containing 60% glycerol at -35°, in a bath of 50% ethylene glycol and dry ice. When the powder was thoroughly dispersed an equivalent volume of a similar buffer containing no glycerol was added. The tube was immersed in an ice-water bath and homogenisation continued for a further 2 min. The homogenate was centrifuged at 12,000 x g for 10 min and the supernatant was assayed for phosphorylase and phosphorylase kinase immediately.

2.4 **Phosphorylase Assay**

The extracts were assayed for phosphorylase in the direction of glycogen synthesis using the micromethod described by Mayer, Cotten and Moran. Duplicate 0.05 ml volumes of a dilution of the extract (usually 10-fold) prepared in 20 mM sodium fluoride, 2 mM EDTA, were incubated with 0.05 ml of the appropriate assay mix for 10 min at 30°.
For the determination of phosphorylase a, the assay mix contained 33 mM purified glucose 1-phosphate and 2% purified glycogen pH 6.1 (-AMP); for the assay of total activity, the assay mix contained the same ingredients as above together with 2 mM 5'-AMP (+AMP). The reaction was terminated by the addition of 1 ml of Buell's phosphate reagent*. Twenty min later, the optical density was read in a 1.5 ml 1 cm cell in a Beckman DU spectrophotometer at 700 nm. Tissue blanks, in which 0.05 ml of the diluted extract was added after the phosphate reagent, were carried in the assay in order to correct for inorganic phosphate and protein in the extract.

Assay in the absence of 5'-AMP (-AMP) gave a measure of phosphorylase a activity, and in the presence of 5'-AMP (+AMP) the total phosphorylase activity, a plus b. The activation of phosphorylase was expressed as the ratio of phosphorylase a to total activity (-AMP/+AMP). A standard solution of inorganic phosphate was prepared from pure disodium phosphate (Na₂HPO₄) to contain 4 μmoles of inorganic phosphate per ml. A standard curve was constructed and all optical density readings obtained in the microassay of phosphorylase have been expressed as μmoles of inorganic phosphate released per g of tissue in 10 min at 30°.

*Buell's phosphate reagent contained 600 mg of Buell's powder, 10 ml of 2.5% ammonium molybdate solution, 0.2 ml of 1% copper sulphate solution, water to 90 ml, and the pH adjusted to 2.3 to 2.5 with 10% perchloric acid.
Buell's powder contained sodium bisulphite 9.5 g, sodium sulphite 0.45 g, and l-amino-2-naphthol-4-sulphonic acid 0.05 g, finely triturated in a mortar.
2.5 **Phosphorylase Kinase Assay**

Phosphorylase kinase was assayed by its ability to catalyse the conversion of rabbit skeletal muscle phosphorylase \( b \) to phosphorylase \( a \) in the presence of ATP and magnesium. The assay system consisted of the following components which were added to tubes kept in an ice bath: 0.2 ml of 0.125 M Tris-HCl/0.125 M \( \beta \)-glycerophosphate buffer at pH 6.8 or 8.5, 0.1 ml of a solution containing 18 mM ATP and 60 mM magnesium acetate, and 0.1 ml of recrystallised skeletal muscle phosphorylase \( b \) containing 10,000 Cori units of phosphorylase activity. The tubes were then placed in a bath at 30° and after a 1 min equilibration period, 0.2 ml of an appropriate dilution (usually 15-fold) of the extract which had been prepared immediately before the assay in 15 mM neutral cysteine, was added to start the reaction. After incubating for 5 min the reaction was stopped by diluting a 0.1 ml aliquot into 1.9 ml of dilution buffer at 4° containing 30 mM cysteine and 40 mM \( \beta \)-glycerophosphate pH 6.8. This solution was assayed immediately for phosphorylase \( a \). A 0.2 ml aliquot of this dilution was incubated for 5 min at 30° with 0.2 ml of a mixture of 32 mM glucose 1-phosphate and 2% glycogen pH 6.8. The reaction was stopped by the addition of 8.2 ml of ammonium molybdate solution*. To each tube was added 0.9 ml of 5 N sulphuric acid followed by 0.5 ml of reducing solution**. Ten minutes later the blue

*Ammonium molybdate solution contained ammonium molybdate 9.15 g, 0.244 N sulphuric acid 750 ml, and water to 3 l.

**Reducing solution contained 1 g of reducing powder in 40 ml of water.
Reduction powder contained sodium bisulphite 1.2 g, sodium sulphite 1.2 g, and 1-amino-2-naphthol-4-sulphonic acid, 0.2 g finely triturated.
colour was read in a Klett colourimeter with a 660 nm filter.

For controls, the diluted muscle extract was added to the kinase assay system, phosphorylase b being omitted. After termination of the reaction by dilution, the appropriate amount of phosphorylase was added and the assay completed in the usual way. This procedure enabled a correction to be applied for protein and inorganic phosphate in the assay system. All assays were performed in duplicate at pH 6.8 and 8.2. One unit of phosphorylase kinase activity was defined as that amount of enzyme which catalysed the formation of 100 units of phosphorylase a in 5 min under the conditions of the assay. The data has been expressed as units of activity per g of tissue at pH 6.8 and pH 8.2, and the degree of activation expressed as the ratio of activity at these two pH values.

2.6 Preparation of Samples and Assay of Cyclic AMP

For the assay of tissue levels of cyclic AMP in diaphragm it was necessary to pool several hemidiaphragms to obtain sufficient tissue with which to work. Six hemidiaphragms provided about 1 g of tissue; each gastrocnemius weighed about 1 g and was assayed individually. Frog sartorius was not assayed for cyclic AMP. Control and stimulated muscles were prepared essentially as described in the experiments for the assay of phosphorylase and phosphorylase kinase. The samples were stored in liquid nitrogen until use. The samples were weighed and reduced to a fine powder under liquid nitrogen in a stainless steel centrifuge tube. When all the liquid nitrogen had boiled off, the tube was immediately immersed in a boiling water bath and 10 ml of boiling water added. The nucleotide was extracted
from the tissue for 5 min with stirring, a further 10 ml of water was added and the extract centrifuged at 30,000 x g for 10 min at 4°. The supernatant was removed, and the residue extracted with a further 5 ml of boiling water for 5 min. The supernatants were pooled. A DEAE-cellulose column (0.7 x 10 cm) was prepared in the carbonate form. The column was generated with 15 ml of 1 M triethylammonium bicarbonate pH 7.5 (prepared from freshly distilled triethylamine), and washed with 30 ml of water. The sample was applied to the cellulose and the column washed with a further 30 ml of water. Cyclic AMP was eluted with 50 ml of 100 mM triethylammonium bicarbonate pH 7.5. The eluate was evaporated to dryness on a rotary evaporator, using octyl alcohol to prevent frothing; any residual triethylammonium bicarbonate was removed by re-evaporation of small quantities of water. The residue was dissolved in water and transferred quantitatively with several washings to a small flask for lyophilisation. This residue was dissolved in 25 mM potassium phosphate buffer pH 7.5. For diaphragm, the final extract was dissolved in 1.0 ml of buffer, and for gastrocnemius, 0.5 ml of buffer.

Cyclic AMP was assayed biologically by its capacity to enhance the ATP driven conversion of nonactivated phosphorylase kinase to the activated form. The latter was assayed by its ability to convert phosphorylase b to phosphorylase a at pH 6.8, and was similar to the phosphorylase kinase assay. A partially purified 100,000 x g supernatant (30s) of nonactivated phosphorylase kinase was prepared from rabbit muscle as described in section 2.1.4. Some kinase preparations failed to show sensitivity to cyclic AMP, and this was attributed to
a lack of protein kinase in the preparation. This protein has been shown by the work of Walsh, Perkins and Krebs\textsuperscript{45} to have an absolute requirement for cyclic AMP for its ability to catalyse the phosphorylation of certain substrates. Protein kinase was partially purified as described in section 2.1.5. By adding 20% by volume of protein kinase to the phosphorylase kinase preparation, the sensitivity to cyclic AMP was markedly increased.

