# THE RELATION OF ADENOSINE-3′,5′-PHOSPHATE AND PHOSPHORYLASE TO THE ACTIONS OF CATECHOLAMINES AND OTHER HORMONES[1]

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## I. INTRODUCTION

Catecholamines are observed to produce effects in many different organs and tissues, these effects being manifest in a number of ways. Thus, the heart will beat more rapidly and more forcefully in response to catecholamines, while smooth muscle may contract or relax depending on its anatomical location, or on its physiological or pharmacological status, or both. The central nervous system

[1] Observations reported by the authors and co-workers as in preparation were supported in part by grants from the U. S. Public Health Service (H-2745 and HTS-5228) and from Eli Lilly and Company.

may respond with evidences of heightened or decreased activity depending on experimental conditions. Many tissues display alterations in metabolism after exposure to catecholamines; an especially prominent metabolic effect is the increased glycogenolysis observed in liver and skeletal muscle. Other effects of catecholamines include changes in the secretory activity of the salivary glands, where a thick but sparse secretion occurs.

The cataloguing, classification, and quantitation of these diverse effects has led to extensive use of the concept of receptors in order to supply a unifying framework upon which to build an analysis of the action of sympathomimetic amines. Usually in these conceptual pictures, the receptor is viewed as a substance in or on a cell, which, when in combination with an active agent, may initiate or modify intracellular events resulting eventually in the observable response. For the most part the intervening events between receptor-agent interaction and observable response have remained obscure, due probably in part to the lack of knowledge of the basic biochemistry and biophysics involved in the observable response, such as muscular contraction, rhythmicity, or secretion. Also hampering such investigations has been the necessity of working with intact cells in order to have an observable response. However, in the case of the glycogenolytic action of epinephrine and related amines, the knowledge of some of the biochemical reactions involved in the control of glycogen breakdown and the ability to obtain observable effects in broken cell systems has led recently to the discovery of adenosine-3′,5′-phosphate (cyclic 3,5-AMP). The formation of this compound appears to be a very early result of the interaction of active agent and receptor.

It will not be a purpose of the authors to review in general the actions of catecholamines. The metabolic effects of epinephrine and related amines were reviewed in detail by Ellis in this Journal in 1956 (38); the effects on the central nervous system have been reviewed even more recently by Rothballer (105). The subject of sympathomimetic amine receptors and their blockage has been reviewed recently in this Journal by Furchgott (46), Ahlquist (2), Nickerson (90), and Slater and Powell (110). Instead, an attempt will be made to survey the available information regarding the formation and properties of cyclic 3,5-AMP, with emphasis upon the relation of this compound to the action of catecholamines. In order to aid in the evaluation of the role of cyclic 3,5-AMP, an attempt will be made to survey the current status of the relation of phosphorylase activation to the glycogenolytic and other actions of catecholamines. Finally, an attempt will be made to discuss some of the implications which arise from consideration of the role of cyclic 3,5-AMP in the action of catecholamines and other hormones.

## II. ADENOSINE-3′,5′-PHOSPHATE

### *A. History and chemical properties*

Adenosine-3′,5′-phosphate (cyclic 3,5-AMP) was discovered in 1957 by two separate groups of investigators. Its formula is shown in Figure 1. Cook, Lipkin and Markham were investigating the hydrolysis of adenosine triphosphate (ATP)

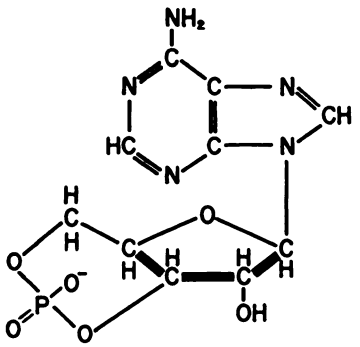

FIG. 1. Adenosine-3′,5′-phosphate

in the presence of barium hydroxide and found several products besides the major products, adenylic acid and inorganic pyrophosphate, one of these being the compound under discussion (23, 73, 74). During the same period of time, a heat stable factor, the accumulation of which was increased by epinephrine or glucagon (94, 100, 118) was isolated from hepatic tissue and was crystallized (118, 119). Both groups determined that the compound had an adenine, ribose, phosphate ratio of 1:1:1 and contained no monoesterified phosphate, and wrote separately to Dr. Leon Heppel for a purified enzyme which might help in the elucidation of its structure. Dr. Heppel informed the groups that the tentative structures proposed were identical, and, as a consequence of this information, samples were exchanged and found to be identical before the final structure was assigned. In an early report (23) it was thought that the compound was a dinucleotide, but subsequent studies showed that the compound was a mononucleotide of adenylic acid with the phosphate attached to the 5-position of the ribose and also to the 3-position (73), as shown in Figure 1.

