PROTEIN PHOSPHORYLATION AND CELLULAR REGULATION, I

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by

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INTRODUCTION

This presentation and the one to be given immediately thereafter will be concerned with the reversible phosphorylation of proteins and the role of this process in biological regulation. In addition to discussing our own early contributions to this field, Ed Fischer and I will describe some of the developments that have occurred subsequently. These developments have led to the realization that protein phosphorylation constitutes a major mechanism by which cellular processes are controlled. My own remarks will review the historical background that provided the setting in which our joint work was carried out in the 1950s and early 1960s. Then I'll turn to a discussion of this work itself, to be followed by comments on the cyclic AMP-dependent protein kinase. Finally I will talk about the intracellular transmission of hormone and growth factor signals through protein kinase cascades.
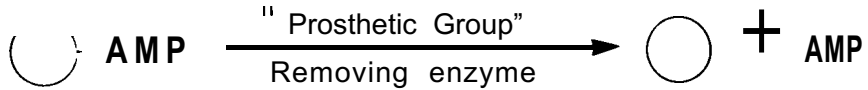
BACKGROUND

By 1940, which is the year in which I entered medical school at Washington University in St Louis, it was well established that the breakdown of glycogen in skeletal muscle and other types of cells occurs by the process of phosphorolysis, catalyzed by the enzyme phosphorylase (for review, see ref. 1). Carl Cori was Professor and Chairman of the Department of Pharmacology at Washington University, when I started my training there, but he was soon to take over the Department of Biological Chemistry. I gradually came to know him, at first distantly, the way medical students usually know their professors, but later on somewhat better after I had become a teaching assistant in biochemistry. In this latter role I also became acquainted with Arda Green, who, together with Carl and Gerty Cori, was purifying rabbit muscle phosphorylase . It was not long until I began to hear about the unusual properties of this remarkable enzyme, which they had found exist-

ed in skeletal muscle in two different forms that they designated a s phosphorylase *b* and phosphorylase *a* (2,3). The *a* form was purified and obtained as a crystalline enzyme. Kinetically, it was shown that phosphorylas e *b* required high concentrations of 5′-AMP for activity whereas phosphorylase *a* was active in the absence of this nucleotide. Since the concentration of 5′-AMP required for the activity of phosphorylase *b* was considerably higher than that found in muscle, this form was considered to be physiologically inactive. Phosphorylas e *a* was thought of as the physiologically active species. Evidence was obtained that the two forms are interconvertible within the cell, and it was postulated by the Coris that interconversion of the forms of phosphorylase constitutes a physiologically significant regulatory mechanism. Resting muscle was reported to contain phosphorylase predominantly in the *a* form, whereas electrically stimulated muscle contained th e *b* form (4). As will be discussed later (see below) the reverse is actually true. Regardless of this latter point, however, the finding that an enzyme might shuttle back and forth between two forms within the cell was a remarkable advance, which set the stage for important later developments.

The Coris were unaware of the chemical nature of the interconversion reactions of phosphorylase, but they did discover an enzyme that would convert phosphorylas e *a* to phosphorylas e *b in vitro.* This enzyme was called the "PR enzyme" based on th e assumption that it acted by removing a prosthetic group from phosphorylas e *a*, which was thought of as a holoenzyme form (2); phosphorylase *6*, lacking the putative group, was considered to be the apoenzyme form. Based on the fact that phosphorylas e *b* could be activated b y 5′-AMP, this nucleotide bound to the enzyme was thought of as the likely candidate for the prosthetic group of phosphorylase a. However, all attempts to identify 5′-AMP as a product formed when phosphorylase a was converted to phosphorylase *b* by the PR enzyme were unsuccessfu l (2,3). Because trypsin treatment of phosphorylase a led to the formation of a phosphorylase b-like form , i.e. an enzyme that could be activated by 5′-AMP (3), it was believed that the PR enzyme might be a protease. No enzyme that could convert phosphorylas e *b* to *a in vitro* in the presence of 5′AMP, or under any other condition that these workers tried, could be demonstrated. This did not seem unreasonable, however, if it were supposed that such a conversion might require peptide bond biosynthesis, because at that time the likelihood that such a reaction could be shown to occur in vitro was considered unlikely. These early concepts with respect to the interconversion reactions of phosphorylase are illustrated in Fig. 1.

Although as a medical student I heard about the research o n phosphorylase, particularly as a result of my friendship with Arda Green, my first "hands on" experience with this enzyme came after World War II had ended, when I started postdoctoral work in the Coris' laboratory (My residency training in internal medicine had been interrupted by the war, and, although at that time I expected to return to clinical medicine in due course, I had decided to work in a basic science department for a year or two before resuming hospital-based training.) The problem that was given

Phosphorylase *b* $\xrightarrow{\textbf{+AMP}}$ **Active Enzyme**
(inactive)

Phosphorylase *a* $\longrightarrow$ **Phosphorylase** *b*
(active)) (requires AMP
for activity)

$\bigcirc$ **AMP** $\xrightarrow[\text{Removing enzyme}]{\text{" Prosthetic Group"}}$ $\bigcirc$ **+** **AMP**

Phosphorylase *a* **Phosphorylase** *b*

Fig. I. Early concepts of the interconversion reactions of muscle phosphorylase . Inactive phosphorylase
b becomes an active enzym e in the presence of 5'-AMP. Phosphorytase a, the physiologically
active form of the enzyme, is inactivated by the prosthetic group removing (PR) enzyme. It was
thought likely that 5'-AMP would be product of this reaction, but this could not be demonstrat-
cd.