The assay system for the activation of phosphorylase kinase by cyclic AMP contained the following components which were added to tubes kept in an ice bath: 0.1 ml of 125 mM Tris-HCl/125 mM β-glycerophosphate pH 6.8, 0.1 ml of 18 mM ATP/60 mM magnesium acetate, 0.1 ml of water, and 0.1 ml of a suitable dilution of the extract in 25 mM potassium phosphate buffer pH 7.5. The reaction was started by the addition of 0.1 ml of a phosphorylase kinase preparation enhanced with 20% protein kinase immediately before the assay. The activation of phosphorylase kinase was carried out at 4° for 30 min. A 0.1 ml aliquot was diluted into 1.4 ml of cold neutral 15 mM cysteine to stop the reaction, and a 0.2 ml aliquot of this dilution was assayed for phosphorylase kinase at pH 6.8.

For the cyclic AMP assays of rat gastrocnemius it was necessary to use a modified procedure. The final extracts were found to contain an inhibitor, similar to that reported by Posner et al.,\textsuperscript{46} which prevented the nucleotide from activating phosphorylase kinase. The effect of the inhibitor was destroyed by incubating the extract with 5 µg of trypsin at 30° for 3 hours. Immediately before assay, 100 µg of soybean trypsin inhibitor was added, maintained at room temperature for 20 min, then chilled in ice. For the assay of cyclic AMP from gastrocnemius, a phosphorylase kinase preparation was made which was
found to be adequately sensitive to the nucleotide without the addition of protein kinase.

Three standard concentrations of cyclic AMP were carried in each assay. All extracts were assayed either in duplicate or using different volumes of diluted extract to ensure that no inhibitory material was interfering with the assay. Previous work had shown that the recovery of cyclic AMP in this assay procedure was quantitative. Results were expressed as μmoles of cyclic AMP per kg of tissue, frozen weight.
3 RESULTS

3.1 Preliminary

The standard curve for the assay of inorganic phosphate is shown in Fig. 1. An optical density of 0.350 was equivalent to 0.10 μmoles of inorganic phosphate. Using this standard the data obtained in the microassay for phosphorylase activity have been expressed as μmoles of inorganic phosphate released per g of tissue in 10 min at 30°. The effect of time on the microassay for phosphorylase is shown in Fig. 2. An extract from diaphragm prepared in the usual way was assayed in the absence of 5'-AMP to give phosphorylase a activity, and with 5'-AMP to give total phosphorylase activity, a plus b. The reaction was linear up to 20 min provided that not more than 15% of the substrate (glucose 1-phosphate) was utilised.

3.2 Studies on Phosphorylase and Phosphorylase Kinase in Rat Diaphragm

The effects of epinephrine and electrical stimulation on phosphorylase and phosphorylase kinase were studied in rat hemidiaphragm in vitro. The activities of the two enzymes under control and stimulated conditions are shown in Tables II and III. The low -AMP/+AMP ratio for phosphorylase and low pH 6.8/8.2 ratio for phosphorylase kinase indicated that the two enzymes were essentially in their inactive forms under conditions of no stimulation (Fig. 3, Bars A, F). Addition of epinephrine (10 μg/ml) to the bath produced a marked increase in the levels of phosphorylase a as shown by the increased -AMP/+AMP ratio (Bar B). The activation was relatively slow and required 5 min to attain this level. Under these conditions the phosphorylase kinase pH 6.8/8.2 ratio increased approximately 4-fold
Fig. 1. Standard curve of inorganic phosphate v.s. optical density for the microassay of phosphorylase.
Fig. 2. Linearity of enzyme reaction with time in the microassay of phosphorylase.
An extract of rat diaphragm was assayed in the presence of 5'-AMP (A) to give total phosphorylase, and in the absence of 5'-AMP (B) to give phosphorylase α, as described in the methods.
TABLE II

Effects of Epinephrine and Electrical Stimulation on Phosphorylase in Isolated Rat Diaphragm

Conditions are as described in the text. n represents the number of samples used. Values are expressed as µmoles of inorganic phosphate released per g in 10 min, ± S.E.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Phosphorylase µmoles Pi/g/10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-AMP</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>18.6 +2.00</td>
</tr>
<tr>
<td>Epinephrine 10 µg/ml for 5 min</td>
<td>12</td>
<td>189 +12.4</td>
</tr>
<tr>
<td>Electrical Stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) direct, 50/sec for 2 sec</td>
<td>14</td>
<td>199 +23.7</td>
</tr>
<tr>
<td>(b) direct, 50/sec for 10 sec</td>
<td>13</td>
<td>271 +36.6</td>
</tr>
<tr>
<td>(c) direct, 20/sec for 10 sec</td>
<td>9</td>
<td>228 +35.8</td>
</tr>
<tr>
<td>(d) via phrenic nerve, 50/sec for 10 sec</td>
<td>15</td>
<td>204 +12.8</td>
</tr>
</tbody>
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TABLE III

Effects of Epinephrine and Electrical Stimulation on Phosphorylase Kinase in Isolated Rat Diaphragm

Conditions are as described in the text. n represents the number of samples used. Values are expressed as units/g, ± S.E.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Phosphorylase Kinase units/g</th>
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<tr>
<td></td>
<td></td>
<td>pH 6.8</td>
</tr>
<tr>
<td><strong>Control</strong></td>
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</tr>
<tr>
<td></td>
<td>19</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±32.9</td>
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<tr>
<td><strong>Epinephrine</strong></td>
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<tr>
<td>10 µg/ml for 5 min</td>
<td>10</td>
<td>1595</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±99.6</td>
</tr>
<tr>
<td><strong>Electrical Stimulation</strong></td>
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<tr>
<td>(a) direct, 50/sec for 2 sec</td>
<td>14</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±24.0</td>
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<tr>
<td>(b) direct, 50/sec for 10 sec</td>
<td>13</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±25.6</td>
</tr>
<tr>
<td>(c) via phrenic nerve, 50/sec</td>
<td>13</td>
<td>264</td>
</tr>
<tr>
<td>10 sec</td>
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<td>±29.3</td>
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Fig. 3. Effects of epinephrine and electrical stimulation on phosphorylase and phosphorylase kinase in isolated rat diaphragm. The bar shadings represent the following: open, control; vertically hatched, epinephrine treatment; cross hatched, electrical stimulation. Bars A and F, control; B and G, epinephrine 10 μg/ml for 5 min; C and H, direct electrical stimulation at 50/sec for 2 sec; D and J, direct stimulation at 50/sec for 10 sec; E and K, stimulation at 50/sec for 10 sec through the phrenic nerve. The vertical lines represent the standard errors. For numbers of tissues used see Tables II and III.
(Bar G), indicating that conversion of the enzyme to its activated form had occurred. It should be noted that since the activated kinase has enhanced activity at pH 8.2, as has been shown in both skeletal and cardiac muscle, the increase in ratio does not reflect the true magnitude of the change at physiological pH. Epinephrine actually increased the activity at pH 6.8 from 235 units/g to 1595 units/g, a 6.5-fold increase (Table III).

When the diaphragm was induced to contract by direct electrical stimulation at 50/sec, phosphorylase was markedly activated, the ratio rising to 0.69 in 10 sec (Fig. 3, Bar D). Activation was achieved remarkably rapidly at this frequency, the ratio indicating nearly full activation in 2 sec (Bar C), this being the shortest time interval which could be conveniently achieved. Stimulation at the lower frequency of 20/sec also produced full activation of phosphorylase in 10 sec (Table II). In order to simulate the physiological conditions more closely, the diaphragm was induced to contract by stimulation of the phrenic nerve. When stimulated at 50/sec for 10 sec, the -AMP/+AMP ratio indicated a marked conversion of phosphorylase b to phosphorylase a (Fig. 3, Bar E). In contrast to the effect of epinephrine, the pH 6.8/8.2 activity ratio for phosphorylase kinase remained essentially unchanged during electrically induced contractions; the total levels of activity also did not change (Table III). At 50/sec, direct stimulation for 2 or 10 sec (Fig. 3, Bars H, J) or neural stimulation for 10 sec (Bar K) all failed to produce any significant change in the phosphorylase kinase ratio. This suggests that during muscle contraction, phosphorylase is activated by a mechanism which does not involve conversion of phosphorylase
kinase to its activated form.

