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ACTIONS OF AUTONOMIC DRUGS ON PHOSPHORYLASE ACTIVITY AND FUNCTION

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LIST OF ABBREVIATIONS

Adenosinemonophosphate—AMP
Adenosinediphosphate—ADP
Adenosinetriphosphate—ATP
Adenosine 3′,5′-monophosphate—cyclic AMP or 3′,5′-AMP
Nicotinamide adenine dinucleotide (DPN)—NAD
Reduced nicotinamide adenine dinucleotide (DPHN)—NADH
Dichloroisoproterenol—DCI
Dimethylphenylpiperazine—DMPP
4-(*m*-Chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride—McNeil-A-343
Lysergic acid diethylamide—LSD-25

INTRODUCTION

Although pharmacology, the study of the action of drugs on living organisms, is essentially an applied science, impetus for research in this field has come not only from clinical medicine but also from the basic medical sciences. Recently, there has been an increasing interest in the biochemical approach to the study of drugs. Refined chemical methods have been used in investigations of the fate of

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drugs in the organism, and in this field spectacular advances have been made in our understanding of drug absorption, transport, binding, and metabolism.

Other investigations have been concerned with the effects of drugs on metabolism *in vivo* and *in vitro*. Some of these studies have been motivated by a desire to use drugs as tools for the elucidation of the nature of cellular biochemical reactions. Others have been concerned primarily with a search for mechanisms by which drugs exert their characteristic actions in the intact organism.

In this article we have chosen to concern ourselves with drugs which mimic or influence the sympathetic and parasympathetic nervous system. We shall emphasize those instances where, in our opinion, there is some reason to believe that a relation may exist between the effects of a drug on metabolism and its actions on cellular functions such as transport of metabolites, electrical phenomena, secretion, and contraction and relaxation.

It has been known for many years that catecholamines, in addition to their characteristic actions on the cardiovascular system, can influence numerous reactions in the metabolism of carbohydrate, fat, and protein. Earlier investigations into the metabolic actions of the catecholamines have been reviewed by Ellis (45), and other reviews which deal in whole or in part with this subject are also available (6, 47, 64a, 158, 192). The most exciting development in the study of the action of the catecholamines was the discovery by Cook *et al.* (24) and Sutherland and Rall (172, 173, 190) of cyclic 3′,5′-AMP and the finding by the latter authors that this nucleotide was an intermediate in the stimulation of phosphorylase by epinephrine. This work made it possible to describe in detail the action of a hormone on biochemical reactions in a cell-free system. Interesting and informative accounts of the investigations up to 1960 of the properties and functions of cyclic AMP can be found in articles by Sutherland and Rall (174, 191, 192, 193).

The great interest on the part of pharmacologists in cyclic AMP and phosphorylase came from the many observations that this system was influenced not only by catecholamines but by other drugs and hormones as well. Particularly intriguing was the possibility that the formation of cyclic AMP and subsequent phosphorylase activation may be involved in mechanisms concerned with the transformation of an impulse into alterations of cellular activity and organ function. Since this review will be concerned so extensively with the action of drugs on cyclic AMP and phosphorylase, it is essential to discuss our present knowledge of the basic chemistry of this complicated and fascinating biochemical system.

I. CHEMISTRY OF THE CYCLIC AMP-PHOSPHORYLASE SYSTEM

A. Glycogen phosphorylase

Glycogen phosphorylase catalyzes the reversible reaction $(\text{glycogen})_n + \text{Pi} = (\text{glycogen})_{n-1} + \text{glucose-1-P}$ [n = glucose units in glycogen; Pi = inorganic phosphate]. In liver the enzyme exists in two forms: phosphorylase itself and a precursor of the active enzyme, dephosphophosphorylase (170, 188, 189, 216). During the conversion of the inactive form to the active enzyme, inorganic

phosphate from ATP becomes attached to the precursor, dephosphophosphorylase, to form the active enzyme (170). In the transformation of phosphorylase to dephosphophosphorylase, inorganic phosphate is split off (216). These interconversions are catalyzed by two specific enzymes, dephosphophosphorylase kinase and phosphorylase phosphatase (170, 216).

The phosphorylase enzymes in other tissues differ in several respects from those found in liver. In a series of classical papers published in 1943 (25, 30, 31, 59), Cori *et al.* demonstrated the existence of two phosphorylase enzymes in rabbit skeletal muscle and described their properties in detail. The form which is the counterpart of liver dephosphophosphorylase was called phosphorylase *b* (31). This form is enzymatically inactive, but it becomes active in the presence of AMP. When phosphorylase activity was measured in the direction of glycogen synthesis and in the presence of excess glucose-1-phosphate and glycogen, the concentration of AMP necessary for half maximal stimulation of crystalline phosphorylase *b* was 5×10^{-5} M (K_M). The counterpart of active liver phosphorylase, phosphorylase *a*, was obtained in crystalline form (59) and shown to be active in the absence of an added coenzyme. However, AMP was not entirely without effect on phosphorylase *a* in that it caused about 25 % further stimulation of activity of the enzyme. The K_M for AMP stimulation of phosphorylase *a* was 1.5×10^{-6} M, a value much lower than the K_M for the activation of phosphorylase *b* by AMP. From observation *in vivo* it appeared that the two phosphorylase enzymes in muscle are readily interconvertible, and an enzyme was demonstrated in muscle extracts which catalyzed the formation of phosphorylase *b* from phosphorylase *a* (31, 32). When the crystallization of phosphorylase *b* was accomplished (26), it became possible to study the phosphorylase *a* to *b* transformation in detail. It was originally thought that the reaction involved the removal of AMP from the active enzyme (31); but no release of adenylic acid from phosphorylase *a* could be detected (32). Keller and Cori (95) later observed that the conversion of phosphorylase *a* to *b* was associated with a halving of the molecular weight of the enzyme, and Graves *et al.* (58) demonstrated that four molecules of inorganic phosphate were removed per molecule of phosphorylase *a*. The reaction can, therefore, be written: phosphorylase *a* $\rightarrow$ 4 inorganic P + 2 phosphorylase *b*.

The conversion of skeletal muscle phosphorylase *b* to phosphorylase *a* was found by Fischer and Krebs (50) to be catalyzed by an enzyme which required divalent metals for activity. In the reaction, phosphate is transferred from ATP and a dimerization of the enzyme occurs (102). The same authors and their co-workers have studied the transformation of phosphorylase *b* to *a* in great detail. Using crystalline phosphorylase *b* (52) from rabbit skeletal muscle, they showed that the reaction proceeds according to the equation: 2 phosphorylase *b* + 4 ATP $\rightarrow$ phosphorylase *a* + 4 ADP (103). Since the process involved the essentially irreversible transfer of a high-energy phosphate group to phosphorylase *b*, the enzyme catalyzing the reaction was named phosphorylase *b* kinase (103).

In an investigation designed to determine the site phosphorylated during the

phosphorylase *b* to *a* conversion, crystalline P^{32} phosphorylase *a* was obtained by incubating crystalline phosphorylase *b* with ATP^{32} in the presence of phosphorylase *b* kinase (53). The labeled phosphorylase *a* was then hydrolyzed by trypsin and radioactive peptides were isolated. Their composition showed that the phosphate group became attached to a serine OH group and that the serine phosphate was situated in the enzyme in the following sequence of amino acids: lysine-glutamine-isoleucine-(serine-phosphate)-valine-arginine. Because all the phosphate incorporated into the enzyme molecule was attached to this particular amino acid sequence, the speculation was made that the change in enzymatic properties during conversion of phosphorylase *b* to *a* was related primarily to phosphorylation at this amino acid site (53).

As part of the studies of the comparative chemistry of the phosphorylase enzymes, Yunis *et al.* (218) crystallized phosphorylase *a* and *b* from human skeletal muscle. Although these enzymes can be distinguished immunologically from the corresponding enzymes from rabbit muscle (220), the enzymes from the two species have almost identical chemical and physical properties (218), and their amino acid compositions do not differ significantly (3). As expected from the close similarity of the enzymes, the site phosphorylated in the phosphorylase *b* to *a* reaction was the same in enzymes obtained from skeletal muscle of rabbit or man (86).

The properties of phosphorylase *b* kinase, which catalyzes the conversion of phosphorylase *b* to *a*, were found on closer examination to be extremely complex (104). The enzyme exists in rabbit skeletal muscle extracts in a form which is inactive at pH 7.0 but shows activity as the pH is increased. Activation of the enzyme at pH 7 can be accomplished by preincubation with calcium ions or with ATP and magnesium; the latter process is accelerated in the presence of cyclic AMP (104). Calcium ions have a dual function in the kinase system in that they cause activation of phosphorylase *b* kinase and are competitive inhibitors with respect to Mg^{++} in the phosphorylase *b* to *a* reaction itself (104). These fascinating but complex relationships have been discussed in an article by Krebs and Fischer (105) in which the authors liken the complexity of the cyclic AMP-phosphorylase system to that of the mechanisms involved in blood clotting.

Experiments with extracts from heart muscle have indicated that the phosphorylase enzymes in this tissue have properties similar to those of the skeletal muscle enzymes (33, 73). Rall *et al.* (171) described the purification of phosphorylase *a*, a phosphorylase phosphatase, and a phosphorylase *b* kinase from extracts of dog heart muscle. Inorganic phosphate was formed during the action of phosphorylase phosphatase on phosphorylase *a*. When the phosphorylase *b* thus formed was incubated with ATP, Mg^{++} and the kinase enzyme, it was reconverted to phosphorylase *a*. More recently, Yunis *et al.* (219) isolated and characterized a number of phosphorylases from extracts of rabbit heart muscle. Surprisingly, three distinct peaks appeared when a partially purified phosphorylase *b* preparation was chromatographed on a cellulose column. The material from one of these peaks was crystallized and found to be indistinguishable from skeletal muscle phosphorylase *b*. On incubation with ATP and phos

phorylase *b* kinase it was converted into phosphorylase *a*. The other fractions separated by column chromatography, both of which were inactive in the absence of AMP, could not be crystallized. However, with one of them it was possible to show that it became active in the presence of AMP, and that it could be converted to a form with properties similar to those of phosphorylase *a*. It was concluded from these studies that phosphorylase *b* is present in heart in a form identical to the skeletal muscle enzyme, but that two isozymes of the enzyme are present also (219). In similar studies with crystalline skeletal muscle phosphorylase *b*, only one form of the enzyme could be detected (219). Subsequent studies (37) led to the finding that brain and smooth muscle from uterus and bladder, like skeletal muscle, contained only one form of phosphorylase *b*. This, however, was different from the enzyme in skeletal muscle and corresponded to one of the isozymes (type 1) in cardiac tissue. The possible role of several different phosphorylase *b* enzymes in heart muscle remains a matter for speculation.

The fact that enzymes catalyzing the same reaction may not be identical in different organs of the same species also was demonstrated in the earlier studies of Henion and Sutherland (72) who showed that phosphorylases from dog heart and liver were immunologically distinct; they also differed to some extent from phosphorylases of other tissues.

Until recently, little was known about the chemistry of the phosphorylase enzymes in smooth muscle. For this reason, Mohme-Lundholm made an investigation of the properties of the phosphorylase system in this tissue (149). Her results led to the conclusion that the *a* and *b* forms of the enzyme, as well as the enzymes catalyzing their conversion, existed in smooth muscle, and that phosphorylase *b* kinase was activated by cyclic AMP. These findings show that the phosphorylase enzymes in smooth muscle are similar to those in skeletal muscle. However, a significant difference was that a higher concentration of AMP was necessary to activate phosphorylase *b* from smooth muscle than for activation of striated muscle phosphorylase *b* when the reaction was studied in the direction of glycogen synthesis.

Bueding *et al.* (16) made an extensive investigation of the properties of phosphorylase *b* from guinea pig taenia coli and found that the enzyme, when studied by immunochemical methods, was different from phosphorylase *b* obtained from skeletal muscle of the same animal. Equally significant was the finding that the kinetic properties of the two enzymes differed markedly. In studies of the phosphorylase reaction in the direction of glycogen breakdown, the dissociation constants and optimal concentrations for glycogen, inorganic phosphate and AMP were 1.4 to 4 times higher for guinea pig skeletal muscle phosphorylase *b* than for the enzyme catalyzing the same reaction in guinea pig intestinal smooth muscle. This interesting study brings out the need for comparative investigations of metabolic reactions in different organs and species and points out the danger in assuming *a priori* that the information about an enzyme in one tissue is applicable to the same enzyme in other tissues.

That the phosphorylases of brain exist in forms that resemble the enzymes from muscle is indicated by the studies of Breckenridge and Norman (13).