The ultraviolet spectrum and the molar extinction coefficient of cyclic 3,5-AMP are essentially the same as those of adenosine-5-phosphate. The compound is very stable chemically (*e.g.*, when compared to adenosine-2′,3′-phosphate) and may be boiled at neutral, or slightly acid or alkaline pH for one-half hour or more with no appreciable loss of activity (118). It has been synthesized by two relatively simple procedures, one by hydrolysis of ATP in the presence of barium hydroxide as mentioned (74), the other by dehydration of adenylic acid in the presence of dicyclohexylcarbodiimide, a procedure used by Khorana *et al.* (62, 126) and by Lipkin *et al.* (73).

### *B. Formation of cyclic 3,5-AMP by tissue preparations*

*1. Preparation and properties of the cyclizing enzyme.* Formation of cyclic 3,5-AMP was demonstrated first when particles from liver were incubated with ATP, magnesium ions, and other additives (100, 119). This finding stemmed from the observation that the effect of epinephrine in homogenates, leading to increased levels of active phosphorylase, was lost if a particulate fraction was removed by centrifugation, even with low gravitational forces (see III, A-1-c).

The material which catalyzes the formation of cyclic 3,5-AMP from ATP will be referred to as the cyclizing enzyme in this discussion, even though more than one enzyme may be involved. A more appropriate name, such as adenyl cyclase, may be used in the future, depending on the outcome of investigations on specificity and on the mechanism of the reaction. Although studied for approximately two years, little published information regarding its properties is available. Preparation of particles containing this enzyme has been described, as well as conditions of incubation for formation of cyclic 3,5-AMP (94). More recent experiments (121) have shown that particles from liver or muscle may be washed in hypotonic solutions, then in hypertonic solutions, and subsequently can be dispersed or solubilized in the presence of 1.8% Triton-X-100. The concentrations of certain other enzymes may be reduced by fractionation on cellulose columns. The cyclizing enzyme, however, is relatively labile, behaves much as a lipoprotein, and has been quite difficult to study because of these characteristics. In addition, a considerable amount of other protein with similar characteristics is present in the Triton solution. All the protein including the cyclizing enzyme is difficult to separate from the detergent. Several other enzymes are known to be present; the only one studied in any detail is an ATPase, which, like the cyclizing enzyme, is readily inactivated by freezing at −20°C, but which may be separated in large part from the cyclizing enzyme with use of cellulose column chromatography.

The usual system employed for production of cyclic 3,5-AMP by tissue preparations contains buffer, ATP, magnesium ions, caffeine, and fluoride ions. The addition of fluoride appears to stimulate maximally the production of cyclic 3,5-AMP and in most cases, little additional effect of epinephrine is observed in the presence of 0.01 M NaF. Frequently, 0.02% to 0.5% of the ATP (0.002 M) is converted to cyclic 3,5-AMP.

The partially purified cyclizing enzyme is often inactivated by slow freezing, or storage at −20°C, even after previous rapid freezing to −70°C or lower. Alcohol-ether extractions of active precipitates cause inactivation, as does heating or incubation with the proteolytic enzymes, trypsin or chyomotrypsin. Partially purified preparations may be frozen rapidly and stored at −70° for days or weeks with little loss of activity.

The mechanism of the cyclization reaction has been explored to some extent, using particulate preparations from dog liver and Triton extracts of particles from dog skeletal muscle. Liver particles catalyzed the formation of $C^{14}$ cyclic 3,5-AMP from 8-$C^{14}$-ATP, with no decrease in specific activity during the process, thus indicating that the purine portion was derived from the added ATP, rather than from tissue sources (94). In other experiments, using extracts of skeletal muscle particles as catalyst, cyclic 3,5-AMP was synthesized from $\alpha$-labeled ATP (*i.e.*, adenine-ribose-$P^{32}$-O-$P^{31}$-O-$P^{31}$), and also from $\beta$- and $\gamma$-labeled ATP (*i.e.*, adenine-ribose-$P^{31}$-O-$P^{32}$-O-$P^{32}$) (98). Cyclic 3,5-AMP, derived from $\alpha$-labeled ATP, contained the isotope in good yield, while cyclic 3,5-AMP prepared from the terminally labeled ATP contained only trace amounts of isotope. These

findings suggest that the cyclization process may involve a relatively simple mechanism, perhaps analogous to the chemical synthesis from ATP catalyzed by barium hydroxide.