3.3 Studies on Phosphorylase and Phosphorylase Kinase in Frog Sartorius

The frog sartorius was found to be the most difficult tissue with which to work because of the high variation in the total activity of phosphorylase and phosphorylase kinase from one sample to another, and by the wide variation in resting levels of each enzyme. The activities of the two enzymes under control and stimulated conditions are shown in Tables IV and V. The resting ratio for phosphorylase of 0.044 (Fig. 4, Bar A) agrees well with the value reported by Danforth, Helmreich and Cori\textsuperscript{36} using similar techniques. Epinephrine, at a concentration of 5 \( \mu g/ml \), produced a slow and modest increase in phosphorylase activity; over 20 min, the ratio had only increased 3-fold (Fig. 4, Bars B, C, D, E). These experiments were performed under anaerobic conditions (gassed with 95\% nitrogen and 5\% carbon dioxide), conditions which would tend to facilitate glycogenolysis and accentuate any changes in the particular enzymes under study. A greater degree of phosphorylase activation was possible as shown by the response to electrical stimulation. Epinephrine produced a slow but significant increase in the pH 6.8/8.2 activity ratio for phosphorylase kinase (Fig. 4, Bars J, K, L, M), although the resting ratio (Bar H) in this tissue was much higher than in diaphragm. In 20 min the ratio had doubled, and the increase in activity at pH 6.8 from 1064 to 1868 units/g in 2 min and 3220 units/g in 20 min was 1.7- and 3-fold respectively (Table V). This activation of phosphorylase kinase by epinephrine occurred at a time when phosphorylase changes were slight.
TABLE IV

Effects of Epinephrine and Electrical Stimulation on Phosphorylase in Isolated Frog Sartorius

Conditions are as described in the text. n represents the number of samples used. Values are expressed as μmoles of inorganic phosphate released per g in 10 min, ± S.E.

<table>
<thead>
<tr>
<th></th>
<th>Phosphorylase</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>μmoles Pi/g/10 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>-AMP</td>
<td>+AMP</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>24.3 ±2.8</td>
<td>556 ±25.2</td>
</tr>
<tr>
<td>Epinephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 30 sec</td>
<td>3</td>
<td>60.1 ±16.5</td>
<td>669 ±142</td>
</tr>
<tr>
<td>(b) 2 min</td>
<td>5</td>
<td>39.4 ±6.8</td>
<td>499 ±44.3</td>
</tr>
<tr>
<td>(c) 5 min</td>
<td>5</td>
<td>64.8 ±18.6</td>
<td>598 ±54.1</td>
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<tr>
<td>(d) 20 min</td>
<td>7</td>
<td>75.0 ±17.1</td>
<td>622 ±59.6</td>
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<tr>
<td>Electrical Stimulation</td>
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</tr>
<tr>
<td>(a) direct, 10/sec</td>
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<td>153 ±23.4</td>
<td>522 ±15.4</td>
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<td>for 15 sec</td>
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<td></td>
<td></td>
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<tr>
<td>(b) direct, 10/sec</td>
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<td>for 30 sec</td>
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</table>
TABLE V

Effects of Epinephrine and Electrical Stimulation on Phosphorylase Kinase in Isolated Frog Sartorius

Conditions are as described in the text. n represents the number of samples used. Values are expressed as units/g, ± S.E.

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<td></td>
</tr>
<tr>
<td>(d) 20 min</td>
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<td>Electrical Stimulation</td>
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<tr>
<td>(a) direct, 10/sec</td>
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</tr>
<tr>
<td>for 15 sec</td>
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<tr>
<td>(b) direct, 10/sec</td>
<td></td>
</tr>
<tr>
<td>for 30 sec</td>
<td></td>
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</table>
Fig. 4. Effects of epinephrine and electrical stimulation on phosphorylase and phosphorylase kinase in isolated frog sartorius. The bar shadings represent the following: open, control; vertically hatched, epinephrine treatment; cross hatched, electrical stimulation. Bars A and H, control; B and J, C and K, D and L, E and M, epinephrine 5 μg/ml for 0.5, 2, 5, and 20 min, respectively; F and N, direct electrical stimulation at 10/sec for 15 sec; G and O, direct stimulation at 10/sec for 30 sec. The vertical lines represent the standard error. For numbers of tissues used see Tables IV and V.
Electrical stimulation of the frog sartorius at 10/sec produced an increase in the phosphorylase ratio after 15 and 30 sec of stimulation (Fig. 4, Bars F, G). Again, as in rat diaphragm, activation of phosphorylase was unaccompanied by any change in the pH 6.8/8.2 ratio for phosphorylase kinase (Bars N, O), indicating that no activation of this enzyme had taken place during contraction.

3.4 Studies on Phosphorylase and Phosphorylase Kinase in Rat Gastrocnemius

The experiments described thus far were performed on isolated tissues. To investigate whether or not similar changes in the activities of the enzymes occurred in vivo, experiments were performed on the rat gastrocnemius in situ. The rat gastrocnemius preparation represents a relatively intact physiological system with an intact nerve and blood supply. The resting levels of phosphorylase in this tissue were much higher than in the other two preparations, with a -AMP/+AMP ratio of 0.26 (Fig. 5, Bar A, for actual values of phosphorylase activities see Table VI). In order to ensure that this was not due to trauma imposed by the surgical procedure, a series of gastrocnemii were assayed which had been prepared from rats in which the skin was left intact. No reduction in the high resting ratio was observed. Posner, Stern and Krebs, in similar studies in rat gastrocnemius, reported resting phosphorylase ratios of 0.10 and 0.16, which are lower than those reported here for rat gastrocnemius, but still considerably higher than those for diaphragm and sartorius. In response to epinephrine, 5 µg/kg injected intracardially, the phosphorylase ratio was markedly increased (Fig. 5, Bar B). The pH 6.8/8.2 activity ratio for phosphorylase kinase was particularly low at
Fig. 5. Effects of epinephrine and electrical stimulation on phosphorylase and phosphorylase kinase in rat gastrocnemius in situ. The bar shadings represent the following: open, control; vertically hatched, epinephrine treatment; cross hatched, electrical stimulation. Bars A and E, control; B and F, epinephrine 5 μg/kg intracardially, muscle frozen in 40 sec; C and G, electrical stimulation at 8/sec for 5 sec; D and H, stimulation at 20/sec for 5 sec. The vertical lines represent the standard errors. For numbers of tissues used see Tables VI and VII.
TABLE VI

Effects of Epinephrine and Electrical Stimulation on Phosphorylase in Rat Gastrocnemius in vivo

Conditions are as described in the text. \( n \) represents the number of samples used. Values are expressed as \( \mu \) moles of inorganic phosphate released per g in 10 min, \( \pm S.E. \)

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</tr>
<tr>
<td>Epinephrine</td>
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</tr>
<tr>
<td>5 ( \mu )g/kg, intracardially, tissue frozen in 40 sec</td>
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<tr>
<td>Electrical Stimulation</td>
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<td>(a) direct, 8/sec for 5 sec</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) direct, 20/sec for 5 sec</td>
<td>11</td>
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</table>
TABLE VII

Effects of Epinephrine and Electrical Stimulation on Phosphorylase Kinase in Rat Gastrocnemius in vivo

Conditions are as described in the text. n represents the number of samples used. Values are expressed as units/g, ± S.E.