to me by the Coris involved solubility measurements on phosphorylase, and
I also studied the effect of protamine and polylysine on the two forms of the
enzyme. It was of interest that in the presence of these polyanionic sub-
stances, inosinic acid, as well o n 5'-AMP, was very effective as an activator of
phosphorylase b. All of these studies on the effects of nucleotides on
phosphorylase were carried out before the revelations of Jacob and Monod
concerning allosterism and protein conformation and at that time were
viewed simply as unexplained phenomenology. Indeed for several years
Carl Cori advised against my publishing these findings unless I could
explain them. Eventually, after Neil Madsen found that protamine could
physically bind to phosphorylase, which was at least a step in the direction of
how it might affect the activity of the enzyme, Cori let me send in my paper.
   Although, as I have pointed out, knowledge with respect to the properties
of phosphorylase as an enzyme increased significantly during th e 1940s, it
was often difficult to explain their physiological importance. This can be
understood if one considers what was known about glycogen metabolism at
that time. The epochal work of Leloir on the mechanism of glycogen
synthesis was still to come, i.e. the role of UDG in polysaccharide synthesis
and the existence of the enzyme, glycogen synthase, were completely un-
known. Hence, it was believed that phosphorylase was involved in glycogen
synthesis as well as in glycogenolysis (Fig. 2). It was assumed that the
direction of the phosphorylase reaction would be determined by the con-
centrations of the components of the reaction, particularly by the concen-
tration of inorganic phosphate. This situation obviously made it difficult to
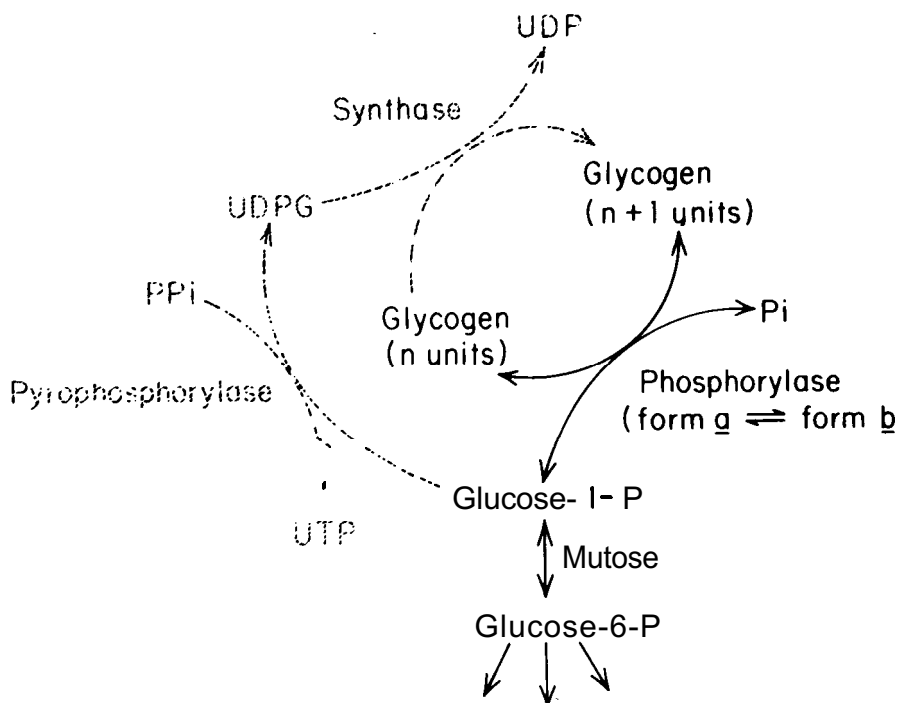interpret findings relating to the interconversion reactions o f phosphory-

Fig. 2. Glycogen metabolism as depicted circa 1945. The stippled portion of the metabolic scheme was unknown. It was assumed that glycogen synthesis and degradation were catalyzed by phosphorylase, the direction of the reaction being determined by the concentration of reaction components. Within the cell, phosphorylase was believed to shuttle back and forth between forms a and b, but the chemical nature of the interconversion reaction was unknown. Conversion of phosphorylase a t o b had been demonstrated *in vitro*.

lase b and a. Nonetheless, in the late 1940s and the early 1950's a clear story was beginning to emerge with respect to the action of the glycogenolytic hormones, glucagon, and epinephrine and their effects on interconversion of the forms phosphorylase as studied in liver cells. Earl Sutherland, working initially in the Coris' laboratory and later independently at Western Reserve University, found that epinephrine and glucagon caused the rapid conversion of liver phosphorylase a to phosphorylas e b (5), and it was also shown that epinephrine caused the same change in the muscle diaphragm (6). Inasmuch as epinephrine and glucagon were known to b e glycogenolytic hormones, Sutherland's findings clearly supported the concept that the activation of phosphorylase is associated with glycogen degradation. Why then, should electrical stimulation and muscle contraction, which were also known to result in glycogenolysis, be associated with the conversion of phosphorylase *a* to the inactive form, phosphorylase b (4).

CONVERSION OF RABBIT SKELETAL MUSCLE PHOSPHORYLAS E b TO PHOSPHORYLASE *a IN VITRO*

In Fall 1953, Ed Fischer joined the Department of Biochemistry at the University of Washington , where I was already a faculty member. As a graduate student in Geneva, Ed had worked on potato phosphorylase, and we thus shared a common interest in this particular enzyme. We discussed some of the puzzling features of phosphorylase and were particularly intrigued by the still unsolved nature of th e 5'-AMP effect, i.e. how 5'AMP activates phosphorylas e b but is seemingly unnecessary for phosphorylase a. It seemed worthwhile, purely as a secondary undertaking for each of us, since we both had problems that we considered as our major areas of concentration, to pool our efforts to see whether or not we could obtain information on this point. The initial experiment that we undertook together was simply to prepare pure crystalline phosphorylase a by the Coris' procedure (7). However , our initial attempts to do this were woefully unsuccessful. The enzyme would not crystallize, a step that was essential in order to obtain pure protein. More puzzling was the fact that the partially pure enzyme, which we did obtain, was always in th e b form rather than the a form. Insofar as we could determine we had followed the Coris' procedure exactly except that we had clarified the original muscle extract by centrifugation rather than by filtration through paper. Although this seemed like a trivial change, we nonetheless included the filtration step in our next preparation. To our surprise, this time we obtained phosphorylase a, which crystallized readily as it was supposed to do. Two conclusions could be drawn that would explain these results. First, "resting" rabbit muscle extracts must contain phosphorylase predominantly in th e b form rather than the a form as had been postulated by the Cori s (4,8), and second, filtration of muscle extracts through paper must trigger conversion of phosphorylase b to a in vitro (9). Subsequently we found that thorough washing of the filter paper before use eliminated conversion of b to a, implying that some component in the paper must have been extracted to account for the effect. It was also found that this conversion failed to occur if the muscle extract were aged prior to filtration. The critical component from the filter paper that was required in th e b to a step was shown to be calcium, and the essential component in the extract that was lost on aging was found to be ATP.