Broken cell preparations have been employed in almost all studies of the formation of cyclic 3,5-AMP. Haynes has reported a study of its formation in slices from beef adrenals and the effect of ACTH on the formation within the slice (53). Column chromatography was employed at times to concentrate the compound, since the concentration in extracts of slices was often below that required for assay. Studies of this type involving slices or whole organs may supply more valuable information in the future, even though the assay and characterization of small amounts of cyclic adenylic acid in tissues appears to be difficult (17).

*2. Location of cyclizing enzyme. a. Distribution in tissues.* The cyclizing enzyme appears to be present in every animal tissue that has been examined with the possible exception of dog blood cells. No systematic and thorough study of distribution has been made, however, and in most cases biological assay alone has been used as evidence for the formation of cyclic 3,5-AMP by tissue preparations. The biological assay is based on the ability of cyclic 3,5-AMP to increase the rate of formation of phosphorylase from dephospho-phosphorylase in tissue extracts (94, 97, 100).

Using not only biological assay, but also isolation and other characterization procedures, formation of cyclic 3,5-AMP has been clearly demonstrated with preparations from liver, heart, skeletal muscle, and brain of the dog (118), with preparations from beef adrenal cortex (53) and with preparations from the flatworm, *Fasciola hepatica* (80). As judged by biological assay, the cyclizing enzyme was present in the spinal cord (44), kidney, intestinal mucosa, intestinal muscle, aorta, uterus, testis, lung, spleen, and omental fat of the dog (121). Active preparations were obtained from the liver of cat, rat and chicken, from the brain of cat, pigeon, ox, pig and sheep, and from blood cells of chickens and pigeons. Some apparent activity was noted in chub minnows and in fly larvae. The annelid, *Lumbricus terrestis*, was quite active in forming cyclic 3,5-AMP, again as judged by biological assay (121). To date, the crude preparations most active in forming the cyclic nucleotide have been from *F. hepatica* and from mammalian brain cortex.

The cyclizing enzyme, therefore, is widely distributed in animal tissue and appears to be present in all four of the phyla that have been investigated. Its presence in the widely separated phyla, Chordata and Platyhelminthes, has been well documented.

*b. Cellular location.* In general, most of the cyclic 3,5-AMP-forming activity of broken cell preparations is found in the particulate fraction collected with low gravitational forces; for example, 600 $\times$ *g* (or 1000 $\times$ *g*) for 17 minutes has been used for collection of active particles from liver. (A variable amount of activity, 20 % to 50 % or occasionally more, may remain in the supernatant fractions above this precipitate.) The precipitate collected at 600 $\times$ *g* or 1000 $\times$ *g* may be washed several times and the activity may be recollected several times at the same low

forces with high recovery of activity. Fractions of this type have been called "nuclear" fractions by many workers, and obviously attention was called to the possibility that the cyclizing enzyme might be associated with the nuclei.

Support for this possibility comes from two sources. Chicken and pigeon erythrocytes contain nuclei and also contain readily demonstrable amounts of cyclizing enzyme, while the erythrocytes of dog are anucleate and contain little or no cyclizing enzyme when tested under the same conditions (121). In addition, a study was made of the specific activities of the "nuclear" fraction, of the mitochondrial fraction, and the mitochondria and microsomes trapped in the first "nuclear" precipitate (120, 121). Guides to fractionation were gravitational forces, microscopic examination, and determination of an enzyme activity that is localized in mitochondria (cytochrome oxidase) and one that is concentrated in microsomes (inorganic pyrophosphatase). Fractionation followed by repeated refractionation of the nuclear fraction showed clearly that cyclic 3,5-AMP-forming activity was not associated with the mitochondria nor with the microsomes, but with some portion of the "nuclear" fraction. Although an early report showed that particulate fractions free of intact nuclei were active (100), it could be proposed that cyclizing activity was still associated with broken nuclei, and that this might even explain the losses of activity into supernatant fractions on initial centrifugation.