<table>
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<th>Phosphorylase Kinase</th>
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<td></td>
<td></td>
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<td>13</td>
<td>211</td>
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<td>±43.8</td>
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<td>±904</td>
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<td>Epinephrine</td>
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<td>18,602</td>
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</tr>
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<td>5 μg/kg, intracardially,</td>
<td></td>
<td>±167</td>
<td></td>
<td>±883</td>
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<td>tissue frozen in 40 sec</td>
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</tr>
<tr>
<td>Electrical Stimulation</td>
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<td></td>
</tr>
<tr>
<td>(a) direct, 8/sec for</td>
<td>7</td>
<td>180</td>
<td>17,307</td>
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<tr>
<td>5 sec</td>
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<td>±21.1</td>
<td></td>
<td>±1,125</td>
</tr>
<tr>
<td>(b) direct, 20/sec for</td>
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<td>205</td>
<td>14,834</td>
<td></td>
</tr>
<tr>
<td>5 sec</td>
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<td>±32.2</td>
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<td>±1,397</td>
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</table>
0.014 (Bar E). With epinephrine, this ratio was increased 9-fold (Bar F). The increase in activity at physiological pH from 211 to 2401 units/g represented an 11-fold increase (Table VII).

When the gastrocnemius was stimulated directly by electrical pulses at 20/sec for 5 sec, the phosphorylase ratio increased to 0.77 (Fig. 5, Bar D). At the slower rate of stimulation of 8/sec, the ratio increased to 0.33 (Bar C). As in the other tissues electrical stimulation failed to alter phosphorylase kinase activity as evidenced by the pH 6.8/8.2 ratio (Bars G, H) essentially identical to the controls. Generally, the total phosphorylase activity (Table VI) and the total phosphorylase kinase activity (Table VII) remained approximately constant, except in those muscles stimulated electrically at 8/sec.

The similarities and differences between phosphorylase activation produced by adrenergic and electrical stimulation have been discussed, and are clearly shown in Figs. 3, 4 and 5. To summarise, in all three tissues, epinephrine produced a significant increase in the -AMP/+AMP ratio for phosphorylase which was accompanied by an increase in the pH 6.8/8.2 activity ratio for phosphorylase kinase. In contrast, during electrical stimulation, although rapid and marked increases in the phosphorylase ratio were produced, no change in the phosphorylase kinase ratio was observed. This was true for direct and neural stimulation, different frequencies of stimulation, in vitro and in vivo, and for two species. It may be concluded that phosphorylase is activated during electrically induced contraction by a mechanism which does not involve conversion of phosphorylase kinase to its activated form.
3.5 **Effect of Epinephrine and Electrical Stimulation on Cyclic AMP Levels**

The foregoing data provide evidence that the activation of phosphorylase by epinephrine is substantially different from that produced by electrical stimulation. Epinephrine is known to enhance cyclic AMP levels in many tissues, and Posner, Stern and Krebs have demonstrated increases in the tissue levels of the cyclic nucleotide in response to epinephrine in frog sartorius and rat gastrocnemius **in vivo**. They also showed that electrical stimulation failed to increase intracellular levels of the nucleotide. In the work of this thesis also, the effects of epinephrine and electrical stimulation were compared on the tissue levels of the nucleotide in rat diaphragm **in vitro** and rat gastrocnemius **in vivo**. Resting levels of cyclic AMP in rat diaphragm were 0.15 μmoles/kg, based on four determinations (Fig. 6, **Bar A**). Each extract was derived from 6 hemidiaphragms. When the diaphragm was treated with epinephrine the tissue levels of the nucleotide increased to 2.17 μmoles/kg in 1 min (**Bar B**), then fell slightly to 1.72 μmoles/kg in 5 min (**Bar C**). Similarly, in the rat gastrocnemius intracardial injection of epinephrine increased the tissue concentration of cyclic AMP from 0.29 to 1.65 μmoles/kg (**Bars F, G**). In contrast, on direct electrical stimulation of the diaphragm at 50/sec for 10 sec no change in the tissue levels of the nucleotide occurred (**Bar D**). It has been demonstrated in cardiac muscle by Drummond, Duncan and Hertzman that epinephrine produced rapid transient changes in cyclic AMP levels. Maximum levels were obtained in 1 sec and were already decreasing in 3 sec. To ensure that similar changes were not occurring during electrical stimulation, the diaphragm
Fig. 6. Effects of epinephrine and electrical stimulation on cyclic AMP levels in rat diaphragm and rat gastrocnemius. The bar shadings represent the following: open, control; vertically hatched, epinephrine treatment; cross hatched, electrical stimulation. Bars A and F, control; B and C, epinephrine 10 µg/ml for 1 and 5 min, respectively; D and E, electrical stimulation at 50/sec for 2 and 10 sec, respectively; G, epinephrine 5 µg/kg intracardially, tissue frozen in 40 sec; H, electrical stimulation at 20/sec for 5 sec. The vertical bars represent the standard errors. 11 to 14 samples were used for gastrocnemius; 4 for diaphragm (each extract was prepared from 6 hemidiaphragms).
was assayed for cyclic AMP after 2 sec of stimulation. No change in the levels was detected (Bar E). In the other tissue, electrical stimulation of the gastrocnemius for 5 sec again produced no change in the tissue levels of cyclic AMP (Bar H). The control levels of cyclic AMP were lower than those reported by either Posner, Stern and Krebs using a similar assay procedure, or by Brooker, Thomas and Appleman using a radioisotope technique. The levels produced in the presence of epinephrine corresponded closely to both of the above reports, so that the change produced on adrenergic stimulation in the present work was of greater magnitude.

3.6 Further Studies on Epinephrine Induced Phosphorylase Activation in Rat Diaphragm

3.6.1 Time Course of Activation

To determine the time required to obtain maximum phosphorylase levels on treatment with epinephrine, the phosphorylase ratio was determined at various times after exposure to the amine. The results are shown in Fig. 7. The much slower time course as compared to that induced by electrical stimulation (Fig. 3, Bars C, D) was probably related to the slow rate of diffusion of epinephrine. There is evidence to suggest that epinephrine induced phosphorylase activation in skeletal muscle in vivo is not as slow as indicated in the diaphragm studies. The phosphorylase ratio was found to attain a value of 0.63 in the present studies in rat gastrocnemius, 40 sec following intracardial injection. In another report of a similar experiment, the ratio reached 0.48, 60 sec following the injection.
Fig. 7. Time curve of epinephrine activation of phosphorylase in rat diaphragm. Conditions are as described in the text. The vertical lines represent the standard errors.
3.6.2 Effect of Epinephrine on Phosphorylase Activation in Potassium Depolarized Rat Diaphragm

The activation of phosphorylase in skeletal muscle during contraction induced by electrical stimulation has been described (sections 3.2; 3.3; 3.4). Some skeletal muscle preparations will undergo contracture in the presence of high potassium concentrations. It was of interest to see whether phosphorylase activation also occurred under these conditions and whether the effect of epinephrine was changed. Danforth and Helmreich reported activation of phosphorylase in frog sartorius associated with potassium induced contracture. They found a linear relationship between the phosphorylase ratio and the logarithm of the extracellular potassium concentration. For the studies in rat diaphragm, sodium chloride in the Tyrode's solution was replaced by potassium chloride to give a final potassium concentration of 140 mM. On exchanging the normal Tyrode's solution for the high potassium solution, there was only a slight contracture, and a low degree of phosphorylase activation. In 30 sec the phosphorylase ratio increased slightly, and returned to control levels in 7 min. Epinephrine treatment of the tissue at this time produced a much lower degree of phosphorylase activation (Fig. 8, Bar C) compared to that produced by epinephrine in normal Tyrode's solution (Bar B). The activation was greater than in unstimulated control diaphragms (Bar A).

In skeletal muscle, Lundholm, Mohme-Lundholm and Vamos reported that potassium depolarization inhibited the activation of phosphorylase by epinephrine. In cardiac muscle, Namm, Mayer and Maltbie found that depolarization of the perfused rat heart with 56 mM potassium produced a transient increase in the phosphorylase
Fig. 8. Effect of various treatments on epinephrine induced phosphorylase activation in rat diaphragm.