Armed with the knowledge that there is an ATP requirement for conversion of phosphorylas e b to phosphorylase a, we thought it likely that we were dealing with a phosphotransferase reaction in which the termina l phosphoryl group of ATP was being transferred either to the protein itself or possibly to a nonprotein component bound to the enzyme. It soon became apparent that a "converting enzyme" , which could be separated from phosphorylase b, was required in the reaction (10). After it had been determined that ADP, as well as phosphorylase a were products of the reaction (1 l), we spoke of this enzyme as phosphorylase b kinase, or simply

phosphorylase kinase, rather than converting enzyme (11). The nature of the Ca^{2+} requirement for the b to a reaction was not apparent immediately, but eventually it was determined by one of our graduate students, William L. Meyer, that the effect of this metal was two-fold. On the one hand Ca^{2+} was needed for the activation of a kinase activating factor, (RAF), but in addition it was also required for the activity of phosphorylase kinase itself (12). RAF was later shown to be a Ca^{2+}-dependent protease by another student, R. Bruce Huston (13). (The current name for this protease is calpain.) We determined the stoichiometry of the phosphorylase b to phosphorylase *a* reaction (11) and also obtained the amino acid sequence surrounding the phosphorylated serine in phosphorylase a (14). It was demonstrated that inorganic phosphate is released in the muscle phosphorylase *a* to b reaction catalyzed by the PR enzyme, which could now be termed phosphorylase phosphatase. Thus, by the late 1950's, it was possible to write specific equations for the interconversion reactions of muscle phosphorylase as follows:

$$\text{phosphorylase b} + 2\text{ATP} \xrightarrow{\text{phosphorylase kinase}} \text{phosphorylase a} + 2\text{ADP}$$
$$(\text{dimer}) \qquad\qquad\qquad\qquad\qquad (\text{dimer})$$

$$\text{phosphorylase a} + 2\text{H}_2\text{O} \xrightarrow{\text{phosphorylase phosphatase}} \text{phosphorylase b} + 2\text{Pi}$$
$$(\text{dimer}) \qquad\qquad\qquad\qquad\qquad\quad (\text{dimer})$$
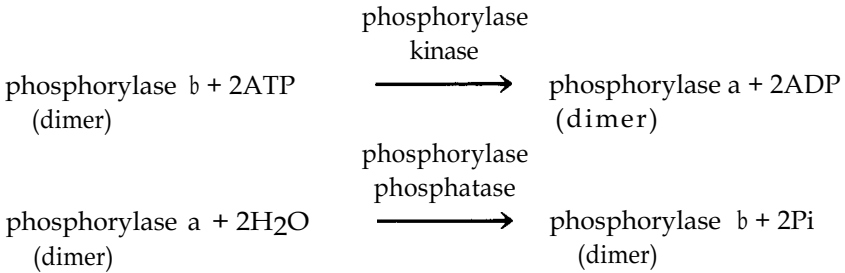
During the same period that our work on the muscle phosphorylase system was being carried out, very similar studies relating to liver phosphorylase were being conducted independently in the laboratory of Earl Sutherland at Western Reserve University. Sutherland's major coworkers in this effort were Walter D. Wosilait and Theodore W. Rall. In an early study (15) these investigators obtained evidence that inorganic phosphate was released when partially purified active liver phosphorylase was incubated with what they referred to as inactivating enzyme, and they also determined that phosphate was incorporated into phosphorylase when liver slices were incubated in the presence of ^{32}P-phosphate. Later they showed that both of the interconversion reactions of liver phosphorylase could be demonstrated in cell free systems (16,17). A monumental "ancillary finding" that grew out of the work on liver phosphorylase by the Sutherland laboratory was, of course, the discovery of cyclic AMP, the first identified "second messenger" of hormone action (18).

It is of interest to consider whether there had been any indications that the phosphorylation and dephosphorylation of proteins might be of regulatory significance prior to elucidation of the muscle and liver phosphorylase

systems. The existence of phosphoproteins had, of course, been known for many years before 1950. Phosphoproteins were classified as "conjugated proteins" and for the most part consisted of proteins of nutritional significance associated with the feeding of the young, e.g   . casein of milk and several phosphoproteins found in egg yolk. Interestingly, it was also appreciated that pepsinogen contained one mole of firmly bound phosphate per mole of enzyme, but the significance of this wasn't (and still isn't) known. It was also known that nonspecific phosphatases could catalyze the release of phosphate from phosphoproteins. In an important study that was carried out at about the same time as the work on phosphorylase was underway, Burnett and Kennedy described an enzyme that catalyzed the phosphorylation of casein (19). These workers were aware of the high rate of turnover of phosphate in proteins and were the first to describe a protein kinase. In general, however, a realization that protein phosphorylation- dephosphorylation is a dynamic process affecting enzymes and important in the control of metabolism had not been forseen.

PROTEIN PHOSPHORYLATION AND THE TRANSMISSION OF EXTRACELLULAR SIGNALS

As we have seen, an interest in the mechanisms of action of epinephrine and glucagon had an important part in work on the interconversion reactions of phosphorylase (5,6) which in turn led to discovery of the dynamic   phosphorylation and dephosphorylation of proteins. Going back even further, however, it can be noted that the original studies of the Coris, which led to the finding of phosphorylase itself, grew out of the longstanding interest of these investigators on the role of epinephrine in the regulation of glycogen metabolism (20). That an interest in hormone action would contribute importantly to the development of the field of protein phosphorylation-dephosphorylation was no accident, since we now know that one of the major functions of protein phosphorylation as a regulatory process is in the transmission of signals that impinge on cells. This is true not only with respect to the transduction of hormone and growth factor signals but for other types of stimuli as well. For example, as mentioned earlier, electrical stimulation of muscle leads to changes in protein phosphorylation within the cell. It is probably more than coincidental that the extent of protein phosphorylation is much greater in eukaryotic cells than it is prokaryotes, particularly in eukaryotic cells of higher animals that are subject to complex forms of external regulation.

It was apparent very early that the relative amounts of phosphorylase   *b* and *a* that would be present within the cell at any particularly time would depend on the relative rates of the phosphorylase kinase and phosphatase reactions, and it was anticipated that one or both of these enzymes must be subject to regulation. Furthermore, two factors capable of influencing the balance between the two forms of phosphorylase had been found. As noted, these were calcium ions, identified as the metal ion that caused phosphory-

lase *a* formation in muscle extracts (9) and cyclic AMP, which promoted phosphorylase a formation in liver cells and homogenates (reviewed in ref. 21). We subsequently showed that cyclic AMP would also cause phosphory-lase a formation in muscle extracts (22). In the muscle system it was determined that the effects of Ca^{2+} and cyclic AMP in stimulating phos-phorylase activation *in vitro* were a result of phosphorylase kinase activation rather than phosphorylase phosphatase inhibition (11).