These investigators found that extracts of mouse brain prepared from brains frozen immediately after decapitation contained a high level of phosphorylase activity in the absence of added AMP. When the brains were frozen at varying periods after decapitation, phosphorylase activity without AMP decreased progressively. It was also shown that addition of AMP to the brain extracts stimulated phosphorylase activity (13). In a later study Drummond *et al.* (42), in agreement with the observations of Breckenridge and Norman (13), found that brain homogenates from decapitated animals showed high phosphorylase activity in the absence of AMP. On incubation of the homogenates at 30°C the enzyme was rapidly converted to a form which showed almost no activity unless AMP was added. When homogenates which had been inactivated in this manner were incubated with ATP and Mg^{++}, the enzyme was reconverted to the form which did not require AMP. These observations strongly suggest that enzymes similar to muscle phosphorylases *b* and *a* exist in brain and that the enzymes catalyzing the transformations of the two forms of phosphorylase are also present in this tissue. This conclusion was strengthened by the finding that a partially purified phosphorylase preparation from brain which was inactive in the absence of AMP could be converted by incubation with ATP and phosphorylase *b* kinase into a form not requiring AMP for activity (42).

To complete the discussion of the chemistry of the phosphorylase enzymes, two additional characteristics deserve to be mentioned: the presence of sulfhydryl groups in the enzyme protein and the occurrence of the coenzyme pyridoxal phosphate bound to the enzyme. Madsen and Cori (131) titrated crystalline phosphorylase *a* from rabbit muscle with *p*-chloromercuribenzoate and found that this compound inhibited phosphorylase activity and caused the enzyme to split into four parts of equal molecular weight. Enzyme activity and recombination of the subunits could be accomplished by incubation of the inactivated enzyme with cysteine. From the amount of *p*-chloromercuribenzoate combining with the enzyme, it was calculated that eighteen sulfhydryl groups reacted with the inhibitor per molecule of enzyme. This is identical to the number of cysteine residues determined by amino acid analysis (3, 202) and shows that phosphorylase *a* does not contain S—S bridges and that all the non-methionine sulfur in the molecule is present in sulfhydryl groups. That not all of these groups are equally important for enzyme activity was demonstrated in the studies by Kudo and Shukuya (106), who observed that inhibition of phosphorylase *a* by *p*-chloromercuribenzoate did not proceed in parallel with mercaptide formation and that four of the eighteen sulfhydryl groups of the enzyme could react with N-ethylmaleimide without change of the activity of the enzyme.

Another interesting property of the phosphorylase enzymes is the presence of pyridoxal phosphate in the molecule. It had been known that muscle phosphorylase contained organically bound phosphate, and it was suspected that this phosphate was part of adenylic acid combined with the enzyme, but no AMP could be detected in phosphorylase preparations. The mystery was solved only when Baranowski *et al.* (7) and Cori and Illingworth (28) found that pyridoxal-5-phosphate was present in the enzyme molecule. It was demonstrated

that muscle phosphorylase *a* and phosphorylase *b* contained four and two moles of the coenzyme, respectively, per mole of enzyme. Pyridoxal phosphate appears to play a vital part in the action of the enzyme since removal of the compound from phosphorylase *a* caused complete loss of activity. Reactivation of the enzyme occurred slowly on incubation with a slight excess of pyridoxal phosphate (28). Spectral properties indicate that the aldehyde group of the pyridoxal phosphate is involved in the combination with the protein part of the enzyme (51), but the exact role of the coenzyme in the catalytic action of phosphorylase remains unknown. The view that the compound is an essential part of the phosphorylase enzymes has received support from the findings that pyridoxine deficiency in the rat (43) and mouse (129) leads to marked decreases in muscle phosphorylase activity. In the rat, liver phosphorylase decreased less markedly and more slowly in pyridoxine deficiency than the muscle enzyme (43). In one strain of mice, severe vitamin B_6 deficiency did cause a small decrease in liver phosphorylase activity, while no enzymatic defect could be demonstrated in another strain (129). When the vitamin B_6-deficient animals were given a diet supplemented with the vitamin, enzyme activity returned to normal (43, 129). In mice receiving a diet enriched with pyridoxal phosphate, muscle phosphorylase activity was greater than normal (129). Adrenal gland phosphorylase activity in rats was reduced by about 30% after 35 days on a diet deficient in pyridoxine (197); adrenal glucose-6-phosphate dehydrogenase was not altered.

The distribution of glycogen and phosphorylase in different areas of the rabbit heart was measured by Jedeikin (93), who found that total phosphorylase activity decreased from endocardium to epicardium in a pattern similar to that observed for glycogen. Using frog and rat hearts, Dhalla *et al.* (40) observed that phosphorylase activity was higher in ventricles than in atria.

Several histochemical studies have appeared dealing with the distribution and localization of glycogen phosphorylase in organs and tissues. Takeuchi and Kuriaki (195) first adapted to animal tissues the method of Yin and Sun (217), which depends on the reaction of iodine with glycogen synthesized from glucose-1-phosphate in the presence of AMP. The presence of phosphorylase was demonstrated in heart, cartilage, epithelial tissue, and skeletal and smooth muscle. Phosphorylase activity was found in the cytoplasm of cells and could not be detected in nuclei. In skeletal muscle, enzyme activity was closely associated with striated structures in muscle fibers.

With the method of Takeuchi and Kuriaki (195), liver cells did not consistently stain for phosphorylase despite the known presence of the enzyme in this organ. The apparent reason for the lack of appearance of phosphorylase with this histochemical method was the fact that in liver most or all of the enzyme was present in the inactive form. The difficulty was overcome by Guha and Wegmann (63), who added ATP and magnesium ions to the incubation medium and in this way converted at least some of the inactive phosphorylase to the active form. Another defect in the otherwise excellent method of Takeuchi and Kuriaki (195) was that, in tissues such as muscle, it was not possible to distinguish between the *b* and *a* forms of the enzyme. What appear to be successful

attempts to differentiate between the two forms of phosphorylase have been made (57, 61, 62) by adaptations of the procedures used by Takeuchi and Kuriaki (195).

In concluding this discussion of the chemistry of the phosphorylase enzymes, it should be emphasized that the existence of two forms of an enzyme that are readily interconvertible in the cell is not unique. There is evidence that phosphofructokinase exists in an active and an inactive form (136, 137, 164, 177, 205), and recently this also has been found to be true for uridine diphosphoglucose-α-glucan-transphosphorylase, the last in the chain of enzymes involved in catalyzing glycogen synthesis. Rosell-Perez *et al.* (180) and Friedman and Larner (55) showed that the enzyme existed in skeletal muscle in two forms, one requiring glucose-6-phosphate for activity, the other independent of this cofactor. The analogy with the phosphorylase enzymes extends also to the fact that a hormone, in this case insulin (203), stimulates the transformation of one form to the other. It may be of wide biological significance that the enzymes concerned with glycogen synthesis and breakdown both are present in active and inactive states and that, therefore, mechanisms are present for the precise control of glycogen metabolism.

B. The adenyl cyclase system

The properties of the adenyl cyclase system, *i.e.*, the enzymes involved in the synthesis of cyclic AMP, have been described in publications from Sutherland's laboratory (38, 39, 98, 154, 175, 194). The enzyme complex is associated with a high density particulate fraction of tissue homogenates and was postulated to be present in the cell as part of the cell membrane (194). In a later investigation by Davoren and Sutherland (39), an extensive effort was made to localize the enzyme system in preparations from pigeon erythrocytes and rat liver. Their results showed that adenyl cyclase activity was indeed associated with fragments of the cell membrane and was not present in nuclei or disintegrated mitochondria.

Up to the present time the enzymes involved in the cyclase system have not been identified, but the reactions, which result in the formation of an internal anhydride of adenylic acid, include the removal of the two outer phosphorus atoms of ATP and the appearance of pyrophosphate (175). Adenyl cyclase activity is determined in cell-free tissue preparations by measuring the amount of cyclic AMP formed on incubation in a Tris buffer containing ATP, Mg^{++}, NaF and caffeine (194). The cyclic AMP is assayed by observing its effect in a system consisting of liver dephosphophosphorylase and fractions of liver homogenates possessing dephosphophosphorylase kinase activity (173). Adenyl cyclase is widely distributed in various tissues, the highest levels occurring in brain, spleen, skeletal muscle, heart, and lung (194).

The accumulation of cyclic AMP in tissue homogenates occurs to a very limited extent in the absence of exogenous catecholamines or methylxanthines, but is markedly increased in their presence. Little is known about the mechanism of action of catecholamines in stimulating cyclase activity, although Belleau (11) has speculated that an ATP-Mg^{++}-catecholamine complex may be formed.

This complex is assumed to be unstable and when it dissociates gives rise to the formation of cyclic AMP.

The mechanism by which methylxanthines increase the accumulation of cyclic AMP in cell-free systems is better understood than the action of catecholamines in this system. Methylxanthines have been shown to inhibit the enzyme, cyclic 3′,5′-nucleotide phosphodiesterase, which hydrolyzes cyclic AMP to form AMP. Phosphodiesterases are widely distributed in nature and have been found in all tissues studied with the possible exception of avian erythrocytes (20). As with adenyl cyclase, the concentration of this enzyme is particularly high in brain (20). By hydrolyzing cyclic AMP the diesterase prevents the accumulation of the nucleotide and plays a role in the termination of the action of cyclic AMP. Thus far it is the only enzyme known to inactivate cyclic AMP.

It should be pointed out that in the intact cell the content of cyclic AMP is at all times extremely small. In working rat hearts for example, even after exposure to epinephrine, the intracellular concentration of the nucleotide is in the range 10^{-7} to 10^{-6} M (162). These values are of a different order of magnitude from the concentrations of ATP, ADP and AMP, which exist in heart in concentrations of 6×10^{-3}, 1×10^{-3}, and 1×10^{-4} M, respectively (54, 56). It is, therefore, possible for the cell to increase its level of cyclic AMP manyfold without appreciable changes in the levels of ADP or ATP.

From the discussion above it is apparent that concentrations of cyclic AMP in the cell may be altered in two ways: 1) by changes in the rate of synthesis of cyclic AMP, as occurs with catecholamines, and 2) by changes in the activity of the phosphodiesterase, as occurs with methylxanthines.

In figure 1 the authors have taken the liberty of picturing cyclic AMP synthesis as a process consisting of two or more separate reactions. If this hypothesis is correct, there may be several sites at which drugs may influence cyclic AMP formation. In addition, one drug may, theoretically, block the action of another at any of the sites involved in the formation and breakdown of cyclic AMP. Effects of drugs on the metabolism of cyclic AMP have been well established, but it is also possible, as indicated in figure 1, that the actions of cyclic AMP in the cell can be influenced by pharmacologically active substances. A more

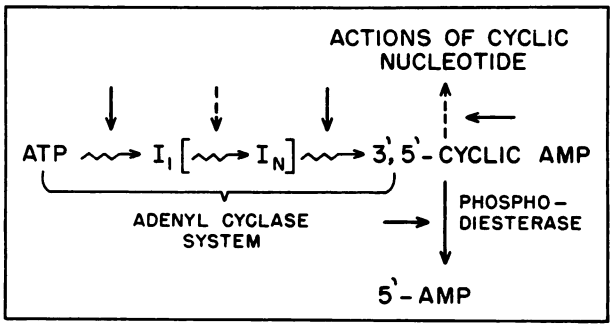

FIG. 1. Possible sites of drug action on cyclic AMP system.

detailed account of the effects of drugs on cyclic AMP will be presented in a subsequent section of this review.

C. Actions of cyclic 3′,5′-AMP

It is well established that cyclic AMP stimulates the transformation of inactive to active phosphorylase *in vitro*. This was demonstrated by Rall and Sutherland (173) with cell-free preparations from rat liver. These investigators incubated a supernatant fraction from liver homogenate plus added purified liver dephospho-phosphorylase with ATP, Mg^{++}, caffeine, and concentrations of cyclic AMP varying from 0 to 2×10^{-7} M. Phosphorylase activity, measured at the end of a ten-minute incubation at 30°C, increased in a linear fashion with the amount of cyclic AMP added. The system forms the basis for the biological assay of cyclic AMP (173). The investigations of Krebs *et al.* (104, 105), discussed earlier, provided evidence that in muscle the action of cyclic AMP in stimulating phosphorylase activity was exerted by an effect on phosphorylase *b* kinase. Fresh rabbit muscle extracts or a fraction obtained by precipitation at pH 5.7 to 5.8 and partially dissolved at pH 7 was used as a source of the enzyme. At neutral pH no phosphorylase *b* kinase activity was present, but enzyme activity slowly appeared on incubation in the presence of ATP and magnesium ions; activation of the enzyme was markedly accelerated by cyclic AMP. It was postulated that phosphorylase *b* kinase exists in two forms, like the phosphorylase enzymes, and that cyclic AMP stimulates the conversion of the inactive to the active form of the kinase (104). Not only does cyclic AMP promote the formation of phosphorylase *a* from phosphorylase *b* by its stimulatory action on phosphorylase *b* kinase, but, as Riley and Haynes (178) have shown in an enzyme system from bovine adrenal cortex, the nucleotide inhibits phosphorylase phosphatase. There are, therefore, two mechanisms by which cyclic AMP may increase phosphorylase *a* activity: by increasing its formation and by inhibiting its destruction.