While the cyclizing enzyme may be closely associated with the nucleus, other possibilities exist, with the cell membrane being a leading contender for consideration. The chicken erythrocyte preparations contained cell membranes, as well as the nuclei. Although the dog erythrocyte preparations contained cell membranes, one could speculate that the membranes from these anucleate cells were relatively inactive at this advanced stage of life. Furthermore, some cell membranes have been noted in liver "nuclear" preparations for some time, although one might expect membranes to sediment with difficulty because of their high lipid content. The recent report of Rajam and Jackson is of considerable interest in relation to this question. They studied the behavior of cell membranes from ascites tumor cells during centrifugation, using antigen-antibody techniques, as well as microscopy. They report that the cell membranes sediment readily at low forces and appear at the very bottom of the tubes after centrifugation (92). Finally, the dispersal of rather large amounts of protein of apparent lipoprotein nature (including the cyclizing enzyme) with high concentrations of Triton-X-100 lead one to suspect that the particle has considerable amounts of lipid material in association.

In summary the particles associated with the cyclizing enzyme sediment readily and are not mitochondria or microsomes. The two most likely candidates for specific association with cyclizing activity are nuclei and cell membranes. Further studies with avian erythrocytes may clarify this perplexing question.

### *C. Enzymatic inactivation of cyclic 3,5-AMP*

Cyclic 3,5-AMP was not attacked by prostatic phosphatase, crude intestinal phosphatase (which contained a phosphodiesterase), Russell's viper venom, ribonuclease or highly purified spleen phosphodiesterase; however, it was rapidly in-

activated by extracts from various tissues (100, 118), as judged by loss of activity in the assay described in III, A-1-c.

An enzyme that inactivated cyclic 3,5-AMP was partially purified from heart of dog and beef (118). This enzyme quantitatively converted cyclic 3,5-AMP to adenosine-5-phosphate and, therefore, was a phosphodiesterase. The enzyme was inhibited by caffeine (9, 118) and by theophylline, which was more active than caffeine as an inhibitor (16).

An enzyme which inactivates cyclic 3,5-AMP and is inhibited by methyl xanthines is widely distributed in animal tissues. To date, such an enzyme has been found in brain, spinal cord, heart, skeletal muscle, liver, kidney, uterus, intestine, spleen, testis, lung, and omental fat of dog (18, 118). Its presence has also been noted in extracts of adrenal cortex of beef (104), *Fasciola hepatica* (80), *Lumbricus terrestis*, fly larvae, and chub minnows (18). The enzyme, when searched for, has been found in all tissues that form cyclic 3,5-AMP, except in chicken erythrocytes where interference by hemoglobin was not excluded.

The phosphodiesterase from heart has been purified considerably by Butcher, and some of its properties have been studied. The enzyme requires magnesium ions for activity and its activity is increased by addition of imidazole. Theophylline is more effective than caffeine in inhibiting the enzyme either in crude or purified preparations. The specificity of the enzyme for substrate has been studied to a limited extent. The purified enzyme caused no release of inorganic phosphate from various non-cyclic adenylic acids, or from ATP or cyclic 2,3-adenosine monophosphate. A sample of cyclic 3,5-uridine monophosphate synthesized by Dr. Khorana was hydrolyzed to uridylic acid at a rate about 60% of that found with cyclic 3,5-AMP, while cyclic 3,5-inosine monophosphate (synthesized by Dr. Lipkin) was hydrolyzed at about the same rate as cyclic 3,5-AMP (18).

Heppel (57) has found that extracts of HeLa cells and of nuclei are capable of degrading cyclic 3,5-AMP; after incubation with HeLa cell extracts, adenosine-5-phosphate has been identified as a product.

Cook *et al.* (23) and Lipkin *et al.* (73) have reported that high concentrations of *Crotalus adamanteus* venom slowly attack cyclic 3,5-AMP. When a purified diesterase fraction of venom was used, the products were adenosine-5-phosphate and adenosine-3-phosphate. It is interesting that the enzyme from heart catalyzes the formation of one product only, while two are formed by the action of snake venom. Since venom is able to attack cyclic 3,5-AMP, albeit slowly, it will be of interest to know if digestive enzymes of various animals possess a phosphodiesterase of a similar or different nature.

We can conclude from these reports that enzymes capable of inactivating cyclic 3,5-AMP are widely distributed in animals. The enzyme of venom appears quite different from the enzyme from heart. It is possible that other enzymes exist which are capable of inactivating cyclic 3,5-AMP, perhaps by mechanisms such as deamination, as well as by phosphodiesterase activity.