The coupling of muscle contraction to glycogenolysis: The finding that the major form of phosphorylase present in resting muscle is phosphorylase *b* (8) rather than phosphorylase *a* (4,7), together with a realization that phosphorylase *b* can be converted to phosphorylase *a* if Ca^{2+} is introduced into muscle extracts containing ATP (9), made it possible to arrive at a rational position with respect to the effect of electrical stimulation on phosphorylase. In 1956 (23) Cori was able to demonstrate that muscle contraction causes conversion of phosphorylase *b* to *a*, rather than the other way around; this was in keeping with the known effect of contraction on glycogen breakdown. The effect of Ca^{2+} on the activation of phosphorylase kinase now fits well into a scheme (Fig. 3) whereby this metal, acting as a messenger substance associated with muscle contraction, could be responsi-ble for the coupling of glycogenolysis (an energy-yielding process) to con-traction (an energy-utilizing process). Of the two different mechanisms by which Ca^{2+} could regulate phosphorylase kinase, i.e. through limited pro-teolysis involving KAF, or as a result of the requirement of phosphorylase kinase for Ca^{2+} per se (12), only the latter was considered to be physiologi-cally significant. The work of Ozawa *et al.* (24), who quantified the effect of Ca^{2+} on the phosphorylase kinase reaction, was critical with respect to our understanding of this process. Many years later the actual mechanism by
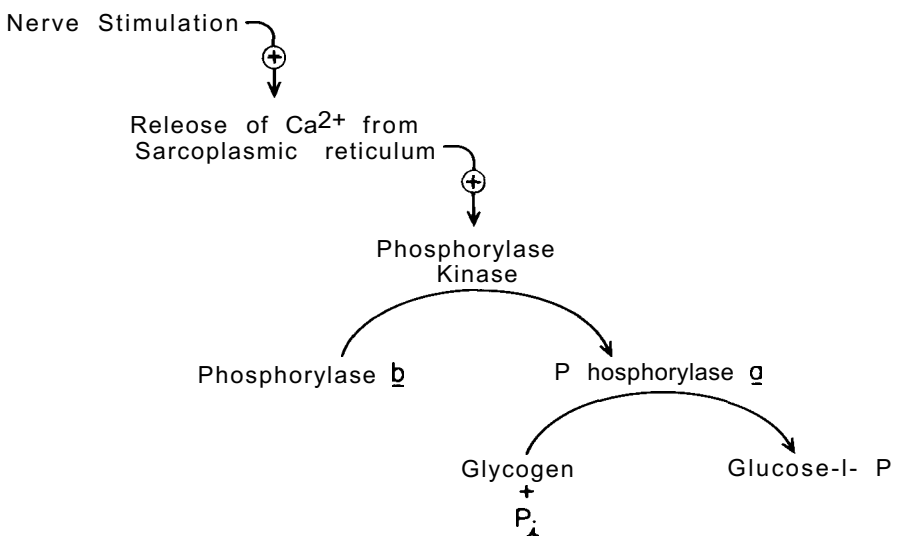

Fig. 3 The role of calcium ions in regulating glycogenolysis (circa 1962).

which Ca²⁺ stimulates phosphorylase kinases became apparent when it was found that calmodulin is a subunit of phosphorylase kinase (25).

The mechanism of action of cyclic AMP and the cyclic AMP-dependent protein kinase: The mechanism by which cyclic AMP causes an increase i   n phosphorylase kinase activity turned out to be more complex than that of Ca2+. Whereas Ca²⁺ is an essential activating component in the phosphorylase b to *a* reaction itself, cyclic AMP causes activation of phosphorylase kinase by promoting its phosphorylation. The first clue that phosphorylation might be involved in the activation of the kinase came with the finding that ATP is required in order to observe the activation reaction and the cyclic AMP effect in rabbit skeletal muscle extracts (22). A key coworker who carried out the initial experiment that revealed this requirement was a graduate student, Donald A. Graves. Activation of muscle phosphorylase kinase was characterized by marked enhancement of its activity as measured at pH 7 or below, and only moderate increases in activity were seen when t   he *b* to *a* reaction was carried out at highe r pH values. The ratio of activity at pH 6.8 to activity at pH 8.2 was found to serve as a useful index of the state of phosphorylase kinase activation (26). Significantly, once phosphorylase kinase had been activated by preincubation with ATP, the presence of cyclic AMP was no longer essential-as might be anticipated if the kinase were being modified covalently in an activation process stimulated by cyclic AMP (22).

Phosphorylase kinase was purified extensively and obtained as a nearly homogeneous high molecular weight protein, (Mr = 1.2   X 10⁶), which still retained the ability to be activated by preincubation wit   h MgATP in a reaction that was strongly stimulated by cyclic AMP. Although there were indications that a second protein kinase might be involved in the activation process (27), the kinetics of the reaction were complex and the work of DeLange et al. (28) suggested instead that an autocatalytic reaction was occurring. However, the existence of a separate "cyclic AMP-dependent phosphorylase kinase kinase" , which accompanied phosphorylase kinase kinase during its purification, was eventually established b   y Donal A. Walsh and John P. Perkins, postdoctoral fellows in my laboratory (29). They succeeded in purifying the new kinase about 200-fold from rabbit muscle extract and separated it completely from phosphorylase kinase. The kinase was referred to as the "cyclic AMP-dependent protein kinase" rather than phosphorylase kinase kinase, because it was found (29) to have a broader specificity than would have been implied by use of the more restrictive name. In retrospect, the cyclic AMP-dependent protein kinase probably represents the same activity reported by Huijing and Larner (30) to be involved in cyclic AMP-stimulated glycogen synthase phosphorylation. The finding of a cyclic AMP-dependent protein kinase separable fro   m phosphorylase kinase made it possible to construct a complete cascade mechanism showing the effect of epinephrine on glycogenolysis (Fig. 4). This represented the first example of a protein kinase cascade in which one protein kinase is phosphorylated and activated by another. Despite the
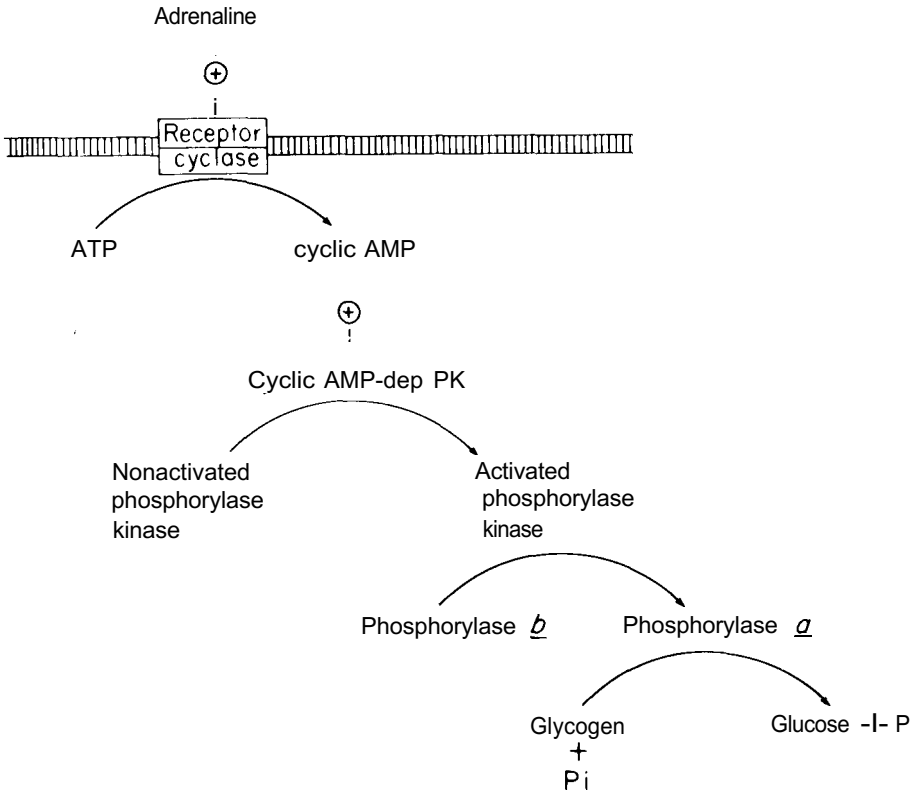
Adrenaline