Another well-established action of cyclic AMP in a cell-free system is the effect of this compound on phosphofructokinase. Mansour originally found (133) that serotonin, which stimulates the rhythmical movements of the parasitic trematode, the liver fluke (*Fasciola hepatica*) (132), also increased the glucose uptake, glycogen breakdown and lactic acid formation of this organism. In contrast to serotonin, epinephrine only slightly increased glucose uptake and had no significant effect on glycogen levels or lactic acid production (133). Subsequently Mansour (135) showed that glycolysis was increased above normal in homogenates obtained from organisms incubated with serotonin. The addition of serotonin or cyclic AMP to homogenates *in vitro* increased glycolysis markedly when glucose, glucose-6-phosphate or fructose-6-phosphate was used as substrate. That stimulation of the enzyme phosphofructokinase was responsible for this metabolic effect was indicated by the observation that the increase in glycolysis was much reduced when fructose-1,6-diphosphate was the substrate. It also was shown that exposure of intact flukes to serotonin decreased the substrate (fructose-6-phosphate) and increased the product (fructose-1,6-diphosphate) of the phosphofructokinase reaction (135). Direct evidence that phosphofructokinase

activation was involved was obtained in studies in which specific assays for this enzyme were performed (136). In fresh homogenates which contained little phosphofructokinase activity, a marked stimulation of enzyme activity was observed in the presence of serotonin or cyclic AMP. The latter compound, but not serotonin, also stimulated phosphofructokinase in extracts freed of particulate material or in concentrated enzyme solutions obtained by precipitation with ammonium sulfate. Finally, evidence for the role of cyclic AMP in the mediation of the serotonin effect was supported by the observation that serotonin caused a large and rapid increase in the production of cyclic AMP by particulate fractions from liver fluke homogenates (134).

Mansour (137) also demonstrated that cyclic 3′,5′-AMP can increase phosphofructokinase activity in mammalian heart muscle. Partially purified preparations of the enzyme from this tissue were inhibited strongly by ATP at pH 6.9. This inhibition could be overcome by the addition of cyclic AMP to the incubation medium. However, the system was found to be extremely complex in that enzyme activity was influenced not only by ATP and cyclic AMP, but also by the concentrations of substrate (fructose-6-phosphate), inorganic phosphate, AMP and ADP.

Several studies with different tissues have shown that phosphofructokinase is a rate-limiting enzyme in the glycolytic pathway (137, 164, 177, 205). Because of the key position that phosphofructokinase holds in glycolysis, the influence of cyclic AMP on the activity of this enzyme assumes added importance.

Effects of cyclic AMP on enzymes other than the phosphorylase and phosphofructokinase systems have not been studied extensively. However, biochemical effects of cyclic AMP have been observed which indicate that the cyclic nucleotide may have other sites of action.

Haugaard and Stadie (67) found that epinephrine and glucagon inhibited the incorporation of C^{14}-acetate into fatty acids in rat liver slices. In this preparation, glucagon also depressed the incorporation of C^{14} from labeled glucose or fructose into fatty acids and increased formation of ketone bodies (68). Implication of cyclic AMP in these changes evoked by epinephrine and glucagon was shown by Berthet (12), who reported that cyclic AMP stimulated ketone body formation and inhibited fatty acid synthesis in hepatic tissue. The mechanism of these effects of cyclic AMP is not clear. It is possible that they are due to a direct action of the nucleotide on fat metabolism, but they also may have been a consequence of alteration in phosphorylase or phosphofructokinase activity.

Studies with adipose tissue have made it abundantly clear that at this site cyclic AMP plays an important role in the regulation of fat and carbohydrate metabolism. A large body of literature has accumulated dealing with metabolic processes in adipose tissue and with the striking effects of many hormones on cellular events in this tissue. This fascinating field has been expertly reviewed by Steinberg and Vaughan (184). Epinephrine, ACTH and glucagon cause a release of free acids from adipose tissue *in vivo* and *in vitro* (184) and activate phosphorylase (200). The action of serotonin is somewhat different in that this substance activates phosphorylase but does not release free fatty acids (200). In their review Steinberg and Vaughan discussed this discrepancy and stated

that "one must conclude either that serotonin has metabolic effects in addition to those of epinephrine, such that changes in triglyceride synthesis and FFA are masked, or that the effects on the 3,5-AMP and phosphorylase systems are actually unrelated to the changes in fatty acid metabolism." Evidence has been obtained recently that the release of free fatty acids produced by epinephrine is caused by an activation of a specific lipase in this tissue (201). From the work of Rizack (179) it appears that the lipolytic action of epinephrine is mediated by cyclic AMP. This investigator demonstrated that cyclic AMP activates lipolytic activity in cell-free extracts of adipose tissue incubated with ATP and magnesium ions. As in the transformation of phosphorylase *b* to phosphorylase *a*, caffeine potentiated the effect of cyclic AMP, and calcium ions could increase enzyme activity in the absence of the cyclic nucleotide.

An unusual action of cyclic AMP was reported by Tarui and co-workers (196). These investigators observed that cyclic AMP mimicked the action of thyroid-stimulating hormone in causing an increase in the penetration of D-xylose and L-arabinose into bovine thyroid slices. Like the findings of Orloff and Handler (160), that cyclic AMP increases the permeability of the toad bladder to water, these results indicate that the cyclic nucleotide alters the properties of the cell membrane. Another component of cells which could be affected by cyclic AMP is the contractile system in muscle. This problem was studied by Uchida and Mommaerts (199), who reported that cyclic AMP at extremely low concentrations prevented the contraction of actomyosin suspended in a medium containing ATP, Mg^{++} and low concentrations of calcium. When $CaCl_2$ was added to the suspension, the relaxing effect of cyclic AMP was overcome and the actomyosin contracted. Honig and Van Nierop (82) reported that cyclic AMP and an unidentified protein component together constituted a cardiac relaxing system. These effects of cyclic AMP were later found not to be easily reproducible (83, 151) and at the present time this important problem remains unsolved.

Cyclic AMP itself appears to penetrate cells only slowly, and the compound, except for producing hyperglycemia (159), has little action when administered to the intact animal. Posternak *et al.* (167) prepared several derivatives of cyclic AMP in attempts to find a substance which would exert a greater biological effect *in vivo* than the parent compound. Acylation of the amino group of cyclic AMP was found to decrease potency in stimulating phosphorylase in a cell-free system, but, in some cases, to lead to greater and more prolonged hyperglycemic action in the intact animal. Among the substances prepared, the N^6-monobutyryl, the N^6-monooctanoyl, and the N^6-2-O-dibutyryl derivatives were all more potent than cyclic AMP in producing and maintaining hyperglycemia when injected intravenously into anesthetized dogs. The increase in the hyperglycemic response produced by these derivatives of cyclic AMP may have been a reflection of a faster entrance of the substances into cells but more likely was due to their greater resistance to hydrolysis by phosphodiesterase.

D. The role of adenine nucleotides in the regulation of phosphorylase activity

In the many studies of the regulation of phosphorylase activity, particularly those concerned with the action of drugs on this system, emphasis has been

placed almost entirely on the transformation of one type of phosphorylase into another and the role that cyclic-3′,5′-AMP plays in this process. However, in tissues in which the enzyme exists in an *a* and *b* form, it has become apparent that both types of phosphorylase are important in the regulation of glycogenolysis. Phosphorylase *b* is inactive in the absence of AMP but becomes active when this coenzyme is present in a sufficiently high concentration. Although the value for half maximal stimulation of the enzyme (K_M) at the concentrations of glycogen, glucose-1-phosphate, and inorganic phosphate present in the cell is not known, the K_M value for phosphorylase *b* from skeletal muscle and heart has been found to be 5×10^{-5} M (0.05 μmol/ml) (25) in the presence of excess glycogen and glucose-1-phosphate. In addition to the uncertainty of the exact value for K_M, another difficulty in estimating the activity of phosphorylase *b* *in vivo* has been the absence of reliable figures for tissue concentrations of AMP. With the advent of rapid freezing techniques (21, 54, 212), it became possible to obtain accurate values for the tissue contents of AMP and the other adenine nucleotides *in vivo*. In heart, the concentration of AMP was found to be 0.1 to 0.2 μmol/g wet tissue (34, 54, 56). If it is assumed that phosphorylase *b* exhibits half maximum activity at a concentration of 0.05 μmol/ml, then most of the enzyme would be active at the concentrations of AMP present in the heart *in vivo*. However, this is incredible, since it would mean that the phosphorylase enzymes in the heart are almost maximally active at all times and that the transformation of phosphorylase *b* to phosphorylase *a* would have little effect on the rate of glycogenolysis in the heart. The answer to the dilemma came with the observation of Parmeggiani and Morgan (163) that ATP was a powerful antagonist to the stimulatory effect of AMP on phosphorylase *b*. This action of ATP has been confirmed in the authors' laboratory in experiments with extracts from rat heart (34). ADP also inhibited the effect of AMP on the activity of phosphorylase *b* (34). Since the cellular concentrations of ADP and ATP are of such magnitude that they would be expected to inhibit the greater portion of phosphorylase *b* activity, it is apparent that phosphorylase *b* *in vivo* is under the restraining influence of these adenine nucleotides. Most recently, Morgan and Parmeggiani (153) found that, in addition to ADP and ATP, the important cellular constituent glucose-6-phosphate is also an inhibitor of phosphorylase *b* in concentrations that may be expected to exist in the cell.

The regulation of cellular phosphorylase activity is then a rather complicated one; figure 2 is a schematic presentation of some of the factors involved. Because of our limited knowledge of all the elements concerned with the activation and inhibition of phosphorylase and the absence of precise information about the cellular concentrations of the substances that inhibit phosphorylase *b*, figure 2 is presented only for illustrative purposes. It does, however, bring into focus rather nicely certain general principles concerning the regulation of phosphorylase activity in the cell.

In figure 2 phosphorylase activity is plotted as a function of the AMP concentration. For simplicity it has been assumed that AMP has no effect on phosphorylase *a*. The activity of this enzyme may, therefore, be presented as a horizontal straight line. Curve A represents the activation of crystalline phos-

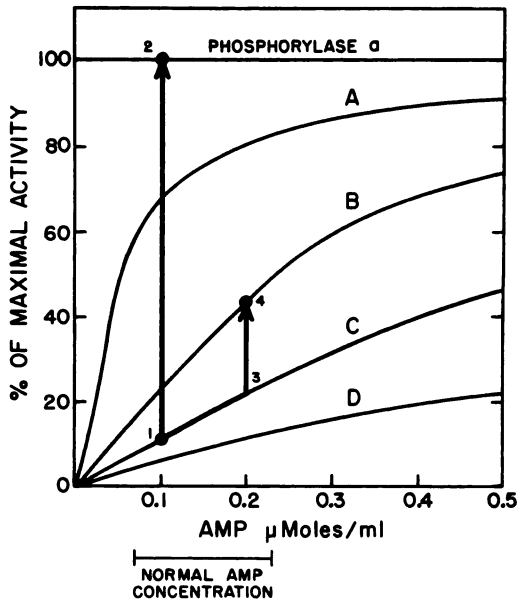

FIG. 2. Regulation of phosphorylase activity in the cell. (Explanation in text.)

phorylase *b* by increasing concentrations of AMP. Curves B, C and D are part of a family of curves representing phosphorylase *b* activation by AMP in the presence of increasing levels of inhibitory cell constituents such as ATP, ADP and glucose-6-phosphate. At the normal AMP concentration (in heart muscle), indicated below the abscissae, crystalline phosphorylase *b* has a high degree of activity. Assuming that the concentrations of inhibitory substances in the cell are such that activation of phosphorylase *b* follows curve C, the activity of the enzyme would be represented by the point labeled 1. It is evident that from point 1 there are two ways in which cellular phosphorylase activity can be increased. First, phosphorylase *b* may be converted to phosphorylase *a*, and this would lead to a marked increase in enzyme activity (point 1 to point 2). Secondly, phosphorylase activity may rise when the concentration of AMP in the cell increases (point 1 to point 3). If at the same time the concentrations of inhibitory substances decline, a further increase in enzyme activity may be expected (point 3 to point 4).