Bar A, control, no epinephrine (25); B, epinephrine 10 μg/ml for 5 min (12); C, epinephrine 10 μg/ml for 5 min to diaphragms depolarized in high potassium Tyrode's solution (8); D, epinephrine 10 μg/ml for 5 min to diaphragms exposed to calcium-free Tyrode's solution to which 4 mM EDTA had been added (10). Vertical lines represent the standard errors. Figures in parenthesis represent the number of tissues used.
ratio and this returned to the control value in 5 min; phosphorylase activation by epinephrine under these conditions was slight and occurred slowly. These results suggest that in both skeletal and cardiac muscle, high potassium interferes with the action of epinephrine in promoting phosphorylase activation.

3.6.3 Effect of EDTA on Epinephrine Induced Phosphorylase Activation

To investigate whether calcium was required for the epinephrine induced activation of phosphorylase, experiments were performed in which calcium was removed from the Tyrode's solution. Diaphragms were prepared in the usual way except that 4 mM EDTA was added to the calcium-free Tyrode's solution. On treatment with epinephrine (Fig. 8, Bar D), the degree of phosphorylase activation obtained was about half that observed in normal Tyrode's solution (Bar B). In studies of the possible role of calcium in phosphorylase activation in hearts, Namm, Mayer and Maltbie found that when perfused with a calcium-free solution, epinephrine caused activation of phosphorylase kinase, but no increase in phosphorylase a levels. It was considered that calcium might be required for the catalytic action of phosphorylase kinase in promoting conversion of phosphorylase b to a. It should be noted that although calcium is chelated by EDTA, magnesium is also removed to some extent. Magnesium, together with ATP, is required for the activation of both enzymes. The results cannot, therefore, at this stage be attributed to the absence of calcium, and in order to investigate the possible involvement of both these ions in phosphorylase activation, it will be necessary to use a perfusion technique.
3.7 Further Studies on the Electrically Induced Activation of Phosphorylase in Rat Diaphragm

In the studies described thus far, it has been shown that the levels of phosphorylase a were increased during electrically stimulated contraction of skeletal muscle. There was no concomitant increase in either the pH 6.8/8.2 ratio for phosphorylase kinase or the tissue level of cyclic AMP. It appeared as if phosphorylase were being activated by a mechanism other than one which involved conversion of phosphorylase kinase to its activated form. No other enzymes are known which can promote the conversion of phosphorylase b to phosphorylase a, and therefore it was of interest to study further the relationship between phosphorylase activation and electrically induced contraction.

3.7.1 Relationship Between Frequency of Stimulation and Degree of Phosphorylase Activation

A series of experiments was undertaken to study the relationship between phosphorylase activation and the frequency of stimulation. Under standard conditions at 37°, the diaphragm was stimulated directly at frequencies from 2/sec to 50/sec. At each frequency, the tissue was stimulated nominally for 2, 5 or 10 sec and the actual time calculated from the polygraph record. These data are presented in Fig. 9. At a stimulation rate of 2/sec, there was virtually no activation of the enzyme and after 20 sec of stimulation, the ratio was only 0.059. With higher rates of stimulation, especially 8/sec and above, there was an initial rapid activation which did not increase further as the duration of stimulation was extended. At 50/sec, the highest rate of
Fig. 9. Relationship between phosphorylase activation and frequency of stimulation in rat diaphragm. Diaphragms were prepared in normal Tyrode's solution at 37° and stimulated directly as described in the text. The numbers on the graph represent the rate of stimulation in pulses per sec. The vertical bars show the standard errors. The actual values of phosphorylase a and total phosphorylase activities, and the number of tissues used, are given in Table VIII.
stimulation used, nearly full activation was reached within 2 sec. Table VIII shows the actual phosphorylase \(a\) and total phosphorylase activities. The latter remained approximately constant with increase in both frequency and duration of stimulation, so that all changes in the ratio can be attributed to conversion of phosphorylase \(b\) to phosphorylase \(a\). Danforth and Helmreich\(^1\) reported that phosphorylase activation in frog sartorius was similarly related to the rate of stimulation and that activation was not dependent upon the external work load. It may be concluded that the degree of phosphorylase activation is intimately coupled to the rate of stimulation. Some event associated with each stimulus initiates both the development of tension, and metabolic changes to ensure that substrates are mobilised to provide for energy utilising processes.

3.7.2 Effect of Temperature upon Electrically Induced Phosphorylase Activation

To investigate further the relationship between increased levels of phosphorylase \(a\) and contraction, the effect of temperature upon phosphorylase activation was studied. A rate of stimulation of 10/sec was chosen and the tissue stimulated directly for 2, 5 and 10 sec at 37°, 30°, 25° and 20°. As shown in Fig. 10, variation in temperature altered the steady state ratio for phosphorylase for the particular frequency; the lower the temperature, the lower the degree of phosphorylase activation observed. Phosphorylase activation was achieved rapidly even at the lower temperature. The total tension developed by the muscle at reduced temperature was not decreased, and as shown in Table IX, the total levels of phosphorylase activity were not altered.
TABLE VIII

Effect of Frequency of Stimulation on Phosphorylase a and Total Phosphorylase Activities during Electrically Induced Contractions in Rat Diaphragm

Conditions are as described in the text. Numbers in parenthesis represent the numbers of samples used. Values are expressed as μmoles of inorganic phosphate released per g in 10 min, ± S.E.

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<td>+AMP</td>
<td>-AMP</td>
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Fig. 10. Effect of temperature on phosphorylase activation during electrical stimulation of rat diaphragm. Diaphragms were stimulated directly at a rate of 10/sec, at various temperatures from 20° to 37° as shown on the graph. Other details are described in the text. The vertical bars represent the standard errors. For activities of phosphorylase a and total phosphorylase, and number of samples used, see Table IX.
Diaphragms were stimulated at a rate of 10/sec at different temperatures for various times of stimulation. Other conditions are described in the text. Values are expressed as μmoles of inorganic phosphate released per g in 10 min, ± S.E.

<table>
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<td>37°</td>
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<td>401 ± 8.4 (4)</td>
<td>7.1 ± 1.8</td>
<td>365 ± 23.5 (6)</td>
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Therefore, the changes were due to a reduction in the degree of conversion of phosphorylase b to phosphorylase a. Whatever event initiates the contractile mechanism also appears to trigger phosphorylase activation, but at a slower rate at reduced temperature. Although lowering the temperature has different effects on the contractile mechanism and phosphorylase activation, this does not imply that the two events are not closely related.

3.7.3 Effect of Chelating Agents (EDTA and EGTA) on Electrically Induced Phosphorylase Activation and Tension

It was considered that calcium, which is known to play an important role in excitation-contraction coupling and the development of tension, may be directly or indirectly responsible for the mechanism of phosphorylase activation during contraction. It is well known that if calcium is removed from a bathing solution, the development of tension of a muscle is impaired. In studies on diaphragm, it was found that the simple exclusion of calcium from the Tyrode's solution had little effect on the mechanical response to electrical stimulation. However, calcium can be removed more effectively using a chelating agent such as EDTA or EGTA. In these experiments, diaphragms were prepared in the usual way and a 2 sec stimulus at 50/sec was applied directly to give an initial tetanus. EDTA was added at various concentrations, usually 1 to 2 mM, and the tissue left for 15 min to equilibrate. A 10 sec stimulus was then delivered to the muscle at the same frequency. The tissues were frozen and homogenised in the usual way and assayed for phosphorylase. The initial and final tension developed by the muscle was calculated, and the final tension expressed as a percentage of the initial tension. Phosphorylase activation was
expressed in the usual way (-AMP/+AMP ratio). The relationship between contractile tension and phosphorylase activation under these conditions is shown in Fig. 11. The regression line has been calculated by statistical methods. Data from the graph was in good agreement with levels of phosphorylase activation found under standard conditions (Fig. 3). In particular, the phosphorylase ratio from the graph at zero load was 0.01, which corresponded closely to the directly determined phosphorylase ratio of 0.052 (Fig. 3, Bar A). At maximum tension development (100%), the phosphorylase ratio from Fig. 11 was 0.58. This is in reasonable agreement with the ratio of 0.69 produced by direct electrical stimulation at 50/sec for 10 sec (Fig. 3, Bar D).