$\oplus$

i

Receptor
cyclase

ATP   cyclic AMP

$\oplus$

Cyclic AMP-dep PK

Nonactivated
phosphorylase
kinase

Activated
phosphorylase
kinase

Phosphorylase *b*   Phosphorylase *a*

Glycogen
+
P i

Glucose -I- P

Fig. 4 The regulation of *glycogenolysis by epinephrine (adrenaline)*

potential usefulness of a cascade device (amplification of the signal, provision of branch points etc.) very few other examples have been reported. Recently, however, a complicated multi-step protein kinase cascade related to the action of numerous growth factors has been detected; this will be discussed at the end of this lecture.

The cyclic AMP-dependent protein kinase has served as a prototype for studies on protein kinases in general. This kinase is made up of regulatory (R) and catalytic (C) subunits and has the general structure , R^2C^2. In the next lecture Ed Fischer will speak about the mechanism by which cyclic AMP regulates the activity of the kinase. The catalytic subunit of this enzyme was the first protein kinase for which the complete amino acid sequence was determine d (31), and it was also the first protein kinase to have had its X-ray crystallographic structure elucidated (32). The cyclic AMP-dependent protein kinase also played a very significant part in our understanding of protein kinase specificity, which has become increasingly important as the number of known kinases has sky rocketed. Although it will not be possible to do more than touch on this latter topic here, it can be noted that the cyclic AMP-dependent protein kinase was the first kinase for which a "consensus" phosphorylation site sequence was clearly recognized (33-37). This sequence, Arg-Arg-X-Ser-X, is found in many, but not all,

substrates for the cyclic AMP-dependent protein kinase. In connection with
the specificty studies that were carried out within my own laboratory, I
would like to call particular attention to the work of David B   . Bylund and
Bruce E. Kemp.

*A protein serine/threonine kinase cascade activated in response to insulin and
related growth factors:* One of the enduring problems in endocrinology has
been the question of the mechanism of action of insulin. Indeed, investiga-
tors have been interested in this area for decades, and over the years
information on this subject gradually accumulated through the application
of whatever tools became available. At first, experiments were carried out
with intact animals, later with perfused organs, eventually with isolated
tissues such as the muscle diaphragm, and finally with cells in culture. Until
comparatively recently it had not been possible, however, to demonstrate a
meaningful insulin response in homogenates or other types of cell-free
system. Approximately ten years ago, however, it was found that the EGF
receptor is a protein tyrosine kinase, and shortly after that it was deter-
mined that this is also true for the insulin recepto r (38,39). Now it is known
that eight or nine different growth factor receptors are protein tyrosine
kinases. These findings ushered in a new era of research with respect to
insulin and related hormones or growth factors. However, although most
workers in the field felt that it would now be a simple matter to identify
meaningful substrates for thes   e receptor/kinases,   and to elucidate the
complete intracellular signal transduction pathway involved, this did not
occur. Several tyrosine-phosphorylated proteins were found in cells treated
with insulin or the other growth factors, but none of these proteins could at
first be readily connected with the known cellular actions of the growth
factors involved. Nonetheless, it was clearly demonstrated that the protein
tyrosine kinase activity of these receptors was essential for their action, and
there was little if any doubt that initiation of their signals must involve
ligand-stimulated tyrosine phosphorylation.

An investigator who is interested in determining the steps of a signal
transduction pathway can either wor k "downstream" from a receptor in
order to identify components of the pathway, or he can work "upstream"
toward the receptor starting with a well established cellular effect of the
growth factor in question. A number of laboratories, including my own,
which are interested in signaling from the insulin or related receptors, have
used the latter strategy in their approach. The particular cellular effect that
these groups have chosen as their starting point has been the activation of
protein serine and threonine kinases, which results from the simulation of
tyrosine kinases. It has long been known that the stimulation of protein
tyrosine kinases in cells results in changes in serine/threonine phosphoryla-
tion as well as in changes in tyrosine phosphorylation, but the mechanism
involved in the coupling of the two types of protein phosphorylation has
been unknown (40).

One of the cellular proteins that is readily phosphorylated on serine
residues in response to the stimulation of receptors having protein tyrosine

kinase activity is ribosomal protein S6, although the role of this phosphorylation insofar as the regulation of protein synthesis is not clear. This phenomenon had been discovered a number of years ago, prior to the involvement of my own laboratory in the problem, and a good start had already been made in identifying components involved in regulating S6 phosphorylation. For example, it had been shown that in adipocytes or Swiss 3T3 cells stimulated by insulin an S6 kinase becomes activated (41,42) and there was good evidence that the activation was due to covalent modification, i.e. phosphorylation. It was determined that the protei n serine/ threonine phosphatases would inactivate the activate d (43,44). Maller and coworkers had shown that an S6 Kinase (S6 Kinase II), purified to homogeneity from *Xenopus laevis* eggs, could also be inactivated by protein phosphatases (45) . Importantly, this latter S6 kinase could be reactivated by a different protein serine/threonine kinase, microtubule-associated protein 2 kinase (MAP kinase), which was known to be stimulated by insulin treatment of 3T3 L1 cells (46). These results strongly suggested the existence of a protein serine/threonine kinase cascade in which one growth factor-activated protein kinase phosphorylated and activated a second protein kinase. Gregory el *al.* (47) confirmed these findings using a phosphatase-treated S6 kinase from rabbit liver and MAP kinase from insulin-stimulated Rat 1 HIRc B cells. Then in this laboratory we showed that an EGF or insulin-stimulated MAP kinase from Swis s 3T3 cells could phosphorylate and activate an S6 kinase obtained from this same cell type (48). MAP kinase, in its active form, appeared to have been activated as a result o f serine/threonine phosphotylation, since it could be inactivated by protein phosphatase 2A. This suggested the existence of even a third protein serine/threonine kinase in this growth factor-stimulated process.