### *D. Effect of catecholamines on the formation of cyclic 3,5-AMP*

*1. Liver.* The effect of catecholamines on the formation of cyclic 3,5-AMP by liver particles has been studied in some detail, although the exact mechanism of

action is unknown. Washed liver particles incubated with ATP, magnesium ions, buffer, and caffeine frequently produce only small amounts of cyclic 3,5-AMP. On addition of a catecholamine or glucagon, significantly increased amounts are found after a few minutes of incubation at 30°C, 15 minutes usually being an optimal time. The amounts formed in the presence of the hormones are frequently in the range from $5 \times 10^{-7}$ to $3 \times 10^{-6}$ M, amounts readily detectable by assay, or identifiable by isolation with use of column chromatography (94, 118).

Approximately $10^{-6}$ M epinephrine solutions are required to produce one-half maximal effects in washed particulate preparations. (Throughout the present review, the term "epinephrine" refers to the levorotatory isomer, as defined by the U. S. Pharmacopoeia.) The relative activities of the sympathomimetic amines were as follows: *l*-isopropylarterenol = 400, epinephrine = 100, *l*-norepinephrine and *d*-epinephrine = 20 (88). These values are approximate and some variation from preparation to preparation has been noted. The action of epinephrine in these preparations is blocked by ergotamine. Ergotamine does not affect significantly the production of cyclic 3,5-AMP in the absence of catecholamines and does not influence the action of glucagon. This is in agreement with effects of ergotamine noted in homogenates (9) and previously observed by Ellis using liver slices and intact animals (40).

The ability of preparations of the cyclizing enzyme from liver to respond to epinephrine with increased formation of cyclic 3,5-AMP remains through all purification procedures, except treatment with Triton-X-100. The particles are routinely washed with the hypotonic solutions, are frozen, and washed again with hypotonic solution and then with hypertonic solutions. At this stage, the enzyme and the response to epinephrine survive vigorous homogenization in a blendor. However, when homogenized in the presence of 1.8% Triton, much of the cyclizing activity remains, but the effect of epinephrine (or glucagon) disappears. The liver differs from brain in this respect, for recently the cyclizing activity of brain has been solubilized in Triton-X-100, fractionated with use of cellulose columns, and continues to respond to epinephrine addition (see II-D-4). This development lends encouragement in the search for conditions where liver and other preparations may be solubilized with retention of ability to respond to hormones. In any event, the addition of fluoride allows measurement of the solubilized enzyme from liver, and in its presence enzyme activity may be followed during fractionation.

*2. Skeletal muscle.* Washed particulate preparations from muscle produce little cyclic 3,5-AMP unless incubated with epinephrine (94, 118) or fluoride. As with other tissues, 0.01 M fluoride is effective in the absence of hormones and epinephrine has little or no additional effect in the presence of this much fluoride. To date, activity in the absence of fluoride is lost when the enzyme is solubilized in Triton-X-100, whether tested in the absence or presence of epinephrine. The enzymatic activity in soluble fractions can be measured in the presence of fluoride (121).

*3. Heart.* Study of the formation of cyclic 3,5-AMP by particulate heart preparations has become of great interest because of the possibility that the effect of

the catecholamines in stimulating the formation of the cyclic nucleotide can be associated with or dissociated from certain effects on the heart such as the inotropic effect. Obviously, rates of penetration and of inactivation of the catecholamines are some factors which may introduce discrepancies when results in broken cell preparations are compared with results obtained from intact hearts or isolated papillary muscles. Nevertheless, important positive or negative correlations might be found which would warrant careful consideration. Since the inotropic potencies of various catecholamines have been studied in numerous species and preparations (see III, A-3), it would be desirable to compare the relative potencies of the catecholamines in stimulating cyclic 3,5-AMP formation with their potencies in producing inotropic effects. Studies with adrenergic blocking agents also would be of interest. In both instances comparison with inotropic effects seems indicated, since the major portion or all of the ventricle and atrium appears to be involved in this response while chronotropic effects may be more localized anatomically. Evaluation of such results might be facilitated if particulate preparations from an organ such as the liver were also studied. This would allow comparison of the response of heart particles with the response of particles from an organ in which glycogenolysis *per se* is accepted as an important physiological phenomenon that is rather well understood.