If one assumes that figure 2 expresses the principles involved in the regulation of cellular phosphorylase activity, it becomes apparent that both phosphorylase *a* and phosphorylase *b* are involved in the regulation of glycogenolysis *in vivo*. Transformation of phosphorylase *b* to the *a* form allows the cell to increase its rate of glycogenolysis rapidly and to a marked degree. Conversely, glycogenolysis may be speedily and drastically reduced by reconversion of the *a* form of the enzyme to phosphorylase *b*. In these processes cyclic AMP is a vital factor. On the other hand, the work of Parmeggiani and Morgan (163) has made it possible to envision a role for phosphorylase *b* in the regulation of glyco-

genolysis. This enzyme, which is usually present in the cell in a greater concentration than phosphorylase *a*, is under restraint from inhibitory cell constituents such as ATP, ADP and glucose-6-phosphate. It is of particular significance that variations in levels of cellular components can exert a regulatory influence on the activity of phosphorylase *b*, as has been illustrated in figure 2. The concentrations of adenine nucleotides and glycolytic intermediates are known to be altered significantly under a variety of conditions associated with changes in cell function, such as in anoxia or during alterations in muscle work; under these circumstances increases or decreases in phosphorylase *b* activity and rate of glycogenolysis can be expected. Conversely, stimulation or inhibition of glycogenolysis, produced by drugs or other agents, will lead to changes in concentrations of cellular components which may profoundly alter electrical, mechanical, or other manifestations of cellular activity. Because of the delicate control that cyclic AMP and the phosphorylase enzymes exert over cell metabolism, the studies of the action of drugs on cell function and on this multi-enzyme system take on great biological and pharmacological significance.

II. PHYSIOLOGICAL AND PHARMACOLOGICAL IMPORTANCE OF PHOSPHORYLASE

The universal presence of phosphorylase and glycogen in animal cells is indicative of their importance to the metabolic economy of the cell (185). Glycogen provides a readily available source of additional energy to be drawn upon when cellular activity is increased. In the supply of energy through glycogenolysis, the phosphorylase enzymes play an essential role. The physiological and pharmacological importance of these enzymes stems from the fact that their activities are easily and rapidly influenced by alterations in cellular environment. In addition to pH, metal ions, oxygen tension, and diet, other factors which influence tissue phosphorylase activity include numerous hormones and drugs. Under some circumstances it has been possible to correlate the enzymatic changes with alterations of organ function. The section to follow will be concerned with a review of the physiology and pharmacology of the phosphorylase enzymes.

A. Skeletal muscle

Evidence that epinephrine increased the content of active phosphorylase in skeletal muscle was obtained by Sutherland (187). The enzyme level was high in rat diaphragm immediately after removal from the animal. On incubation of the tissue, phosphorylase activity decreased to a low value; however, when epinephrine was added in a subsequent period of incubation enzyme activity was restored within a few minutes. G. T. Cori and Illingworth (33) observed that intravenous or subcutaneous injection of epinephrine into rats led to an increase in skeletal muscle phosphorylase *a*. Rat gastrocnemius muscle *in situ* when stimulated electrically through its motor nerve to fatigue had a very low content of phosphorylase *a*. Previous injection of epinephrine prevented the depletion of phosphorylase *a* during stimulation; injection of epinephrine after stimulation greatly accelerated the reappearance of phosphorylase *a*. These metabolic effects of epinephrine may be related to the known action of the drug to increase the

work capacity of skeletal muscle as shown, for example, in the studies of Bülbring (18) with the phrenic nerve-diaphragm preparation.

Krebs and Fischer (101) showed that, in resting skeletal muscle, phosphorylase existed mainly in the *b* form. One of the earliest studies demonstrating a correlation between a change in the activity of phosphorylase and muscle function was done by C. F. Cori (27). In his experiments frog gastrocnemius muscle weighted with 5 grams was stimulated electrically through its motor nerve at different rates. As the frequency of stimulation increased, the level of phosphorylase *a* rose.

Several years after these studies by Cori, another group of investigators (181) performed experiments designed to show the influence of varying periods of stimulation upon the activation of phosphorylase. Using excised anterior tibial muscles of the mouse, Rulon followed the change in phosphorylase *a* activity after ½, 1½, 3, 15 and 30 seconds of stimulation. The greatest rise in phosphorylase activity occurred with the first 1½ to 3 seconds, and prolonging the stimulation beyond 3 seconds had little further effect. The authors concluded that phosphorylase is activated with sufficient speed to bring about an increased rate of glycogenolysis within a period sufficiently short to be important in muscle contraction.

An unusual and interesting study of the possible relation between muscle activity and glycogen metabolism was made by Leonard and Wimsatt (118) using hibernating and non-hibernating bats. Determinations of skeletal muscle and liver glycogen concentrations and phosphorylase activities were made in bats hibernating at 3° to 5°C and 20 hours after arousal at room temperature. The salient points of their study may be summarized as follows: 1) Liver glycogen in aroused bats was approximately one-half that found in hibernating bats, while, as anticipated, liver phosphorylase activity was markedly increased after arousal. 2) In contrast to the changes observed in liver, the glycogen levels in muscle increased in the animals upon arousal as did phosphorylase *a*. Since phosphorylase is usually considered to be involved mainly in catalyzing the breakdown of glycogen, these findings would indicate that the activities of the glycogen-synthesizing enzymes also were increased, or that phosphorylase *b* activity was inhibited, or both. 3) Epinephrine injection failed to alter tissue glycogen levels or muscle phosphorylase in hibernating bats, but caused an elevation in per cent phosphorylase *a* in skeletal muscle from bats in the aroused state. It is intriguing to speculate that the tissue content of adenyl cyclase was low in the hibernating animals but increased after awakening.

Effects of epinephrine *in vitro* and *in vivo* on phosphorylase, cyclic AMP, and phosphorylase *b* kinase have been determined separately in various tissue preparations. Until 1962 no one had measured simultaneously these three parameters *in vivo*. With rat gastrocnemius muscle, Posner *et al.* (166) demonstrated the action of epinephrine on cyclic AMP, phosphorylase *b* kinase, and phosphorylase *a* formation. In response to epinephrine injection in the rat, there followed an increase in the levels of cyclic AMP, an augmentation in phosphorylase *b* kinase activity, and a rise in the ratio of phosphorylase *a* to total phosphorylase. This was the first direct evidence that an increase in skeletal muscle phosphorylase *a*

caused by epinephrine could be correlated with alterations in the tissue cyclic AMP level and phosphorylase *b* kinase activity.

The mechanisms involved in bringing about the rise in phosphorylase *a* activity in skeletal muscle stimulated electrically or exposed to epinephrine were compared by Danforth *et al.* (36). During this investigation it became clear that the events resulting in an increase in enzyme activity were quite different in the two circumstances. Experimental evidence for this conclusion was based on the observations that: 1) the increase in phosphorylase *a* during incubation of frog sartorius muscle with epinephrine at 30°C had a half-time of 370 seconds as compared to 0.7 second during isometric contraction following electrical stimulation of the muscle itself, and 2) dichloroisoproterenol inhibited the effect of epinephrine or norepinephrine and hastened the reversal of epinephrine action, but had no effect on the increase in phosphorylase *a* associated with muscle work.

Guillory and Mommaerts (64) studied the effect of electrical stimulation of frog sartorius muscle at different temperatures. At 0°C, tetanic stimulation for 15 seconds only slightly increased the activity of phosphorylase *a*; when stimulation was terminated, enzyme activity continued to rise. In contrast, tetanization of the muscle at 25°C resulted in a two- to threefold increase in phosphorylase *a*, and enzyme activity fell promptly on cessation of stimulation.

In view of the effects of electrical stimulation of skeletal muscle on phosphorylase activity, one would expect alterations of this enzyme system following denervation. Studies of this problem have been made, but the results have been inconclusive up to this time (4, 87, 88, 140). However, glycogen synthesis in pigeon breast muscle, as measured by the incorporation of C^{14}-glucose into glycogen, was depressed after denervation (22).

It appears that skeletal and cardiac muscle phosphorylases respond somewhat differently to subcutaneous injections of epinephrine. Hornbrook and Brody (85) found that epinephrine administered by this route in rats caused an increase in skeletal muscle phosphorylase *a* but had no effect on cardiac phosphorylase. Accompanying the increase of the enzyme in skeletal muscle, there was a fall in glycogen content. When vasoconstriction was prevented by pretreatment with phenoxybenzamine, administration of epinephrine resulted in higher plasma concentrations of the catecholamine. In the presence of the adrenergic blocking agent phosphorylase *a* levels rose in both skeletal muscle and cardiac tissue after epinephrine injection. The results of this study indicate that skeletal muscle phosphorylase is more sensitive to low levels of circulating epinephrine than is that of the myocardium.

There are several reports in the literature suggesting a direct relationship in man between the amount of phosphorylase in skeletal muscle and the ability to perform muscle work. For example, Schmid and Mahler (182) and Pearson *et al.* (165) have published extensive studies of patients with a myopathy which involved an unexplained limited capacity for muscular exercise. It was eventually found that there was a complete absence of phosphorylase in the skeletal muscles of these patients. As a result of the enzyme deficiency, muscle glycogen stores were four to five times those found in normal persons. Because the patients had no

access to the energy derived from the breakdown of glycogen, their ability to perform muscular work was markedly curtailed. The rise in blood sugar following epinephrine infusion (165) was normal, indicating that the phosphorylase enzyme system in the liver was intact.

Pertinent to these studies in man is the work of Lyon and Porter (130) on muscle glycogenolysis in two inbred strains of mice. In one strain phosphorylase *a* and phosphorylase *b* kinase were almost absent in skeletal muscle, while in the other strain the levels of these enzymes were normal. In spite of the striking differences in enzyme content, both strains of mice had an equal capacity to perform muscle work, and in both an increased glycogenolysis followed exercise or epinephrine administration. The marked differences in enzyme content found in skeletal muscle were not observed in heart, and in both strains of mice increases in cardiac phosphorylase *b* kinase and phosphorylase *a* could be induced. This interesting study calls attention to the importance of factors other than phosphorylase *a* in the control of glycogenolysis in muscle.

B. Cardiac muscle

In view of the importance of the phosphorylase enzymes in the regulation of glycogen metabolism, the state of these enzymes in a constantly active muscle, such as the heart, is a problem of exceptional interest. During his investigations on the activity of phosphorylase in striated muscle, Cori (27) also studied this enzyme in cardiac tissue. Phosphorylase *a* activity in hearts from rabbits or rats was found to be considerably higher than that observed in skeletal muscle. With heart slices Ellis *et al.* (46) showed that epinephrine increased glycogenolysis, phosphorylase activity, and the concentration of glucose-6-phosphate. The effects of cardiac stimulating drugs on the intact heart phosphorylase were first investigated by Hess and Haugaard (73). When epinephrine or aminophylline was infused into the isolated, perfused rat heart, there was an increase in enzyme activity concomitant with the familiar positive inotropic effect of these substances. It was suggested that a correlation existed between the inotropic actions of cardioactive drugs and their effects on phosphorylase. This idea received support from the studies of Kukovetz *et al.* (107), who found that infusion of isoproterenol, norepinephrine or epinephrine into the isolated, perfused rat heart produced a dose-dependent increase in force of contraction and activity of phosphorylase *a*. No significant changes in heart rate were observed in these experiments. Sympathomimetic amines with no action on heart contractility exerted no effect on phosphorylase. Belford and Feinleib (8) observed with the isolated rat auricle driven at a constant rate, that exposure to epinephrine resulted in significant increases in the force of contraction and activity of phosphorylase *a*. Mayer and Moran (142) with the open-chest dog preparation also studied the effect of sympathomimetic amines on contractility and cardiac phosphorylase activity. These investigators observed that stimulation of the heart by catecholamines and ephedrine was accompanied by augmentation of the activity of phosphorylase *a*.

In the studies of Kukovetz *et al.* (107) with the rat, the potency of *dl*-isopro-

terenol was about five times that of *l*-epinephrine when contractility or enzyme activity was measured; *l*-norepinephrine was twice as potent as *l*-epinephrine. Mayer and Moran (142) found in the dog that *dl*-isoproterenol was approximately ten times more potent than *l*-epinephrine or *l*-norepinephrine. It is of interest that in later studies with the adenyl cyclase system from particulate fractions of dog heart Murad *et al.* observed the same relative potencies of the catecholamines in stimulating cyclic AMP accumulation (154).