 A finding of major significance from Sturgill's laboratory was the fact that MAP kinase in its active form contains phosphotyrosine as well a s phosphothreonine, and that in addition to being inactivated by protein phosphatase 2A, it can also be inactivated by CD45, a protein tyrosine phosphatase (49). These investigators went on to show that phosphorylation of MAP kinase on tyrosine and threonine is essential for its activity, and it seemed likely that two different types of protein kinase, i.e. a protei n serine/threonine kinase and a protein tyrosine kinase, would be involved as upstream components involved in its activation. There was no evidence, however, that a receptor-type protein tyrosine kinase could serve as either one of these kinases. It was thus of great interest to search for an enzyme or enzymes, MAP kinase kinases, that would catalyze MAP kinase phosphorylation and activation.

 In this laboratory Natalie Ahn et *al.* (50) discovered two separable activating factors from Swiss 3T3 cells that catalyzed the activation of an inactive form of MAP kinase *in vitro*. Each of these factors required the presence of MgATP in order to bring about activation of MAP kinase, and it did not matter whether the latter had first been inactivated by a protei n serine/ threonine phosphatase or by a protein tyrosine phosphatase. This made it

appear probable that the factors wer e "dual specificity" kinases capable of phosphorylating proteins or serine and threonine residues as well a s tyrosine residues. The existence of dual specificity had, in fact, been found in yeast. At first we hesitated to call the activating factors "MAP kinase kinases", because in work carried out by Rony Seger et al. (51) we had determined that MAP kinase can undergo a slow but distinc t autophosphorylation reaction, i.e. that it can catalyze its own phosphorylation, and that in this reaction threonine and tyrosine residues are phosphorylated. Moreover, autophosphorylation resulted in activation of the kinase. This result made it appear possible that a MAP kinase kinase as such might be unnecessary and that the next upstream component could be a protein factor that simulated autophosphorylation. Eventually it was shown, however, that the MAP kinase activating factors were, in fact, protein kinases capable of phosphorylating a mutant form of MAP kinase that lacked enzymic activit y (52,53). Moreover, amino acid sequence data obtained in our laboratory and elsewhere, showed that the activating factors contain protein kinase amino acid sequence motifs. Later, similar "activators", i.e. MAP kinase kinases, were found in PC1 2 cell stimulated by nerve growth factor or bradykinin (54,55). Gomez and Cohen showed that activated MAP kinase kinase could be inactivated by a protein serine/threonine phospha-
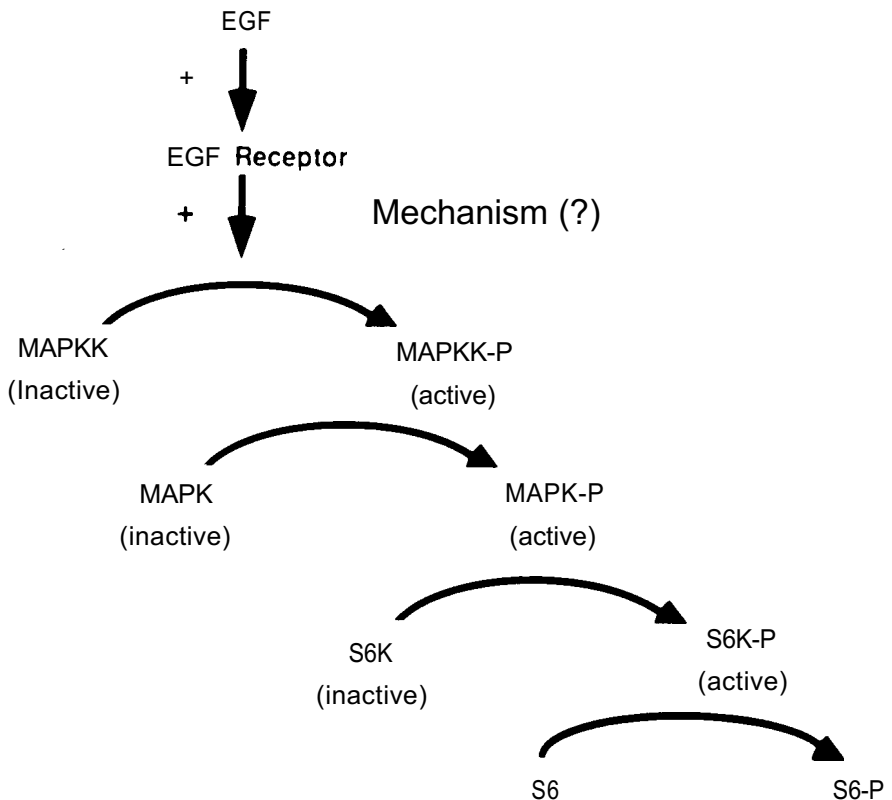

Fig. 5 Th e MAP kinase cascade.

tase but was not affected by protein tyrosine phosphatases (54). This latter result indicated that an additional upstream protein serine/threonin e kinase, i.e. a MAP kinase kinase kinase, was probably a component of the pathway. The present status of the MAP kinase cascade, without the putative MAP kinase kinase kinase, is shown in Fig. 5.

Intense effort on the part of many different laboratories is currently centered on the question of the identity of MAP kinase kinase kinase and the mechanism by which such an enzyme might be regulated. It was reported by Kyriakis *et al.* that Raf-1 activates MAP kinase kinase and thus might itself be a candidate for the kinase kinase kinase (56). This result was confirmed by Dent *et al.* (57). Other studies have implicated the possibility that cdc2 might be an upstream component of the MAP kinase cascade but not an immediate kinase kinase kinase itself (58). Finally, there is considerable evidence that p21 ras is a component acting at some site between the receptor and the components of the cascade as currently identified (59-62). A somewhat neglected area of study with respect to the MAP kinase cascade relates to the question of its precise function and the reasons for the existence of such a complicated scheme. It is probable that numerous substrates may exist for each of the protein kinases in the cascade, and in this connection it is noteworthy that a number of transcription factors appear to be targeted by MAP kinase and by the S6 kinase. The different kinases may also have specific metabolic enzyme targets that have not as yet been identified. Finally, some of the kinases may branch off and regulate still other kinases (63).