Some preliminary results regarding the effect of catecholamines on particulate preparations of heart have been reported (94, 95, 96) and this area is currently under investigation by Murad *et al.* (88, 89). The formation of the nucleotide was increased nearly 4-fold in the presence of maximally active amounts of catecholamines. *l*-Isopropylarterenol was most potent in stimulating the formation of cyclic 3,5-AMP by preparations from dog ventricle, being roughly 5 to 10 times more potent than epinephrine or *l*-norepinephrine. Epinephrine and *l*-norepinephrine were about equipotent with variation from one preparation to another, so that at times *l*-norepinephrine appeared about twice as active as epinephrine, while at times the reverse ratio was observed. The potency of *d*-epinephrine was about $\frac{1}{20}$ that of epinephrine or *l*-norepinephrine; amphetamine was inactive. These relative potencies are remarkably similar to those reported when inotropic effects have been studied (see III, A-3). The relative potency of *d*-epinephrine on intact dog heart was not found in the publications surveyed in this review. Since the free base preparations of this amine tend to deteriorate on storage, potency determinations may tend to be underestimated.

When liver homogenates were used and phosphorylase activation was measured, *l*-norepinephrine was much less active than epinephrine, while *d*-epinephrine was as active as *l*-norepinephrine (see III, A-1). These relative potencies were greatly different from those found when cyclic 3,5-AMP formation by heart particles was studied, as noted above. In order to explore this point further, particulate preparations from heart and liver were prepared from the same dog. The liver particles responded much like the liver homogenates, *i.e.*, epinephrine was more active than *l*-norepinephrine while *d*-epinephrine had about the same activity as *l*-norepinephrine (88). Thus, it appears that the relative potency of catecholamines is similar when cyclic 3,5-AMP formation by heart particles is com-

pared with inotropic potencies, but the relative potencies found in heart and liver preparations differ considerably even when the preparations are derived from the same animal.

Use of particulate preparations with study of cyclic 3,5-AMP formation may help in the analysis of the action of certain other chemical agents which act on the heart, especially those the action of which is associated with changes in glycogenolysis. An important and interesting example is acetylcholine. Vincent and Ellis (132) have reported that acetylcholine opposes the glycogenolytic action of epinephrine on guinea pig heart (see III, A-3). Preliminary studies by Murad *et al.* (89) with acetylcholine and carbachol on the accumulation of cyclic 3,5-AMP by heart particles indicate that these two agents tend to depress the accumulation of cyclic 3,5-AMP.

Studies with adrenergic blocking agents on cyclic 3,5-AMP formation have been limited. Murad *et al.* (89) have reported that dichloroisoproterenol (DCI) behaves as a partial agonist when tested alone, as well as an antagonist when tested with heart particle preparations in the presence of epinephrine.

*4. Other tissues.* Preliminary studies with particulate preparations from brain showed no clear effect of epinephrine on the production of cyclic 3,5-AMP and it was reported that "conditions have not been devised as yet whereby an effect of added epinephrine on the formation of cyclic 3,5-AMP could be observed" (94). Subsequent studies by Klainer *et al.* (63) have indicated that consistent and significant increases in cyclic 3,5-AMP formation occurred in the presence of $10^{-5}$ M U.S.P. epinephrine, using broken cell preparations of various portions of dog or cat brain. Although the cortex was the most active portion of the brain in both species, the epinephrine effect was most prominent in preparations of cat cerebellum in which increases in cyclic 3,5-AMP formation up to 80% were observed. Preparations of brain are capable of forming unusually large amounts of the nucleotide in the absence of added hormones or NaF; this tends to obscure hormone effects, although the "absolute" stimulation, in terms of extra nucleotide formation, may be high in comparison to that observed in preparations of other tissues. Another problem may be the occurrence of relatively high rates of hormone destruction in broken cell preparations of brain.

Recently, the preparative procedures for obtaining cyclizing enzyme activity from liver and muscle (see II, B-1) have been applied to beef, sheep, and pig brain (121). As a result, it has been possible to obtain preparations from all three species which are dispersed or solubilized in 1.8% Triton-X-100 (non-sedimenting or non-floating at 40,000 $\times$ *g*, 30 minutes) and which formed up to 50% more cyclic 3,5-AMP in the presence of epinephrine than in its absence. It is hoped, therefore, that brain tissue will provide the long-awaited soluble preparations that are responsive to hormones, allowing further dissection of the mode of hormonal action.