Because of the marked effects of exogenous catecholamines on heart phosphorylase, it would be expected that enzyme activity also would increase as a result of electrical stimulation of the cardioaccelerator nerves. That such was the case was shown in the intact dog by Mayer and Moran (142). Dichloroisoproterenol prevented both the positive inotropic and the phosphorylase activating effects of either epinephrine administration or sympathetic nerve stimulation (142).

1. Relation of phosphorylase activity to cardiac function. Kukovetz *et al.* (107) did not find any dissociation between the effects of catecholamines on contractility and activity of phosphorylase *a* in the isolated, perfused rat heart. However, Mayer and Moran (141, 142) and Mayer *et al.* (145) using the open-chest dog preparation observed a significant increase in myocardial force of contraction after extremely small doses of epinephrine with no measurable change in phosphorylase activity, whereas, with doses of epinephrine greater than 1 μg/kg (141, 145) or with electrical stimulation of the cardiac sympathetic nerves there was an increase in enzyme activity along with the positive inotropic response. Hess *et al.* (75) reinvestigated the action of epinephrine on contractility and phosphorylase activity in the perfused rat heart. With small doses of epinephrine, within the limitations of the methods used, no dissociation between the functional and metabolic effects of the drug was evident. Most recently Drummond *et al.* (42) studied the effect of epinephrine on the force of contraction and cardiac phosphorylase activity in the perfused rat heart and the open-chest dog preparation. At very low concentrations of epinephrine (0.025 and 0.4 μg total dose) these authors did not observe a significant augmentation in the activity of phosphorylase *a*, although the force of contraction was increased. At higher doses of epinephrine there was a sharp increase in phosphorylase activity and a greater positive inotropic effect. In the open-chest dog preparation, doses of epinephrine of 0.1 and 0.2 μg/kg produced a positive inotropic action but had no effect on phosphorylase activity. When epinephrine was administered in greater amounts, both enzyme activity and contractility increased markedly.

Support for an association between increased force of cardiac contraction and phosphorylase activity was obtained by Øye *et al.* (162). These investigators did not attempt to establish a dose-response relationship, but found that the addition of epinephrine to the recirculating fluid of a perfused rat heart caused a prompt and simultaneous rise in heart rate, systolic pressure, cyclic AMP, and phosphorylase *a*. All of the studies published thus far are in agreement that doses of catecholamines well below those required to produce maximal mechanical response also give rise to an increase in cardiac phosphorylase *a*.

The discrepancy in results between the different laboratories is apparent primarily in studies of the effects of small doses of catecholamines. The reviewers would like to point out that small changes in cardiac phosphorylase activity are difficult to measure and that the techniques used in tissue sampling, freezing and analysis are subject to error. For example, in open-chest preparations it is not certain whether the phosphorylase activity measured in the tissue sample truly represents the activity of the enzyme in the entire ventricles. It is also conceivable that changes in phosphorylase activity may occur during freezing and thawing or during storage of the frozen tissue sample.

Whether or not a dissociation between the effects on function and phosphorylase activity exists when the heart is exposed to very small concentrations of catecholamines must await further investigation. It is likely that the solution to this problem will come not from studies of phosphorylase activity alone, but rather from investigations which include determinations of a number of the products of glycogenolysis.

With drugs other than the catecholamines, a dissociation between mechanical and metabolic effects has been demonstrated. For example, Mayer and Moran (142) reported that ouabain, theophylline, serotonin, and phenoxybenzamine all exerted a positive inotropic action on the dog heart *in situ* but had no effect on cardiac phosphorylase activity. A similar finding was obtained by Nardini and Giotti (156) using guinea pig heart stimulated by histamine. Studies of the action of cardiac glycosides on isolated guinea pig auricles were done by Belford and Feinleib (8). K-Strophanthin at a concentration which produced an increase in the height of contraction also caused a rise in phosphorylase *a*. However, at concentrations of the glycoside which depressed the contractility of the preparation, enzyme activity was also well above control values. In the open-chest cat preparation (8) there was no increase in cardiac phosphorylase activity after administration of digitoxin in amounts sufficient to produce arrhythmias. In the opinion of the reviewers, no definitive conclusions can be made concerning the action of cardiac glycosides on heart phosphorylase because of the paucity of information and diversity of the preparations studied up to the present time.

The action of calcium ions on heart function and phosphorylase is of special interest because of their known stimulatory effect on phosphorylase *b* kinase in cell-free systems (104, 105). With isolated guinea pig auricles, calcium chloride at a concentration which increased contractility also elevated phosphorylase *a* activity. At a concentration which depressed auricular contractility the enzyme activity was also markedly increased. In the studies of Mayer and Moran (142) with the open-chest dog, low doses of calcium chloride which greatly stimulated the contractile force of the heart did not significantly change cardiac phosphorylase. With larger doses of calcium chloride there was an increase in both enzyme activity and force of contraction. To understand more thoroughly the mechanism of the calcium effect, it would be important to determine whether the changes in phosphorylase activity are associated with alterations in the level of cyclic AMP.

The studies with cardiac glycosides and calcium showed that depression of

myocardial contraction was not associated with a decrease in the level of phosphorylase *a*. With the cardiac depressants, quinidine and pentobarbital, phosphorylase *a* activity of isolated rat auricles was unchanged following doses of these substances which markedly inhibited contractility (8). In the isolated, perfused rat heart dinitrophenol, quinidine or chloroquine all produced profound depression of the myocardium, but no alteration of phosphorylase activity (80).

The effect of anoxia on cardiac phosphorylase was studied in three different preparations. In isolated rat auricles (8) and in the perfused rat heart (80), prolonged anoxia did not significantly change enzyme activity although contractility was severely curtailed. In the open-chest dog preparation the immediate effect of anoxia, produced by severing the aorta, was an increase in the activity of phosphorylase *a* (100, 213, 214, 215); approximately 30 seconds after the arrest of blood flow (213, 214, 215) the enzyme activity began to return to normal. Wollenberger *et al.* (215) showed that the *beta* adrenergic blocking agent pronethalol (nethalide) partially blocked the stimulation of phosphorylase; this finding indicates that a sympathetic component was involved. That enzymes other than phosphorylase *a* exhibit an altered activity during anoxia was demonstrated by Regen *et al.* (177). These authors observed an increase in phosphofructokinase activity during anoxia in the perfused rat heart and pointed out that changes in the cardiac adenine nucleotides would be expected to lead to an increase in the activity of phosphorylase *b*.

2. Role of the autonomic nervous system in the regulation of heart phosphorylase activity. Mention has been made of the increase in heart phosphorylase *a* activity accompanying the positive inotropic effect seen during electrical stimulation of the cardioaccelerator nerves (142). The influence of sympathetic activity on cardiac phosphorylase was demonstrated also by Hess *et al.* (74) using various drugs known to depress the sympathetic system. General anesthesia produced by ether or pentobarbital prior to decapitation greatly reduced the elevated enzyme levels usually observed in hearts frozen immediately after removal from decapitated animals. Pretreatment with other drugs which interfere with sympathetic transmission (hexamethonium, bretylium or reserpine) decreased cardiac phosphorylase in hearts from animals which had been decapitated (74). The effect of reserpine in lowering the phosphorylase *a* activity in hearts from decapitated rats was also observed by Lacroix *et al.* (111) and by Belford and Feinleib (9). Iproniazid pretreatment did not prevent the diminution of phosphorylase *a* in hearts from reserpinized animals (9).

Lacroix and Leusen (110) reported that reserpine abolished the positive inotropic effect and increase in phosphorylase activity that follow infusion of tyramine into the perfused rat heart. In contrast, the effects of norepinephrine were not abolished, but rather increased when the drug was infused into hearts from animals pretreated with reserpine. The administration of iproniazid had no effect on the action of large doses of tyramine (110) but potentiated the activation of phosphorylase by smaller doses of this drug (112). As in the open-chest dog experiments of Mayer and Moran (142), dichloroisoproterenol inhibited both the stimulatory action of norepinephrine on phosphorylase activity and its

positive inotropic effect. Unexplained as yet is the increase in phosphorylase *a* activity in perfused hearts from rats pretreated only with reserpine, described by Lacroix and Leusen (110) and confirmed by Hess *et al.* (77).

Elevation of cardiac phosphorylase can also be elicited indirectly by several drugs in the presence of atropine. If acetylcholine, nicotine, dimethylphenylpiperazine (DMPP) or sebacyldicholine is infused in the presence of atropine into the isolated, perfused rabbit heart, there is a marked rise in phosphorylase *a* activity concomitant with the cardiac stimulation (108). The increase in phosphorylase activity following nicotine does not agree with the previous findings of Buffoni and Giotti (17). They found that nicotine had no effect on the phosphorylase of guinea pig atrium; perhaps the difference in species and experimental preparation could explain the divergent results. Further experiments with ganglion-stimulating drugs showed that intravenous injection of DMPP or McNeil-A-343 (76) markedly increased blood pressure and heart phosphorylase *a* activity in the open-chest rat preparation. Both the hypertension and the elevation of myocardial phosphorylase *a* caused by the latter drug were smaller when the animal had been injected with bretylium prior to the experiment (90).

Not only catecholamines or substances releasing endogenous norepinephrine have been found to stimulate heart phosphorylase, but also theophylline, one of the methylxanthines. Hess and Haugaard (73) originally observed an increase in phosphorylase *a* activity in perfused rat hearts infused with aminophylline. The probable mechanism of this effect came from the studies of Sutherland *et al.* (173, 190) when they found that the methylxanthines inhibited the phosphodiesterase responsible for the hydrolysis of cyclic AMP. Administration of one of these compounds, such as theophylline, would be expected to lead to increased tissue levels of cyclic AMP and subsequent activation of phosphorylase. Although Mayer and Moran (142) failed to observe an increase in cardiac phosphorylase activity following intravenous injection of theophylline in the intact dog, this action was noted by Hess *et al.* (77) in the perfused rat heart. Indirect evidence for a stimulatory effect of theophylline on cardiac phosphorylase can be found in the studies of Vincent and Ellis (204) and Belford and Feinleib (10). The former investigators observed that theophylline increased the breakdown of glycogen in the isolated, perfused guinea pig heart. Belford and Feinleib (10) showed that aminophylline injected intravenously in the open-chest cat preparation led to an increase in the glucose-6-phosphate content of the myocardium. A report by Kukovetz and Pöch (109) described stimulatory effects of caffeine, theophylline and euphyllin on cardiac phosphorylase and an inhibition of the actions of these compounds by imidazole. The effect of imidazole may be related to the ability of this compound to increase the activity of phosphodiesterase (20).

As discussed in an earlier section, an accumulation of cyclic AMP in the cell may be brought about by an increase in its rate of formation, as with epinephrine, or by a decrease in its rate of inactivation, as with theophylline. Therefore, under proper circumstances theophylline and other methylxanthines would be expected

to potentiate the biochemical and functional effects of small doses of catecholamines. Potentiation of the inotropic effect of norepinephrine by theophylline was indeed demonstrated by Rall and West (176), who showed that the increase in the force of contraction of electrically driven rabbit atria produced by norepinephrine was augmented when theophylline, aminophylline or caffeine was also present. Theophylline and aminophylline were about equally potent and effective in potentiating the effect of norepinephrine, while the action of caffeine was less pronounced and not predictable. Subsequent to the work of Rall and West (176), the results of combined infusion of theophylline and epinephrine into the isolated, perfused rat heart were reported by Hess *et al.* (77). In this preparation, theophylline did not increase the effects of epinephrine on force of contraction, but did potentiate the increase in cardiac phosphorylase *a* seen after infusion of epinephrine. Potentiation of the effect of epinephrine on phosphorylase activity by theophylline was observed also in the rat diaphragm *in vitro*, although theophylline alone had no effect (77). In addition to potentiating epinephrine action, theophylline accentuates the stimulatory effect of cyclic AMP on blood sugar (159).

The numerous observations that heart phosphorylase was influenced by the sympathetic nervous system and sympathomimetic drugs led to an interest in the action of the parasympathetic system on cardiac phosphorylase. In the open-chest rat preparation it was found that injection of acetylcholine or electrical stimulation of the vagus nerve decreased heart phosphorylase *a* activity along with the familiar hypotension and bradycardia associated with either of these two procedures (76). In addition, the hypertension and phosphorylase stimulation produced by the ganglion-stimulating drug, McNeil-A-343, was antagonized by injection of acetylcholine.