CONCLUSION

In this talk I have reviewed the early work on the interconversion reactions of the two forms of glycogen phosphorylase and phosphorylase kinase. A third enzyme that played an important part in early work on protein phosphorylation-dephosphorylation was glycogen synthase. Much of the original research on that enzyme was carried out by Joseph Larner and his associates during the early 1960s. These investigators found that, in contrast to the effect of phosphorylation on phosphorylase and phosphorylase kinase, phosphorylation caused a decrease in the activity of the synthase. Because the "field" of protein phosphorylation during the first ten years was dominated by those interested in glycogen metabolism, some even expressed the idea that perhaps regulation of enzymes by phosphorylation-dephosphorylation might be restricted to this area. However, the finding of a multifunctional cyclic AMP-dependent protein kinase, which was almost immediately shown by Kuo and Greengard (64) to be very widespread in nature, served to change this concept. Another important finding that dispelled the idea that protein phosphorylation was very limited in scope was the report that pyruvate dehydrogenase is regulated by phosphorylation (65). As can be noted in Fig. 6, the number of enzymes reported to undergo regulation as a result of this being phosphorylated started growin g precipi-
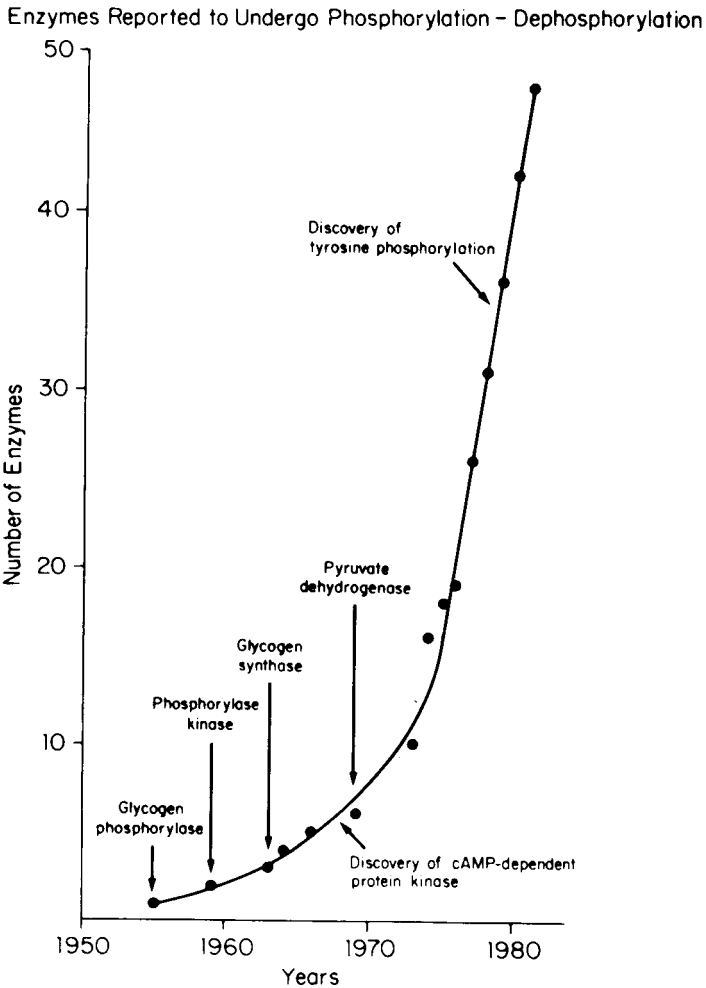
Fig. 6 *Growth in an awareness of protein phosphorylation as a mechanism for regulating enzymes.* The total number of enzymes reported to undergo phosphorylation dephosphorylation as a function of time is plotted against years.

tously in 1970. If reports on protein phosphorylation involving nonenzymic proteins were included, the growth curve in Fig. 6 would be even steeper. In the next talk Ed Fischer will trace some of the developments that have contributed to this explosion. I anticipate that he may also "correct" some of the errors that I may have made in my historical account of our work together.

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I would like to dedicate this lecture to my parents who had the education of their children as a major goal in their lives and to my wife, Deedy, who has always been extremely supportive of my work. In addition, I recognize the

very important contributions made by numerous talented unnamed students and postdoctoral fellows,.some but not all of whom I have mentioned in this article, who carried out the actual experiments reported here and in addition helped to provide direction for many of the studies that are reported.

REFERENCES

1. Cori, C.(1939) *Cold Spring Harbor Symposia on Quantitative Biology*, V*ol.* VII, p. 260 − 268.
2. Cori, G. T. and Green, A. A. (1945) J. *Biol. Chem. 158*, 32 1 − 332.
3. Cori, G. T. and Cori, C F. (1943) J. *Biol. Chem., 151*, 31-38.
4. Cori, G. T. (1945)J. *Biol. Chem.,* 158, 333-339.
5. Sutherland, E. W. and Cori, C. F. (1951) J. *Biol. Chem., 188*, 531-543.
6. Sutherland, E. W. (1950*)* Recent Progress in Hormone Research, Proceedings of the I.aurentian Hormone Conference, Vol. 5, Academic Press, New York, p. 441463.
7. Green, A. A. and Cori, G. T. (1943) J. *Biol. Chem., 151,* 21-29.
8. Krebs, E. G. and Fischer, E. H. (1955) J. *Biol. Chem., 216 ,* 113 −120.
9. Fischer, E. H . and Krebs, E . G. (1955) J. *Biol. Chem.,* 216, 121 −132.
10. Krebs, E. G. and Fischer, E. H. (1956) *J. Biochim. Biophys. Acta, 20,* 150 −157.
11. Krebs, E. G., Kent, A. B. and Fischer, E. H. (1958) J. *Biol. Chem., 231,* 78-83.
12. Meyer, W. L., Fischer, E. H. and Krebs, E. G: (1964) *Biochemistry, 3,* 1033 − 1039.
13. Huston, R. B. and Krebs, E. G. (1968) *Biochemistry, 7,* 2116-2122.
14. Fischer, E. H., Graves , D. J., Crittenden, E. R. S. and Krebs, E. G. (1959) J. *Biol. Chem., 234 ,* I698 − 1704.
15. Sutherland, E. W. and Wosilait, W. D. (1955) *Nature, 175,* 169.
16. Rall, T. W., Sutherland, E. W. and Wosilait, W. D. (1956) J. *Biol. Chem., 218,*
17. Wosilait, W . D. (1958) J. *Biol. Chem. 233,* 597.
18. Sutherland, E. W. and Rall, T. W. (1958) J. *Biol. Chem.*, 233, 1077-1091.
19. Burnett, G. and Kennedy, E. P. (1954) J. *Biol. Chem., 211*, 969 -988.
20. Cori, C. F. and Cori, G. T. (1928) J. *Biol. Chem., 79, 309 -355.*
21. Sutherland, E. W. (1962) *The Harvey Lectures, Series 57,* Academic Press, New York, p. 17-33.
22. Krebs, E. G., Graves, D. J. and Fischer, E. H. (1959) J. *Biol. Chem. 234:2867-2873.*
23. Cori, C. F. in O. H. Gaebler, ed., Enzymes: *Units of Biological Structure and Function,* Academic Press, New York, 1956, p. 573.
24. Ozawa, E., Hosoi, K., and Ebashi, S. (1967) J. *Biochem. 61,* 531-533.
25. Grand, R. J. A., Shenolikar, S. and Cohen, P. (1981) Eur. J. *Biochem.* 113, 359 − 367.
26. Posner, J. B., Stern, R. and Krebs, E. G. (1965) J. *Biol. Chem., 240,* 982 − 985.
27. Krebs, E. G., DeLange, R. J., Kemp, R. G. and Riley, W. D. (1966) P*harmacol. Rev.,* 18, 163-171.
28. DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. and Krebs, E. G. (1968) *J. Biol. Chem., 243,* 2200−2208.
29. Walsh, D. A., Perkins, J. P. and Krebs, E. G. (1968) J. *Biol. Chem., 243,* 376 3−3765.
30. Huijing, F. and Larner, J. (1966) *Biochem. and Riophs Res. Commun., 23,* 259-263.