Because of the lack of specificity of EDTA in chelating both calcium and magnesium, the results could be attributed to the absence of either ion. Although it was improbable that magnesium was required for phosphorylase kinase, since activation of this enzyme has been shown not to occur during electrical stimulation, the ion was still required for the phosphorylase b to a conversion. To obviate any criticism concerning the absence of magnesium, a second series of experiments were performed using EGTA which has a much higher affinity for calcium than magnesium. The magnesium level in the Tyrode's solution was increased to 5 mM to ensure that there was no lack of this ion. The stimulation was conducted essentially as described for EDTA, except that a 2 sec tetanus was given both initially and after treatment with EGTA. This avoided the problem of variable tension over a 10 sec tetanus. The data are represented in Fig. 12 and show a similar correlation to that obtained with EDTA in Fig. 11. The data
Fig. 11. Effect of EDTA on phosphorylase activation and tension in rat diaphragm.

Diaphragms were prepared in calcium-free Tyrode's solution to which EDTA was added. The final tension developed by the muscle after exposure to EDTA is expressed as a percentage of the initial tension. Other details are described in the text. The regression line has been calculated by standard statistical methods.
Fig. 12. Effect of EGTA on phosphorylase activation and tension in rat diaphragm. Diaphragms were prepared essentially as described in Fig. 11 and in the text, except that EGTA was used. Tension is the final tension expressed as a percentage of the initial tension before exposure to EGTA. The regression line has been calculated by standard statistical methods.
with EGTA were also in good agreement with the levels of phosphorylase activity obtained under standard conditions: for resting phosphorylase ratios, the value was 0.052 (Fig. 3, Bar A) under standard conditions, and 0.05 from the graph (Fig. 12); for the maximum tension developed by a 2 sec tetanus at 50/sec, the phosphorylase ratio under normal conditions was 0.50 (Fig. 3, Bar C) and from the graph, 0.45 (Fig. 12). In both these experiments in which the development of tension has been reduced by restricting the availability of ionised calcium using chelating agents, it has been shown that there is a correlation between phosphorylase activation and the development of muscle tension.
4 DISCUSSION

4.1 Methodology

From the literature it can be seen that studies on the mechanisms of phosphorylase activation have been made difficult by the instability and high activity of the enzymes involved in the activation reactions. Parts of the system are activated during sampling, homogenisation and extraction procedures and have given erroneous values for the activities of some enzymes. This has resulted in a failure to detect certain changes and in difficulties in interpretation of the data. In the original work of Cori\(^{34}\) in skeletal muscle resting phosphorylase ratios up to 0.80 were found, but Cori considered that these high values were the result of inadequate methodology. With the advent of better techniques and improved understanding of the systems involved, it was shown that resting levels of phosphorylase \(a\) in tissues were much lower. By fixing tissues in isopentane cooled to \(-160^\circ\), Rulon, Schottelius and Schottelius\(^{35}\) showed that the average resting ratio of phosphorylase in the excised tibial muscle of the mouse was 0.18. In studies on rat and frog muscle, Posner, Stern and Krebs\(^{37}\) found resting phosphorylase ratios to be between 0.10 and 0.21. In these studies the muscles were removed from the animals and then immersed in cold isopentane, so that there was a delay of at least 0.5 sec between sampling and arrest of all enzyme activity. In the work described in this thesis, it was considered that freezing the tissue with tongs prechilled in liquid nitrogen could effect an even more rapid and complete fixation of the tissue; the enzyme activities determined would be a true reflection of the conditions in the tissue.
at the time the sample was taken.

Danforth, Helmreich and Cori\(^3\) have demonstrated that immediate arrest of all enzyme activity at temperatures of liquid nitrogen or cooled isopentane does not necessarily give low resting ratios of phosphorylase. Although sodium fluoride and EDTA are used in homogenisation buffers to inhibit the phosphatase and kinase respectively, the kinase is still sufficiently active during homogenisation and extraction procedures to give erroneously high levels of phosphorylase. Homogenisation of frog sartorius at 0\(^\circ\) gave a mean resting phosphorylase ratio of 0.10 (range, 0.03 to 0.32) but at -35\(^\circ\) using a glycerol containing buffer, the ratio was considerably reduced with a mean value of 0.028 (range, 0.01 to 0.05).\(^3\) Homogenisation at 0\(^\circ\) was probably responsible for the high resting phosphorylase ratio in the tibial muscle of the mouse.\(^3\) In the present studies, to prevent activation of phosphorylase, homogenisation was carried out at -35\(^\circ\) and care was taken to ensure complete dispersal of the powdered tissue in the buffer. Using these techniques, resting ratios of around 0.05 were determined in rat diaphragm and frog sartorius; with these precautions, it was unexpected to find resting ratios of 0.26 in rat gastrocnemius in vivo.

One of the purposes of the present investigation was to establish whether activation of phosphorylase kinase, as measured by increased pH 6.8/8.2 ratios, was occurring during muscle contraction. Since the maximum kinase activation which could be produced was low, 0.18 in diaphragm, 0.23 in sartorius and 0.13 in rat gastrocnemius (on epinephrine stimulation), a problem arises as to how precisely the control ratios can be determined. This in turn depends upon adequate
tissue blanks to correct for inorganic phosphate and protein in the assay system. In the assay of phosphorylase kinase in the present work, the precautions described by Drummond, Duncan and Hertzman were used. (For details see section 2.5.)

4.2 Epinephrine Induced Phosphorylase Activation in Skeletal Muscle

The results obtained in the work reported here on epinephrine activation in skeletal muscle are in agreement with the current hypothesis on the mechanism by which epinephrine causes activation of glycogen phosphorylase in other tissues. The upper half of Fig. 13 shows the sequence of reactions which has been established for some tissues. In skeletal muscle it has been found that epinephrine induced activation of phosphorylase was accompanied by an increase in the intracellular levels of cyclic AMP (Fig. 6), and an increase in the pH 6.8/8.2 ratios for phosphorylase kinase (Figs. 3, 4, 5). In in vitro studies, DeLange et al. have shown that the activation of phosphorylase kinase occurs by a phosphorylation reaction requiring ATP and magnesium. Cyclic AMP was found to increase both the rate of activation and phosphorylation, but not at the same rate. Riley et al. showed that phosphorylation and activation were linked, since a phosphatase, designated phosphorylase kinase phosphatase, catalysed a dephosphorylation and a loss of activity resulted. The fact that cyclic AMP can stimulate a reversible activation of phosphorylase kinase can be taken as evidence for the physiological significance of the nucleotide in the adrenergic activation of phosphorylase (Fig. 13).

DeLange et al. suggested that cyclic AMP may be activating
Fig. 13. Mechanisms by which epinephrine and electrical stimulation increase the levels of phosphorylase $b$ in skeletal muscle.
phosphorylase kinase by stimulation of second enzyme, because they failed to detect any significant binding of the nucleotide to phosphorylase kinase. Walsh, Perkins and Krebs have reported on an enzyme that may act in this manner which they have called protein kinase. This enzyme has been shown to have an absolute requirement for cyclic AMP to phosphorylate casein and protamine, with Km values of $1 \times 10^{-7}$ and $6 \times 10^{-8}$ M, respectively. These values agree well with the Km of $7 \times 10^{-8}$ M for cyclic AMP determined by DeLange et al. in the phosphorylation of nonactivated phosphorylase kinase; this reaction probably occurred through the presence of protein kinase as a contaminant.