In line with these studies is the work reported by Vincent and Ellis (204), who used the isolated, perfused guinea pig heart. When the heart rate was not controlled, acetylcholine obliterated the glycogenolytic effect of epinephrine and reduced the positive inotropic effect of the drug. In rate-controlled hearts, epinephrine caused the usual increase in glycogenolysis and force of contraction; following acetylcholine injection in these experiments epinephrine-induced glycogenolysis was markedly inhibited, but the increase in force of contraction persisted. The results of the experiments in which heart rate was controlled provide evidence that the metabolic effect of acetylcholine is a direct cellular action of this compound and not a consequence of a change in the mechanical activity of the heart. Although there was no clear-cut separation between the mechanical and glycogenolytic effects of epinephrine in the experiments in which heart rate was not controlled, the latter group of experiments does indicate that epinephrine influences glycogenolysis and force of contraction by independent mechanisms.

Further evidence for an inhibitory action of acetylcholine on cardiac phosphorylase activity was provided by the experiments of Kukovetz (108). When atropine was infused into the isolated rabbit heart, there was a small but significant increase in the activity of phosphorylase *a* as well as in the rate and force

of contraction. The author concluded that the isolated heart was restrained by some cholinergic activity, which caused depression of phosphorylase and was removed by atropine.

That parasympathomimetic drugs exhibited biochemical effects in cell-free systems was shown by Murad *et al.* (154), who reported that the synthesis of cyclic AMP by a particulate preparation from dog heart was inhibited 20 to 30% by acetylcholine or carbachol. This inhibition was prevented by atropine. The changes in phosphorylase activity seen with acetylcholine or atropine are probably a reflection of effects of these substances on the synthesis of cyclic AMP.

We have discussed in considerable detail drug-induced changes in myocardial activity and related alterations in heart phosphorylase. Relevant to this discussion is the work of Klarwein *et al.* (99) concerning the effect of atrial and ventricular fibrillation and ventricular tachycardia on cardiac carbohydrate metabolism. These arrhythmias alter coronary blood flow and myocardial oxygen consumption and, therefore, some of the experiments were done with the coronary circulation artificially maintained. When the coronary flow was not supported, active phosphorylase increased, then diminished during ventricular and atrial fibrillation and during ventricular tachycardia. The biphasic response of the enzyme was attributed by the authors to changes in pH which occur during the arrhythmia. At the onset of ventricular tachycardia and fibrillation, heart muscle becomes alkaline; this favors formation of phosphorylase *a*. With time, lactic acid accumulates, lowering the pH and depressing phosphorylase *a* content. As one would expect, myocardial concentration of lactate and glucose-6-phosphate rose, while that of glycogen diminished, as the arrhythmia continued. No significant changes occurred in the concentration of fructose-1,6-diphosphate, dihydroxyacetone phosphate, or pyruvate. When the coronary circulation was maintained, no changes in the concentration of carbohydrate intermediates or in phosphorylase *a* activity were observed. The results indicate that the enzyme and metabolic alterations measured in heart muscle during the arrhythmias were the consequence of myocardial anoxia.

In addition to the influence of pH on the phosphorylase enzymes, temperature has also been shown to be a factor in the regulation of phosphorylase activity. This was alluded to earlier in reference to the work of Leonard and Wimsatt (118) with hibernating and non-hibernating bats. More recently, Mayer *et al.* (145) studied in more detail the effects of temperature on the response to catecholamines. In the open-chest dog made hypothermic by surface cooling, intravenous injection of norepinephrine produced a triphasic response of cardiac contractility. Force of contraction was first increased briefly, then decreased, and finally increased for a relatively long period. Phosphorylase activity was measured in the second and third phases of the heart's response, and was higher during the depressed stage than during the later period of stimulation. It is interesting that when norepinephrine was administered to a hypothermic animal previously given ouabain, there was a decrease in contractile force, but an increase in phosphorylase activity. An explanation for the mechanism of the curious negative inotropic effect of norepinephrine in the digitalized, hypothermic animal awaits additional experimentation.

Nayler and Wright (157) also studied the influence of temperature on the response of normo- and hypothermic hearts to epinephrine, but they used two different species for their investigations. As a poikilothermic animal the toad was used, and as a homeothermic, the rat was chosen. The hearts from each animal were isolated and perfused at different temperatures and drugs added at appropriate times. In rat hearts as the perfusion temperature was decreased, heart rate and the amplitude of contractions fell, and there was a progressive decrease in the amount of phosphorylase present in the *a* form. When the perfusion temperature reached 12°C, epinephrine no longer exerted a positive inotropic effect, and although there was still an increase in phosphorylase *a* activity, it was considerably less than that obtained at 35°C.

In hearts from the poikilothermic animals, lowering the perfusion temperature from 22°C had a moderate stimulatory effect on contractile force and enzyme activity (157). At 2°C the toad hearts no longer exhibited a mechanical response or a rise in phosphorylase *a* after administration of epinephrine. It was found also that the high proportion of phosphorylase in the *a* form in toad hearts perfused at 2°C could be lowered either by dichloroisoproterenol or by prior administration of reserpine. The authors proposed that the latter finding may mean that poikilothermic hearts respond to cold by releasing their endogenous catecholamines and that this may be the reason that such hearts are able to function at low temperatures.

Another variation in the investigation of temperature effects on phosphorylase activity is that of Nakatani (155). He noted that there were seasonal changes in total phosphorylase and phosphorylase *a* in rat hearts; the content of the enzymes was highest in January and February (winter) and decreased progressively through July. The changes in per cent phosphorylase *a* of total were not as large, although there was a slight decrease during the period of April–June. Confirming the findings of Mayer *et al.* (145), Nakatani also reported a negative inotropic response when epinephrine was injected intravenously into rats with a body temperature lower than 35°C. This depression of contractility was accompanied by an increase in phosphorylase *a* activity.

With regard to the duration of the elevation in phosphorylase *a* activity following epinephrine, Mayer *et al.* (145) obtained results different from those of Nakatani. The former reported that prolonged infusion of epinephrine did indeed increase active phosphorylase, but that the enzyme returned to control levels of activity when contractile force was still maintained above normal. On the other hand, Nakatani observed an increase in enzyme activity long after the positive inotropic effect of epinephrine had disappeared and suggested that the long-lasting elevation of heart phosphorylase *a* activity may be related to the uptake and storage of epinephrine in cardiac tissue.

3. General interpretations. The many studies of the actions of drugs on cardiac phosphorylase activity illustrate the variety of drugs and physiological conditions which influence this enzyme in the heart. Many drugs have been demonstrated to have rapid and specific effects on phosphorylase activity in this tissue. Among the numerous factors which influence the level of cardiac phosphorylase is the activity of the sympathetic nervous system. Increases in the activity of

the enzyme have followed the injection of catecholamines, electrical stimulation of the cardioaccelerator nerves, and administration of drugs which cause release of norepinephrine. Decreases in phosphorylase activity have been demonstrated when sympathetic transmission has been interfered with by general anesthesia, ganglion blocking drugs, agents which interfere with the release or storage of neurohumoral transmitter, or drugs which block the receptor site.

The action of the parasympathetic system on cardiac phosphorylase has not been studied extensively and is not well understood. Effects of acetylcholine on phosphorylase activity in the heart have not thus far been shown to follow a definite dose-response relationship. In the open-chest rat, large doses of acetylcholine are necessary to depress cardiac phosphorylase activity (76), and in the isolated, perfused heart no diminution in enzyme activity was demonstrated (123). Since the action of acetylcholine is most evident when cardiac phosphorylase activity has been stimulated (76), the lack of effect of acetylcholine in the perfused heart may be due to the low sympathetic tone in this preparation. Although it has not been possible to correlate the action of acetylcholine on phosphorylase with changes in heart function, there is further evidence that the drug exerts a biochemical effect in myocardial cells. Vincent and Ellis (204) showed that acetylcholine in the perfused heart diminished the glycogen content, and Murad *et al.* (154) demonstrated a 30% depression of cyclic AMP synthesis in cell-free heart preparations.

That glycogenolysis is indeed increased in the heart stimulated by epinephrine has been demonstrated not only by measurements of alterations in phosphorylase activity, but also by observations of other biochemical changes in the myocardium. The administration of epinephrine to the isolated, perfused heart produces a decrease in glycogen content (204, 209), an increase in the concentration of glucose-6-phosphate (10, 144), and a rise in the amount of lactate appearing in the perfusion fluid (209). Recently, the elegant fluorometric method of Chance *et al.* (23) has made it possible to follow continuously the concentration of reduced nicotinamide adenine dinucleotide (NADH) in the intact, beating heart. Williamson and Chance's (210) observation that a marked rise in NADH occurred after administration of epinephrine to the perfused heart indicated that glycogenolysis was increased.

The approach of Williamson and Chance (210) utilizing the fluorometric technique of Chance *et al.* (23) permits continuous measurement of contractile force and the concentration of one important constituent of the cell, NADH, so that the time sequence of mechanical and biochemical events during drug action can be followed. Since the concentration of NADH in the cardiac cell is dependent upon several factors, *e.g.*, glycogenolysis, ADP formation as a result of increased work, and interaction with the lactate-pyruvate system (210), the interpretation of changes in fluorescence is difficult. However, results with this method have indicated that a rise in NAD, caused by the increased work of the heart, precedes the glycogenolytic effect during the inotropic action of epinephrine (209, 210).

The most important question to be answered is, what is the relationship of

phosphorylase activation and glycogenolysis to the increased force of cardiac contraction caused by catecholamines. Unfortunately we do not understand the nature of muscle contraction itself, nor are we aware of all the biochemical changes in the heart stimulated by epinephrine. It is, therefore, impossible to answer satisfactorily the question we have posed. The suggestion has been made (145, 192) that cyclic AMP formed in response to epinephrine stimulates cardiac contraction and glycogenolysis by separate mechanisms. If this be true, a separation of inotropic and glycogenolytic effects can be expected if the concentration of cyclic AMP necessary for an action on contractile processes is smaller than the concentration required for activation of phosphorylase. It has been proposed also that hexosephosphates, accumulating as a result of increased glycogenolysis, can influence the contractile mechanism (47). So far, we have no adequate explanation of how such an effect could be brought about. A further consideration is that metabolic effects of cyclic AMP other than phosphorylase activation, such as stimulation of phosphofructokinase (137), may play a role in the contractile effect of epinephrine. The reviewers do feel that, despite the considerable evidence against it, the possibility should not be dismissed that the augmentation of the force of contraction produced by epinephrine is a result of an increased production of high-energy phosphate bonds following a rise in phosphorylase activity. It should be pointed out that at this time no definite effect of cyclic AMP on the contractile mechanism in muscle has been established and phosphorylase activation appears to be the most prominent action of cyclic AMP in the heart.

C. Smooth muscle

Our discussion thus far has been primarily concerned with the phosphorylases of heart and skeletal muscle, but no review describing the effects of drugs on phosphorylase activity should neglect the excellent work which has been done with smooth muscle. One of the earlier publications which contains information about smooth muscle phosphorylase is that of Leonard (117), who used uteri of rats. He found that injection of estradiol into spayed rats markedly increased uterine weight during the first 6 hours after hormone administration; in this period there was a sharp decrease in total phosphorylase activity per unit weight of tissue. The per cent changes in uterine weight and enzyme activity were approximately equal; this finding indicated that no enzyme was synthesized during this time. From 6 to 48 hours after estradiol was given, there was only a small increase in the weight of the uterus and a progressive, large increase in total phosphorylase activity. At the end of 48 hours the enzyme activity per unit weight was the same as that found at the beginning of the experiment. The proportion of phosphorylase in the *a* form rose subsequent to estradiol injection, the largest increase occurring in the first 6 hours. In a later paper Leonard (121) presented evidence that the changes in weight and phosphorylase activity of uteri during the first 6 hours after estradiol were due to the release of endogenous histamine.

The effects of intraperitoneal injection of posterior pituitary extract, epi-

nephrine or norepinephrine into estrogen-primed rats were studied also (117) One hour after administration of any one of these substances, phosphorylase *a* activity in the uterus was decreased but total phosphorylase remained unchanged. It is interesting that posterior pituitary extract, known to have an effect opposite to that of epinephrine on uterine motility, like epinephrine, decreased the activity of phosphorylase *a*. In interpreting these observations it should be remembered that a relatively long time elapsed between drug administration to the intact animal and the removal of a tissue sample for measurement of enzyme activity. Therefore, the results obtained may not reflect direct actions of the drugs. Serotonin (119), like epinephrine, tended to decrease the activity of uterine phosphorylase *a* when the enzyme was assayed a long interval after administration of the drug. When *in vitro* experiments were performed, it was found that both epinephrine and serotonin increased uterine phosphorylase *a* within a few minutes after addition of either drug to the bath (122). Because of the opposite effects of epinephrine on uterine phosphorylase when studied *in vivo* and *in vitro*, Leonard (120) restudied this problem. He confirmed the finding that epinephrine decreased uterine phosphorylase activity in spayed estrogen-primed rats and also found a similar effect of epinephrine in spayed animals not given estrogen. From the observation that epinephrine caused constriction of uterine blood vessels, and the finding that ligation of the blood vessels to the uterus could reproduce the epinephrine action on phosphorylase, it was concluded that the depression of phosphorylase *a* by epinephrine *in vivo* was due to uterine ischemia (120).