31. Shoji, S., Parmelee,   D. C., Wade, R. D., Kumar, S., Ericsson,   L H., Walsh, K. A., Neurath, H., Long , G. L., DeMaille, J. G., Fischer, E. H. and Titani, K. (1981) Proc. Natl. Acad. Sci. USA, 78 **,** 848-851.

32. Knighton, D. R., Zheng, J., Eyck, L. F. T., Ashford, V. A., Xuong, N.-H., Taylor, S. S. and Sowadski, J. M. (1991) Science, *253*, 407-414.

33. Kemp, B. E., Bylund, D. B., Huang, T. S. and Krebs, E. G. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 344 8—3452.

34. Humble, E., Berglund, L., Titanji, V., Ljungstrom, O. Edlund, B., Zetterquist , O. and Engstrom, L. (1975) *Biochem. Biophys. Res. Commun.* **66**, 614– 62 1.

35. Daile, P., Carnegie, P. R. and Young, J. D. (1975) Nature, **257 ,** 416-418.

36. Zetterquist, O., Ragnarsson, U., Humble, E., Berglund, L. and Engstrom, L. (1976) *Biochem. Biophys. Res. Commun.* **70**, 696–703.

37. Kemp, B. E., Graves, D. **J.,** Benjamini, E. and Krebs, E. G. (1977) J. *Biol. Chem.*, ***252, 4888— 4894.***

38. Cohen, Carpenter, G. and King,   L. E., Jr. (1980) J. B*iol. Chem.*, ***255, 4834—4842.***

39. Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A.,   Haring, H. U., and Kahn, C. R. (1982) J. Biol . Chem., 257, **9891-9894.**

40. Denton, R. M. (1986) *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (P. Crecngard and G. A. Robinson , Eds.) Raven Press, New York, p. **293-341.**

41. Cobb, M. H. and Rosen, O. M. (1982) J. Biol. Chem., *258*, 12472-12481.

42. Novak-Hofer, I., and Thomas, G. (1984) J. B*iol*. Chem., 259, 5995-6000.

43. Ballou, L. M., Jeno, P. and Thomas, G. (1988) J. *Biol. Chem.*, ***263***, *1188*- 1194.

44. Ballou , L. M., Siegmann, M. and Thomas**,** G. (1988) *Prac. Natl. Acad. Sci. USA*, ***85,7154-7158.***

45. Andres, J. L. and Mallet-, J. L. (1989) J. B*iol*. Chem., ***264*,** 151 — 156.

46. Sturgill, T. W., Ray, I,. B., Erickson, E. and Mallet-, J. I.. (1988) *Nature,* ***334*,** 715-718.

47. Gregory, **J.** S., Boulton, T. G., Sang, B.-C., and Cobb, M. H. (1989) J. Biol. Chem., ***264*** , 18, 397– 18, 401.

48. Ahn, N . G. and Krebs, E.   G. (1990) J. B*iol. Chem.*, ***265,*** 11495- 11501.

49. Anderson, N.   G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) *Nature*, ***343***, *651-653.*

50. Ahn, N . G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N.K. and Krebs, E. G. (1991) *J. Biol*. Chem., ***266***, 4220 — 4227.

51. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziqjewska, E., Ericsson, I,.. Bratlien, R. L.**,** Cobb, M. H. and Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. USA,* **88,** ***6142-6146,***

52. Posada, J. and Cooper, J. A. (1992), Science, **255,** **212-215.**

53.. Seger, R., Ahn, N. G., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H., and Krebs, E. G. (1992) *J. Biol.* Chem., 267, 14373 — 14381.

54. Gomez, N. and Cohen, P. (1991) *Nature, 351, 69-72.*

55. Ahn, N. C. , Robbins, D. J., Haycock, J. W., Seger, R., Cobb, M. H., and Krebs, E. G. (1992) *J. Neurochemistry,* **147**– 156.

56. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R., Avruch, J. (1992) Nature, 3***58,417-421.***

57. Dent, P., Haser, W., Haystead, T. A. J., Vincent, L. A., Roberts, T. M., Sturgill, T. W. (1992) *Science*, **257,** 1404– 1406.

58. Matsuda, S., Kosako, H., Takenaka, K., Moriyama, K., Sakai, H., Akiyama, T., Cotoh, Y., Nishida, E. (1992) *EMBO J.*, 11, 973- 982 **.**

59.   Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S., Brugge, J. S. (1992) Cell, 68, 1031-1040.

60. Wood, K. W., Sarnecki, C., Roberts, T. M., Blenis, J. (1992) Cell, 6**8,** **1041**–1050.

61. deVries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J., Bos, J. L. (1992) Nature, 357, 602-604.

62. Robbins, D. J., Cheng, M., Zhen, E., Vanderbilt, C. A., Feig, L. A., Cobb, M. H. (1992) *Proc. Natl. Acad. Sci. USA, 89, 6924-6928.*

63. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C. and Cohen, P. (1992) *EMBOJ., 11,*3985-3994.

64. Kuo, J. F. and Greengard, P. (1969) J. *Biol.* Chem., **244**, 3417-3419.

65. Linn, T. C., Pettit, F. H., Hucho, F. and Reed, L. J. (1969a) *Proc. Natl. Acad. Sci. USA, **64**,227 − 234.*