The work on protein kinase implies that phosphorylase kinase preparations which exhibit sensitivity to cyclic AMP will contain the enzyme as a contaminant. In the work reported here, it was found that some preparations of phosphorylase kinase were not sensitive to the nucleotide until a partially purified preparation of protein kinase was added. The quantity of contaminating protein kinase would also explain the wide variation in sensitivity to cyclic AMP among preparations of phosphorylase kinase. It has yet to be shown that protein kinase is physiologically important in mediating the effects of epi-nephrine in phosphorylase activation, but its use in the biological assay of cyclic AMP supports this hypothesis. This enzyme may serve as the link between stimulation of adenyl cyclase, through increased intracellular levels of cyclic AMP, and the activation of phosphorylase kinase.

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*Unpublished observations from the laboratory of Dr. G.I. Drummond*
Several investigators have noted the low degree of activation of phosphorylase kinase which can be demonstrated both in vivo and in vitro, with pH 6.8/8.2 ratios rarely exceeding a value of 0.25 to 0.30. The control values may be fairly high, and in the present work, the control and epinephrine stimulated ratios were as follows: rat diaphragm, 0.04 and 0.18 (Fig. 3), frog sartorius, 0.11 and 0.23 (Fig. 4) and rat gastrocnemius, 0.01 and 0.13 (Fig. 5). The reason for such a narrow range of activation is not known, but it appears to be sufficient to achieve full activation of phosphorylase. There seems to be a large quantity of phosphorylase kinase in the cell, and DeLange et al.\textsuperscript{50} have calculated that the kinase comprises approximately 1% of the soluble protein in rabbit skeletal muscle. With a phosphorylase \textsubscript{b} content of about 2% the ratio of phosphorylase to phosphorylase kinase is between 10 and 15 to 1, based upon molecular weights of 185,000 and $1 \times 10^6$, respectively. With such large quantities of kinase there appears to be no need for a higher ratio of activation of the kinase than those observed. These considerations imply that small but physiologically significant changes would be difficult to detect. Krause and Wollenberger\textsuperscript{52} found the phosphorylase kinase of dog heart to be activated under conditions of ischaemia to a ratio of 0.69. It would therefore be interesting to see if the kinase from the skeletal muscle of this species could be activated to such a degree.

4.3 Activation of Phosphorylase During Electrically Induced Contraction

In the work reported here, no increase was detected in the pH 6.8/8.2 ratio for phosphorylase kinase during electrically induced....
contraction. No increase in the tissue levels of cyclic AMP occurred either, and both of these were unchanged at a time when phosphorylase was markedly activated. The data strongly suggest that phosphorylase is activated during contraction by a mechanism other than conversion of phosphorylase kinase to its activated form. Rapid activation of phosphorylase on electrical stimulation was reported by Danforth, Helmreich and Cori in studies on frog sartorius. In a kinetic analysis of the rate of activation and inactivation of phosphorylase during muscle contraction, the rate of the phosphatase reaction was found to remain constant. They concluded that phosphorylase activation was due to changes in the activity of phosphorylase kinase, although no direct measurements of this enzyme were carried out. It had been shown previously by Krebs, Graves and Fischer that the kinase could be activated in vitro by calcium ion, but the reaction was irreversible. It was suggested that calcium ions liberated during muscle contraction were responsible for the activation of phosphorylase kinase under these conditions.

Subsequent work by Meyer et al. in skeletal muscle and Drummond and Duncan in cardiac muscle showed that activation of phosphorylase kinase by calcium required a protein factor called kinase activating factor (KAF). Again it was not possible to show a reversible activation of the enzyme in the calcium/KAF activation. Furthermore, the concentration of calcium required for kinase activation was too high to have physiological significance with respect to calcium levels required for muscle contraction. Further work by Huston and Krebs and Drummond and Duncan showed that the calcium/KAF activation of kinase occurred by a proteolytic mechanism with the release of
peptide material. Kinase activation and peptide release were found to correspond closely in both studies but the two groups of workers concluded that such a mechanism would probably have no physiological function; the activation would be too slow to account for the rate of phosphorylase activation during contraction.

Posner, Stern and Krebs\textsuperscript{37} were the first to measure phosphorylase kinase activity during contraction. They reported that electrical stimulation of frog and rat skeletal muscle did result in increased pH 6.8/8.2 ratios for kinase. In contrast, in the present work no activation of phosphorylase kinase, as measured by changes in the pH 6.8/8.2 ratios, was detected (Figs. 3, 4, 5). It was noted earlier that there appears to be a large excess of kinase for the activation of phosphorylase, and it is conceivable that small changes in kinase activation were not detected. However, variation in both frequency and time of stimulation, direct and neural stimulation, all failed to reveal any change in the three tissues studied. This was considered to be a good indication that the activation of phosphorylase kinase was not occurring during contraction. From the differences in tissue levels of cyclic AMP and phosphorylase kinase activities produced on adrenergic and electrical stimulation, it may be concluded that phosphorylase is activated by different mechanisms under these conditions. Additional evidence that electrically induced phosphorylase activation is occurring by a non-adrenergic mechanism is provided in studies with beta adrenergic blocking drugs such as dichloroisoprenaline (DCI) and pronethalol. Several reports indicate that these drugs inhibit epinephrine stimulated phosphorylase activation\textsuperscript{36,57,58} but not the electrically induced increase in phosphorylase a.\textsuperscript{36}
4.4 Further Considerations of the Electrically Induced Increase in Phosphorylase Activity

Although the calcium/KAF activation of phosphorylase kinase as a mechanism for phosphorylase activation has been discounted (section 4.3), evidence for the possible involvement of calcium in other ways must be considered. In particular, the relationship to calcium in muscle contraction will be discussed and a mechanism is proposed to account for the electrically induced increase in phosphorylase activity.

The studies with EDTA and EGTA described in this thesis support a role for calcium in both phosphorylase activation and the mechanical response of the muscle. These agents chelate and thus remove calcium ions from the medium. When these agents were added to the tissue bath the mechanical response was impaired, and a correlation was shown to exist between the tension developed and the degree to which phosphorylase became activated (Figs. 11, 12). The studies in Fig. 9 provide further support for the involvement of calcium in these processes. Here it was shown that a close relationship existed between the rate of stimulation and the degree of phosphorylase activation. For any given frequency, there was an initial rapid activation of phosphorylase to a plateau which was characteristic of that frequency. The ratio did not increase with further stimulation. Danforth and Helmreich reported similar observations in frog sartorius, although the rates of stimulation were much slower, and the time for phosphorylase activation to reach a steady state was longer.

More direct evidence for the possible involvement of calcium in phosphorylase activation has been provided by studies in cardiac
muscle. Friesen, Allen and Valadares\textsuperscript{28} showed that on perfusion of rat hearts with high calcium medium, phosphorylase was activated. Further studies on perfused hearts revealed that the activation of phosphorylase was dependent upon the calcium concentration gradient rather than the absolute concentration in the medium.\textsuperscript{*} However, in all cases, activation of phosphorylase was preceded by an increase in the force of contraction, and it is not clear whether phosphorylase activation was the result of the inotropic response or increased calcium levels. In experiments in which calcium was removed from the perfusion medium, Namm, Mayer and Maltbie\textsuperscript{29} showed that epinephrine administration to perfused rat hearts resulted in increased pH 6.8/8.2 ratios for phosphorylase kinase but no phosphorylase activation. On the other hand, when the hearts were perfused with a calcium-enriched medium (no epinephrine) phosphorylase was activated but no increase in the activity ratio for kinase was detected. These experiments provide strong evidence that calcium is involved in phosphorylase activation, and for the possible site of action of calcium in this effect.

It was concluded that calcium was not required for the conversion of phosphorylase kinase to its activated form, but was needed catalytically for either activated or nonactivated phosphorylase kinase to effect the conversion of phosphorylase $b_1$ to the $a_1$ form.\textsuperscript{29} If calcium does act in this manner on nonactivated kinase in skeletal muscle, it could account for the electrically induced increase in phosphorylase activity described in this thesis.