It should be pointed out that the changes in phosphorylase in the uterus produced by ischemia are opposite to the alterations in enzyme activity observed by Klarwein *et al.* (100) and Wollenberger *et al.* (213, 214) in the ischemic heart. One factor which could account for the difference in results is the time element. In the experiments with uterine muscle (120), phosphorylase was measured 10 minutes after arrest of blood flow, while Wollenberger and Krause (213), in experiments with dog hearts, observed an increase in phosphorylase within seconds following severance of the aorta. In a brief report Diamond and Brody (41) also described stimulatory effects of catecholamines on uterine phosphorylase activity. Results of studies with adrenergic blocking agents led to the suggestion that catecholamines acted in this tissue in two ways: by a direct stimulation of β-receptors and by anoxia through stimulation of α-receptors.

Important investigations on the relation of smooth muscle function to metabolism were done by Axelsson *et al.* (5). These workers were particularly interested in the possible correlation of electrical activity of smooth muscle, in this case taenia coli, and metabolic reactions occurring within the tissue. Measurements of spike discharge, conduction of impulses, and membrane potential were made on muscles paired with those used for determinations of phosphorylase activity. Briefly, their results may be summarized as follows: in smooth muscle bathed in oxygenated Krebs solution epinephrine increased phosphorylase activity at the same time that the drug stopped spontaneous spike activity, abolished a conducted response to electrical stimulation, and caused hyper-

polarization. In glucose-free medium the stabilizing effect of epinephrine on the cell membrane became less, *i.e.*, spike activity was only temporarily absent; hyperpolarization also decreased. After prolonged exposure to the glucose-free solution when the glycogen depletion of the tissues was severe, the effect of epinephrine was reversed, *i.e.*, the drug caused depolarization and initiated spikes. In the presence of iodoacetic acid, the inhibitory action of epinephrine also was abolished. From the results of their experiments the authors felt that the hyperpolarizing action of epinephrine on the cell membrane may be related to the ability of the catecholamine to increase the rate of energy production, as manifested by the rise in phosphorylase activity.

From the results of later experiments, Timms *et al.* (198) came to conclusions different from those drawn in the paper just discussed. In confirmation of the earlier work, they found that epinephrine at a concentration of 5.4×10^{-7} M caused a relaxation of taenia coli with a simultaneous increase in the activity of phosphorylase *a*. The authors also observed that the glycogen level of the muscle decreased from 1.46 to 1.01 mg/g (wet wt) during a two-minute incubation with epinephrine at 5.4×10^{-7} M. However, since dichloroisoproterenol blocked phosphorylase stimulation without interfering with the relaxation produced by epinephrine, they concluded that relaxation of the muscle was not dependent on phosphorylase activation. This conclusion was strengthened by the additional observation that decrease in muscle tone could be obtained at a concentration of epinephrine (2.7×10^{-8} M) which produced no effect on phosphorylase activity or glycogen concentration.

A further examination of the effects of epinephrine on taenia coli was carried out by Bueding *et al.* (15). On minimizing handling and manipulation of the taenia coli after they were removed from the bath, they could observe no increase in phosphorylase *a* after exposure to 5×10^{-7} epinephrine. On the other hand, if the muscles were drawn over a glass plate or the inside of an homogenizer, then the taenia coli showed an increase in phosphorylase activity; the increase was larger in the muscles which had been incubated with epinephrine. It was hypothesized that manipulation of the tissues after removal from the bath allowed sufficient time and proper conditions for the activation of phosphorylase by cyclic AMP formed in the intact, incubated muscles. Therefore, the authors concluded that an activation of phosphorylase does not occur in the tissues while the physiological effect takes place. Their earlier results were ascribed to a conversion of phosphorylase *b* to phosphorylase *a* during the period between removal of the tissues from the epinephrine-containing medium and the assay of enzymatic activity. It would be of interest to know whether the cyclic AMP content of the taenia coli was increased during the exposure to epinephrine, and to investigate the mechanism by which phosphorylase was activated when the tissue was drawn over a glass plate.

The relation between carbohydrate metabolism and function in smooth muscle has been the subject of a long series of interesting publications by Mohme-Lundholm and Lundholm. The former investigator found that relaxation produced by epinephrine in various smooth muscle preparations was directly related

to an increase in lactic acid production (146, 147, 148). In a later study Lundholm and Mohme-Lundholm (124) showed that in tracheal muscle, coronary arteries, and uterus the relaxing effect of epinephrine and the increase in lactic acid production were also associated with a decrease in glycogen. However, the authors pointed out that since the production of lactate could not be completely accounted for by the decrease in glycogen, epinephrine, besides its known effect on phosphorylase, must have influenced carbohydrate metabolism at additional sites.

The effect of adrenergic blockade on the action of epinephrine on the isolated rabbit stomach preparation was studied by Mohme-Lundholm (150). Epinephrine produced contraction of the muscle and an increase in lactic acid. When Dibenamine was added, epinephrine caused muscle relaxation, but lactic acid production remained unchanged.

A dissociation between increase in muscle tone and lactic acid production was demonstrated by Lundholm and Mohme-Lundholm (126) in bovine mesenteric arteries. In the presence of glucose, dihydroergotamine selectively inhibited the contractile effect of epinephrine without influencing its stimulatory action on lactic acid production. Replacement of the original bath solution (Tyrode) with 6% dextran resulted in inhibition of the contractile effect of epinephrine, but lactic acid production was unaffected. Contraction of the muscle, occurring as a result of increasing the potassium concentration of the bath solution, was accompanied by a decrease in lactic acid formation rather than an increase, as seen with epinephrine. From these observations and from the results of later experiments (125), the authors concluded that, in smooth muscle, drugs that stimulate contractile processes can increase carbohydrate metabolism by a separate mechanism.

In further studies (127), the action of drugs and electrical stimulation of smooth muscle were investigated, and it was found that under some experimental conditions the amount of lactic acid formed could not be accounted for by the decrease in tissue glycogen. It appeared that substantial quantities of lactic acid could be formed from carbohydrate intermediates other than glycogen.

The fascinating and complex relationships between changes in smooth muscle tone and alterations in carbohydrate metabolism demonstrated by the Swedish investigators were further explored in studies in which phosphorylase activity was also determined (149). Although epinephrine could activate phosphorylase of tracheal muscle, there was not a good correlation between alterations in enzyme activity and muscle relaxation. In studies with mesenteric artery in which epinephrine caused contraction, there was no effect on phosphorylase.

The investigations by Mohme-Lundholm and Lundholm may be summarized as follows: epinephrine produces distinct changes in carbohydrate metabolism in smooth muscle; relaxation of this tissue in response to epinephrine is better correlated with the formation of lactic acid than with glycogen breakdown or phosphorylase stimulation; contraction of smooth muscle is not always accompanied by an increase in glycogenolysis or lactic acid formation; and the effects of epinephrine on contraction and carbohydrate metabolism appear to be exerted by different mechanisms.

D. Brain

Cori and Cori (29) first described a phosphorylase enzyme in brain which required AMP for activity. Later studies, referred to earlier in this review, showed that the phosphorylase enzymes exist in brain in forms that are similar to phosphorylase *a* and *b* in skeletal muscle.

Because of the observations that many drugs influenced phosphorylase activity in other tissues, several investigators became interested in the possibility that agents which affect the central nervous system might alter the enzyme in brain. It is difficult to study this problem because there are inherent difficulties in obtaining samples of tissue from this organ without producing major alterations in the state of the phosphorylase enzymes. For example, Breckenridge and Norman (13) found that in mice decapitation produced a large increase in the level of brain phosphorylase *a*. It is apparent also that in obtaining frozen samples of brain the particular technique used is extremely important. This is illustrated by the findings of Breckenridge and Norman (13) that low levels of phosphorylase *a* could be obtained with a technique that allowed rapid freezing of the outer layers of brain cortex frozen *in situ*. In the usual procedure, which consisted of dropping the decapitated head into freon cooled with liquid nitrogen, much higher values for phosphorylase *a* were observed.

Several drugs have been studied for their action on brain phosphorylase, but only a few have been found to have consistent effects (9, 13, 91). Among the drugs which influence brain phosphorylase *a* is insulin, which when injected in near-convulsant doses in mice (13) or rats (91) caused a significant increase in the activity of the enzyme in the brain. Reserpine administration to rats was observed by Belford and Feinleib (9) to depress cerebral phosphorylase *a* in one series of experiments, but this effect of the drug could not be confirmed in a second group of experiments. Breckenridge and Norman (13) reported experiments in which the injection of reserpine into mice led to a decrease in brain phosphorylase *a*.

An intriguing study of the effects of meprobamate on the electrical activity of the brain and phosphorylase activity in various portions of the brain was done by Kajtor *et al.* (94). Chronic administration of meprobamate brought about a decrease in the rhythmic waves of the electroencephalogram, which was accompanied by a fall in cerebral phosphorylase activity. Postmortem examination of the brains from treated dogs showed a diminution in phosphorylase activity of about 20 % in the cortices and pyriform lobes and a 31 % decrease in enzyme activity in the thalamic-hypothalamic areas. No correlation could be established between the magnitude of the change in phosphorylase activity and previously noted bioelectric changes in the brains.

Finally, in a rather speculative paper by Iriye and Simmonds (92), the effect of several psychotropic drugs on rat brain phosphorylase levels was measured. The hallucinogenic agents, mescaline and LSD-25, plus the anxiety-producing drugs, amphetamine and epinephrine, depressed phosphorylase activity in the brain. Insulin, a drug used in the treatment of mental illness, caused an increase in the enzyme, and BOL-148, a non-psychogenic analogue of LSD-25, had no

effect on brain phosphorylase. The authors suggested that the level of phosphorylase activity in the brain may be implicated in mental illness.

The studies of the action of drugs on brain phosphorylase are in their early stages. With improvements in techniques, it may be anticipated that investigations of brain phosphorylase will ultimately lead to results of great significance with regard to normal and disturbed brain function.

E. Effects of adrenergic blocking agents on phosphorylase activity

Nickerson (158) in his comprehensive review pointed out that the classical adrenergic blocking agents inhibited the hyperglycemia but had little effect on the increase in blood lactic acid produced by catecholamines. When Powell and Slater (168) studied dichloroisoproterenol (DCI) and found that this substance blocked the inhibitory actions of epinephrine and isoproterenol, a new tool was added to explore the metabolic and functional effects of sympathomimetic amines. In contrast to the α-adrenergic blocking agents, DCI inhibited both the increase in blood glucose and the lactacidemia produced by epinephrine (143). Lundholm and Svedmyr (128) also found that DCI inhibited the increase in lactic acid produced by epinephrine and in addition reported that the calorigenic action of epinephrine in the intact rabbit was depressed by DCI. These studies led to investigations of the actions of DCI and other adrenergic blocking agents on tissue phosphorylase activity. Ali *et al.* (1, 2) observed that DCI abolished the effect of epinephrine on phosphorylase in rat liver slices and diaphragm *in vitro* and that dihydroergotamine or phentolamine inhibited phosphorylase stimulation by epinephrine in liver but not in diaphragm. In their studies of the actions of adrenergic blocking agents *in vivo* Hornbrook and Brody (84) found that the activation of liver phosphorylase following intravenous infusion of epinephrine into the rat was completely inhibited by ergotamine and partially blocked by phenoxybenzamine, DCI or pronethalol. Phosphorylase stimulation was markedly depressed in skeletal muscle by DCI or pronethalol but unaffected by ergotamine or phenoxybenzamine (84).

The studies of the action of adrenergic blocking agents on blood glucose, lactic acid and tissue phosphorylase indicate that *alpha-* and *beta*-adrenergic blocking agents generally prevent the metabolic effects of catecholamines in the liver; in skeletal muscle only *beta*-adrenergic blocking drugs are inhibitory. However, the precise sites of action of the different adrenergic blocking agents in these tissues are still unknown.