As discussed in a previous section (II, B-2-a), broken cell preparations of a number of tissues other than liver, heart, skeletal muscle and brain have appeared capable of forming cyclic 3,5-AMP. The nucleotide formation was usually quite small and was identified solely by use of the phosphorylase activation assay sys-

tem (121). In most of these cases, hormonal effects on cyclic 3,5-AMP formation were not observed. There are at least three possible reasons for this: 1) the nucleotide formation in the particular tissue was not actually subject to hormonal regulation; 2) the amount of nucleotide formed in the absence of NaF and in either the presence or absence of hormone was below the limits of detection; 3) optimal conditions for the preparation and testing of different tissues had not been used. It would appear that reason 1 could apply only after exhaustive examination of reasons 2 and 3. For example, by changing preparative and testing conditions and/or by decreasing the lower limits of detection, it has been possible to observe in preparations of omental fat of the dog and of the epididymal fat pad of the rat, that the formation of cyclic 3,5-AMP is increased from an undetectable amount to a small but detectable amount in the presence of epinephrine (88). It is hoped that such observations can be magnified and extended to other tissues.

The formation of cyclic 3,5-AMP by preparations of the epididymal fat pad raises the question of whether the nucleotide is responsible for the prominent lipolytic effects of epinephrine on this organ (47, 75, 135). If this should be so, then there would be a possibility that ACTH also produces its lipolytic effects on this organ (75, 128) *via* the nucleotide.

*5. Possible mechanism of action of catecholamines on particulate preparations.* The exact mechanism of action of the catecholamines remains unknown. Several possibilities, however, are apparent at this early stage. The cyclizing enzyme itself may be stimulated, or an inhibitor of the enzyme may be neutralized in some fashion. If the actual reaction involves more than one enzyme, the same considerations would apply to one or more of the participating enzymes. Another possibility is that some intermediate is formed and that this intermediate is protected by the hormone. The cyclizing enzyme might catalyze the formation of some compound other than cyclic 3,5-AMP as the major product of its enzymatic activity, *e.g.*, formation of a 3,5-bond in a polynucleotide. In this case the major reaction might be diverted by the hormones into the formation of cyclic 3,5-AMP. Or perhaps, the cyclizing enzyme is embedded in a lipoprotein complex in close proximity to another enzyme utilizing ATP. If the activity of the neighbor were to be suppressed by catecholamines, the cyclizing enzyme could have better access to the substrate (ATP) and thus form more cyclic 3,5-AMP. In such a case the "receptor" would be the neighboring enzyme and not the cyclizing enzyme. At the present time, such speculations are of interest, primarily because they indicate possible experimental approaches.

It is unlikely that the stimulation of the accumulation of cyclic 3,5-AMP by catecholamines is due to a protection of cyclic 3,5-AMP itself. Attempts to show effects of catecholamines on phosphodiesterase preparations that inactivate cyclic 3,5-AMP have been uniformly negative. Furthermore, preparations forming cyclic 3,5-AMP and responding to addition of catecholamines have been purified to the stage where essentially all phosphodiesterase activity was absent. In these and cruder preparations no effect of the catecholamines on cyclic 3,5-AMP concentration has been noted, unless ATP was present in the media. Therefore, unless some unknown ATP-dependent inactivating system was present, the

effect of the amines would be at some other site. Although the presence of such an inactivating system appears to be an unlikely possibility, it should be noted that an ATP-stimulated adenylic acid deaminase has been described by Mendicino and Muntz (83) and by Waitzman (134).

### *E. Effect of other hormones on formation of cyclic 3,5-AMP*

Glucagon stimulates the accumulation of cyclic 3,5-AMP by liver particles (94, 100); it is inactive with skeletal muscle preparations (94). On a molar basis, glucagon is more active than the catecholamines and its action is not blocked by ergotamine.

Adrenocorticotrophic hormone (ACTH) has been shown by Haynes (53) to cause an accumulation of cyclic 3,5-AMP in slices from adrenal cortex. This effect on adrenal slices was specific for ACTH and was not imitated in these slices by epinephrine, insulin, or glucagon; in the same studies it was shown that cyclic 3,5-AMP, as well as ACTH, increased the phosphorylase activity of adrenal cortical tissue. Previous observations by Haynes and Berthet (54) had shown that ACTH increased the phosphorylase levels of adrenal cortical tissue slices.

Mansour *et al.* (80) showed that 5-hydroxytryptamine (serotonin) markedly stimulated the accumulation of cyclic 3,5-AMP by particulate preparations from the parasitic flatworm, *Fasciola hepatica*. Here, again, specificity was apparent, and the catecholamines were inactive when tested with these preparations.