The mechanisms of phosphorylase activation in skeletal muscle are shown in Fig. 13. The upper part of the scheme shows the sequence

\textsuperscript{*}Friesen, personal communication
of reactions which occur on treatment with epinephrine as discussed earlier (section 4.2). In the lower half of the diagram, an attempt has been made to indicate how phosphorylase activity might be increased during muscle contraction. The hypothesis with regard to electrically induced phosphorylase activation may be summarised as follows: an impulse to the muscle causes an influx of calcium which ultimately causes the release of calcium ions into the myoplasm. This calcium is responsible for initiating the contractile mechanism and also acts as a transient cofactor for cytoplasmic phosphorylase kinase. No conversion to the activated form occurs, but the nonactivated phosphorylase kinase/calcium catalyses the conversion of phosphorylase b to phosphorylase a. The degree of phosphorylase activation depends upon the frequency of stimulation, and the mean intracellular calcium level determines the degree to which nonactivated kinase causes the activation of phosphorylase. Calcium is not only involved in excitation-contraction coupling but rather excitation-contraction-biochemical coupling, to ensure that metabolic events are guaranteed for energy utilising processes.

If calcium does act in the manner predicted, it should be possible to study these effects in vitro. Using purified phosphorylase kinase, Ozawa, Hosoi and Ebashi\textsuperscript{59} found that the enzyme was activated by calcium ion at pH 6.8, at calcium concentrations compatible with those required for muscle contraction. By complexing calcium with EGTA they were able to demonstrate a partial reversal of calcium induced kinase activation. It would be necessary to achieve complete reversibility of this reaction to show that the increased activity was not associated with a calcium/KAF mechanism. These authors\textsuperscript{60} have
also reported an absolute requirement for calcium in the ATP/magnesium activation of phosphorylase kinase, but it was not clear where the calcium was required.

A strong indication that phosphorylase kinase may be required for the electrically induced increase in phosphorylase \( a \) is provided by the observations on I strain mice. Lyon and Porter\(^{61} \) have shown that these mice are genetically deficient in phosphorylase kinase, and that phosphorylase in skeletal muscle is always in the \( b \) form. Danforth and Lyon\(^{62} \) compared glycogenolysis in skeletal muscle from normal and I strain mice during tetanic contraction. Although glycogenolysis occurred in both muscles, no phosphorylase \( a \) was formed in those muscles which contained no phosphorylase kinase. These observations support the hypothesis that nonactivated kinase is required in the activation of phosphorylase during contraction. It should be realised that although a deficiency of kinase has been shown, where a genetic deficiency exists other proteins which may be required in these reactions could be modified or absent. A recent report shows, for instance, that although adenylyl cyclase is present in both strains of mice, the formation of cyclic AMP in response to epinephrine differs in the two strains.\(^{63} \)

In the present work, it has been demonstrated that two completely different mechanisms exist for the activation of phosphorylase in skeletal muscle: on adrenergic stimulation, cyclic AMP and pH 6.8/8.2 ratios for phosphorylase kinase were increased, whereas on electrical stimulation, no change in the above two parameters occurred. It is interesting to relate these findings to studies in cardiac muscle. In the latter, epinephrine produces both an increase in force of
contraction (inotropic response) and an activation of phosphorylase. Cyclic AMP levels increase extremely rapidly in response to epinephrine and reach peak levels before either phosphorylase activation or the inotropic response. Considerable effort has been expended in trying to implicate the increased levels of cyclic AMP to the inotropic effect of epinephrine. In skeletal muscle epinephrine produces no mechanical response, and this observation might be considered as negative evidence for cyclic AMP being the mediator of the inotropic response. Whether phosphorylase can be activated in cardiac muscle by electrical events alone, i.e. by non-adrenergic mechanisms, is not known, but this certainly does occur in skeletal muscle. Cyclic AMP appears to be involved in the mediation of adrenergic stimulation and calcium may be important in the electrically stimulated phosphorylase activation. Although from these considerations, cyclic AMP and calcium appear to have separate functions, Rasmussen and Tenenhouse have proposed that the action of the nucleotide is to increase the permeability of cell membranes to calcium. This hypothesis tends to ignore the observations in vitro in which cyclic AMP has been shown to increase the activity of some enzymes, notably protein kinase. The theory could be modified, however, to account for the biochemical effects by considering an ATP/calcium complex as the substrate for adenyl cyclase. On stimulation, the enzyme would form cyclic AMP which would be responsible for the metabolic events, and the liberation of calcium could account for the physiological changes.
4.5 Control of Glycogenolysis and Glycolysis

The mechanisms of phosphorylase activation as described in this thesis will now be considered in relation to the control of glycogenolysis and glycolysis in skeletal muscle. The enzymes of glycogen metabolism and the interrelationships between the various activating mechanisms are shown in Fig. 14. Glycogenolysis can occur in the following ways (Fig. 14, lower right. For more detail refer also to Fig. 13). Muscle contains phosphorylase b which is normally inactive. Phosphorylase kinase, which is also normally inactive, can be converted to an activated form on adrenergic stimulation (through cyclic AMP and protein kinase), which can then convert phosphorylase b to phosphorylase a, the active form of this enzyme. Activated phosphorylase kinase can be inactivated by phosphorylase kinase phosphatase. Phosphorylase b can be converted to phosphorylase a by the catalytic action of calcium on nonactivated phosphorylase kinase. Finally, phosphorylase b itself can become active, without conversion to the a form, in the presence of 5'-AMP. These actions of the nucleotides have greater significance in the control of glycolysis by modulating the activity of other key regulatory enzymes, notably phosphofructokinase. These effects of the nucleotides, particularly 5'-AMP and ATP, in regulating the activity of enzymes have been elaborated by Atkinson⁶⁵ into the adenylate control hypothesis. ATP is a negative effector for certain enzymes and in resting muscles where ATP levels are high, the enzymes are under restraint. 5'-AMP is a positive effector of these enzymes. During muscle work, the ATP levels fall removing the restraint, while 5'-AMP levels rise. Through the change in the nucleotide ratio the activities of these enzymes are
Fig. 14. Relationships between the enzymes catalysing the formation and breakdown of glycogen. The dotted lines show where forms of the enzymes, which are normally inactive, become active in the presence of the agent indicated.
increased. Such rapid control is well suited to accommodate the sudden demand for energy during muscle work.

Conversion of phosphorylase \textit{a} to phosphorylase \textit{b} is catalysed by phosphorylase phosphatase (Fig. 14, upper right). Evidence has recently been provided from Leloir's laboratory,\textsuperscript{66} using skeletal muscle phosphorylase phosphatase from pigeon breast, that this enzyme may also be subject to metabolic and adrenergic control. They found that the activity of the enzyme was increased on incubation with ATP, phosphocreatine and magnesium, and decreased with ATP alone, an effect which was accelerated by cyclic AMP. They concluded that phosphorylase phosphatase from pigeon breast muscle has at least two interconvertible forms. Similar control properties have been reported from studies of phosphorylase phosphatase from bovine adrenal cortex.\textsuperscript{67}

If multiple forms of this enzyme do exist, nothing is yet known of the enzymes responsible for the interconversion reactions.

The enzymes of glycogen synthesis have been studied, notably by Larner\textsuperscript{68} (Fig. 14, left). Glycogen synthetase has been shown to exist in two forms: synthetase I, which is independent of glucose 6-phosphate for activity, and synthetase D, which is dependent upon glucose 6-phosphate for activity. The two forms of the enzyme are interconvertible by transferase phosphatase, a dephosphorylating enzyme which produces the active form, and transferase I kinase, a phosphorylating enzyme which gives the inactive form; the latter reaction is accelerated by cyclic AMP.

In response to adrenergic stimulation, the whole system is integrated to promote glycogenolysis. As phosphorylase kinase is
converted to its activated form to catalyse the phosphorylase $b$ to $a$ reaction, phosphorylase phosphatase is converted to its less active form and at the same time the glycogen synthesising system is inactivated. As a result of this integration, glycogen is not synthesised from the precursors which are produced as a result of glycogen catabolism. Although the interconverting reactions probably mediate the effects of epinephrine on this system, these interconversions probably do not mediate metabolic regulation in the moment to moment activity of living tissue.
5 BIBLIOGRAPHY