The action of adrenergic blocking drugs on the heart was studied by Moran and Perkins (152) who found that DCI selectively inhibited the inotropic and chronotropic effects of catecholamines. Later Mayer and Moran (142) showed that the activation of cardiac phosphorylase also was blocked by this compound. In agreement with these findings, Kennedy and Ellis (96) observed that DCI inhibited the glycogenolytic effects of epinephrine in the myocardium.

Moran and Perkins (152) in their original paper on the action of DCI in the heart briefly reviewed previous reports of cardiac adrenergic blockade. They pointed out that the effects of *alpha*-adrenergic blocking agents were very inconsistent and obtained only with high doses of drug.

There is considerable evidence that, in addition to the usual adrenergic blocking agents, certain sympathomimetic amines also can produce adrenergic blockade. Curtis (35), for example, described a decrease in the hypertensive response to epinephrine in the cat and found that the relaxation of the guinea pig uterus produced by epinephrine was not present when ephedrine had been previously added to the organ bath. These observations led Curtis to suggest that ephedrine in some way interfered with the action of epinephrine on its receptor. Other instances of an inhibition of the action of catecholamines by ephedrine were reported by Finkleman (49), using a rabbit intestinal preparation, and by West (206), who showed that high doses of ephedrine antagonized the action of epinephrine and norepinephrine in the Straub frog heart preparation. A recent report by Imai *et al.* (89) described an inhibition by methoxamine of the stimulatory effects of catecholamines on the heart.

Antagonism to epinephrine by ephedrine also has been observed in experiments in which metabolic effects of sympathomimetic amines were investigated. Ellis (44a) showed that ephedrine itself did not alter the blood glucose concentration in the rat, but abolished the hyperglycemia that follows the administration of epinephrine.

Additional evidence that certain sympathomimetic amines inhibit the metabolic effects of epinephrine was provided by Ali *et al.* (2). These investigators found that methoxamine inhibited stimulation of phosphorylase activity by epinephrine in isolated rat diaphragm, and in liver and heart slices. Ephedrine, which was studied only in the diaphragm, produced a small activation of the enzymes in this tissue and inhibited the effect of epinephrine on phosphorylase.

Inhibition of metabolic actions of epinephrine by methoxamine was also demonstrated by Burns *et al.* (19). Their studies showed that methoxamine and a derivative of this compound, N-isopropyl methoxamine, markedly diminished the hyperglycemia and the increase in plasma-free fatty acids that followed injection of epinephrine into the intact dog. N-Isopropyl methoxamine blocked the activation of phosphorylase in liver slices caused by epinephrine.

It is apparent from the discussion above, and from numerous studies in the literature not cited here, that adrenergic blocking agents can interfere not only with the functional effects of catecholamines but with their biochemical actions as well. Little is known about the mechanisms involved in the blockade of the functional and metabolic actions of epinephrine. However, recent studies by Northrop and Parks (159) indicate that dissimilar adrenergic blocking drugs exert their actions at different metabolic sites. It was shown that dihydroergotamine inhibited the hyperglycemia that followed the administration of either epinephrine or cyclic AMP in the intact rat. DCI, on the other hand, blocked the effect of epinephrine on blood glucose but did not prevent the hyperglycemia following cyclic AMP. The authors interpreted their results to mean that DCI exerts its primary effect prior to the synthesis of cyclic AMP in the liver cell and that DCI does not influence the metabolic reactions regulated by the cyclic nucleotide. They suggested that dihydroergotamine acts subsequent to the release of cyclic AMP, perhaps by blocking the action of this compound or by increasing its rate of inactivation.

F. The influence of hormones on phosphorylase activity

One of the most exciting developments in the studies of the cyclic AMP-phosphorylase system has been the realization that many hormones have marked effects on the cellular concentration of the cyclic nucleotide and on phosphorylase activity. It is of particular significance that the hormones exhibit some degree of organ specificity in their biochemical action. Epinephrine, for example, has no effect on the phosphorylase activity of the adrenal cortex (70) despite its well-known stimulatory effect in most other tissues. On the other hand, adrenocorticotropin does not activate hepatic phosphorylase (70) but does increase phosphorylase activity in the adrenal cortex (70) and adipose tissue (200). In several instances a single hormone will increase phosphorylase activity in different organs, but the dose necessary to produce a given effect varies from one organ to another. This has been shown to be true in the case of epinephrine (192).

The relation of cyclic AMP and phosphorylase to the physiological actions of hormones is a challenging field of research in which there is currently much interest. For accounts of these investigations, the reader is referred to the reviews of Sutherland and Rall (174, 192, 193) and Haynes *et al.* (71). Studies dealing with the action of adrenocorticotropin on adrenal cyclic AMP and phosphorylase may be found in the publications by Haynes (70), Riley and Haynes (178) and Ferguson (48). The synthesis of cyclic AMP by slices of corpus luteum has been demonstrated by Williams *et al.* (208), who also studied the effect of hormones on this process (208). Marsh and Savard (138) showed that luteinizing hormone stimulated phosphorylase activity of luteal slices. Orloff and Handler reported that cyclic AMP, theophylline and vasopressin were similar in that all three compounds increased the permeability of the toad bladder to water (160). Additional studies comparing the action of posterior pituitary hormones and cyclic AMP were subsequently performed (65, 66, 161), and it was found that both vasopressin and cyclic AMP stimulated phosphorylase activity in toad bladder and kidney slices (161). Although the effects both on metabolism and on permeability of vasopressin and cyclic AMP on the toad bladder are well established, the relationship between these actions is obscure. It has been shown, for example, that neither vasopressin nor cyclic AMP caused an elevation in oxygen consumption or phosphorylase activity in a sodium-free medium although permeability changes were still noted (183). That vasopressin may act by increasing the formation of cyclic AMP is suggested from the work of Strauch and Langdon (186), who observed a marked diminution of the effect of vasopressin on water permeability of the toad bladder by dichloroisoproterenol. In contrast, the adrenergic blocking agent caused only a slight decrease in the permeability change induced by cyclic AMP. The remarkable sensitivity of the toad bladder to hormones is also illustrated by the findings that thyroxine increases water movement in this tissue (60) and may potentiate the action of vasopressin (139).

It is interesting that hormones not only affect permeability to water but also influence active sodium transport in the toad bladder (114, 115), but the importance of cyclic AMP and phosphorylase stimulation in ionic transport remains

to be elucidated. Recently, direct proof for the formation of cyclic AMP in response to vasopressin was obtained by Brown *et al.* (14). When dog kidney cortical or medullary homogenates were incubated with arginine-vasopressin, there was an increase in the production of the cyclic nucleotide.

Since the major concern of this review is the action of autonomic drugs, no attempt has been made to discuss all of the outstanding investigations on the effects of hormones on cyclic AMP and phosphorylase activity. In the remaining portion of this section emphasis will be placed on reviewing those studies which pertain to modifications of the actions of catecholamines by hormones.

One of the first to study the action of adrenal cortical hormones on phosphorylase activity was Kerppola (97), who found that intramuscular injection of cortisone in rabbits decreased the activity of the enzyme in muscle and liver. When epinephrine was given to the cortisone-treated animals, phosphorylase activity returned to approximately normal values. Extensive studies on the effect of adrenalectomy and adrenal cortical hormones on liver phosphorylase were carried out by Willmer (211) and Eisenstein (44). The results obtained by the latter author were not in agreement with those of Kerppola in that glucocorticoid hormones were found to increase liver phosphorylase activity and adrenalectomy was associated with a decrease in enzyme activity.

Investigations into the action of adrenal cortical hormones and epinephrine on phosphorylase in muscle were made by Leonard (116). In contrast to the results of Kerppola, chronic administration of cortisone did not change muscle phosphorylase activity, although there was an elevation of glycogen in this tissue. When epinephrine was injected into cortisone-treated rats, phosphorylase activity increased to a greater extent than in normal animals. A potentiation of the action of epinephrine by adrenal cortical hormones was demonstrated also by Hess and Shanfeld (78). In the open-chest rat preparation the stimulatory effect of epinephrine on cardiac phosphorylase *a* was greater than normal when the animals had been pretreated with cortisol or corticosterone. The potentiation of epinephrine action appeared to be limited to the glucocorticoid steroids, since deoxycorticosterone was without effect.

More recently, thyroid hormones have been found to influence phosphorylase activity and the action of catecholamines on this enzyme. Hornbrook and Brody (85) reported that chronic injection of thyroxine in rats caused an increase in cardiac phosphorylase *a* and a decrease in cardiac glycogen; no changes in skeletal muscle phosphorylase *a* and glycogen content were noted. In a subsequent publication Quinn *et al.* (169) described the influence of thyroid hormones on the action of norepinephrine on myocardial phosphorylase and glycogen content. Both thyroxine and triiodothyronine potentiated the action of infused norepinephrine in elevating cardiac phosphorylase *a* levels and decreasing glycogen stores. Reserpine abolished, and *beta*-adrenergic blocking agents reduced, the thyroxine-induced rise in phosphorylase *a*.

In studies in which the open-chest rat was used, Hess and Shanfeld (79) also observed that chronic administration of thyroxine or triiodothyronine led to an increase in cardiac phosphorylase *a* activity. When epinephrine was given to the

thyroxine-treated rat, there was a further increase in phosphorylase *a* activity so that extremely high values of enzyme activity were obtained. As in the experiments of Quinn *et al.* (169), reserpine prevented the rise in cardiac phosphorylase activity associated with injection of thyroxine. In addition, it was demonstrated that the intravenous administration of acetylcholine or pronethalol to the thyroxine-treated animal caused an immediate decrease in heart rate and phosphorylase *a* activity.

Wollenberger and his co-workers studied the metabolic changes produced by ischemia in hearts from normal, hypothyroid and hyperthyroid dogs (214, 215). The extent and time course of the stimulation of phosphorylase seen after cessation of coronary flow were influenced by the thyroid hormones. In the hypothyroid animal the increase in enzyme activity was delayed and of smaller magnitude than in normal controls. The initial rise in phosphorylase *a* activity following anoxia in hyperthyroid dogs was almost identical to that seen in the untreated animals. However, in thyroxine-treated dogs the enzyme level in the heart remained elevated for a considerably longer period of time, and reconversion to phosphorylase *b* was greatly slowed. From studies in which pronethalol was used, it was concluded that part of the increase in phosphorylase *a* following anoxia was due to the release of endogenous catecholamines and the remainder was caused by an effect of anoxia *per se*. Both of these components of the anoxic stimulation of phosphorylase were augmented by thyroxine.

III. CONCLUDING REMARKS

As far as is known, the cyclic AMP-phosphorylase system is unique among enzymes in that it responds to a great variety of drugs at concentrations which result in discrete alterations in cell function. In particular, the sympathetic nervous system and sympathomimetic drugs produce effects on phosphorylase activity and function that show a remarkable correlation. The influence of the parasympathetic nervous system on cyclic AMP or phosphorylase is not fully understood, and the effects of acetylcholine on phosphorylase activity are not well correlated with the functional changes produced by the drug. It is of interest in this connection that acetylcholine has been reported to influence enzyme systems other than cyclic AMP and phosphorylase, in particular the incorporation of inorganic phosphate into phospholipids (81, 113). In the rat superior cervical ganglion preparation Larrabee *et al.* (113) found that stimulation of the preganglionic nerve was accompanied by a large increase in the incorporation of phosphate into phosphatidyl inositol, while other phospholipids were unaffected. One aspect of the regulation of phosphorylase activity in the cell which has received little attention is the effects of ions on the enzymes of the cyclic AMP-phosphorylase system. It would be of great interest, for example, to explore the possibility that calcium, liberated during cellular excitation, stimulates phosphorylase activity by activating phosphorylase *b* kinase. Potassium and sodium ions, which so markedly influence membrane phenomena, have been almost ignored in studies of phosphorylase. Investigations of the actions of these ions are urgently needed.

That phosphorylase *b* is of importance in the control of the rate of glycogenolysis *in vivo* was suggested by Haugaard (69), who also proposed that this enzyme may play a unique role in the rhythmical production of energy in the heart. With the findings of Parmeggiani and Morgan (153, 163) that ATP and glucose-6-phosphate markedly inhibit the activation of phosphorylase *b* by AMP, it became apparent that the regulation of phosphorylase activity was more complex than originally thought. In addition, it was realized that the cyclic AMP-phosphorylase system was unusually well adapted to make acute or longterm adjustments to the energy requirements of the cell. Since it is known that enzymes other than phosphorylase are present in cells in active and inactive states, it is possible that their activities also vary with cell function and are influenced by drugs. However, at the present time the cyclic AMP-phosphorylase system stands out as the most likely mediator for the transfer of impulses from the autonomic nervous system to cell function.

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