### *F. Action of cyclic 3,5-AMP*

*1. Activation of phosphorylase.* It is clear that cyclic 3,5-AMP promotes the accumulation of active phosphorylase from inactive phosphorylase in a number of tissues. This has been demonstrated in homogenates and extracts of liver and in extracts of heart of the dog (94). This effect of cyclic 3,5-AMP has also been observed in extracts of rabbit muscle by Krebs, Graves and Fischer (65) and in extracts of adrenal glands by Riley and Haynes (104). The effects in extracts or homogenates are produced by final concentrations of cyclic 3,5-AMP in the $10^{-7}$ M range (94). These concentrations are near the concentrations of catecholamines required for the stimulation of active phosphorylase formation in liver homogenates (see III, A-1-c).

The effect of cyclic 3,5-AMP on active phosphorylase concentration has been observed with two preparations that contained intact cells, liver slices (111, 118) and adrenal slices (53). In both cases relatively large amounts of the cyclic compound were required for a consistent effect. With liver slices, concentrations in the $10^{-5}$ M range were effective, while with adrenal glands ever. higher concentrations were required ($8 \times 10^{-4}$ M). These findings have been interpreted as indicating that the cyclic nucleotide enters cells very poorly, since much lower concentrations were effective in broken cell preparations. Two experiments carried out by Dr. Roger Jelliffe and the authors with anesthetized dogs showed only a slight rise in blood sugar following the intravenous injection of up to 4 mg/kg of cyclic 3,5-AMP. Part of the injected cyclic 3,5-AMP was found in the urine of the dog.

Experiments with adrenal sections from rats have been reported by Haynes, Koritz and Peron, in which the effect of cyclic 3,5-AMP on steroid production was studied. Clear-cut and large effects were observed using $2 \times 10^{-3}$ M solutions of cyclic 3,5-AMP (55), but were not observed using five related nucleotides. According to the theory proposed by Haynes (53), the increased steroid production would be related to the increased levels of adrenal phosphorylase on incubation with cyclic 3,5-AMP.

In the studies mentioned above, attempts have been made to evaluate the specificity of the response to cyclic 3,5-AMP. A number of related compounds have been inactive in the various systems. When tested with liver preparations and using phosphorylase activation as the indication of activity, cyclic 3,5-uridylate, synthesized by Khorana *et al.*, and cyclic 3,5-inosinic acid, prepared by Lipkin by deamination of cyclic 3,5-AMP, were found to have about 0.4% and 2% of the activity of cyclic 3,5-AMP, respectively (99). Thus, the system responding to cyclic 3,5-AMP by activation of phosphorylase seems to be much more sensitive to changes in the base than was the phosphodiesterase.

The exact mechanism of action of cyclic 3,5-AMP on the activation of phosphorylase remains unknown. The activation of phosphorylase is brought about by transfer of phosphate from ATP to inactive phosphorylase (dephosphophosphorylase) catalyzed by a kinase which is discussed in detail in III, A-1-c. The activation of phosphorylase can proceed in the apparent absence of the cyclic nucleotide; ATP, but not added cyclic 3,5-AMP, appears essential. When kinase preparations that catalyze the formation of active phosphorylase from inactive phosphorylase are purified, they continue to require ATP for activity, but are not affected by addition of the cyclic nucleotide. In cruder preparations, such as homogenates or extracts, the kinase appears to be restrained by an unknown factor(s), and when this restraint is operative, effects of cyclic 3,5-AMP can be observed. In liver extracts, prepared by collecting the supernatant fraction of homogenates after centrifugation at $11,000 \times g$, effects of cyclic 3,5-AMP are prominent. After centrifugation at $100,000 \times g$, the effects of cyclic 3,5-AMP may be less prominent, because the kinase activity without cyclic 3,5-AMP is greater; the absolute increase in kinase activity due to cyclic 3,5-AMP addition, however, may be as great as that noted in extracts prepared by centrifugation at $11,000 \times g$ (100). Thus, one restraining factor, removable by centrifugation at $100,000 \times g$, although not essential for the action of 3,5-AMP, apparently may magnify its action. Krebs and Fischer (65, 65a) have reported that phosphorylase *b* kinase from skeletal muscle may be extracted in a form which is completely inactive at pH 7.0 or below, but active at higher pH. Cyclic 3,5-AMP plus ATP or calcium ions activate the inhibited or inactive kinase. In liver extracts (93) zinc ions and, to a lesser extent, copper ions, increased the kinase activity, while trypsin in small amounts is a very active stimulant (94). Makman *et al.* (77) have noted a factor in plasma that increases phosphorylase activation in liver extracts. It is obvious that a number of agents influence the rate of phosphorylase activation by the kinase and the relation of these to the action of cyclic 3,5-AMP is not clear. Possibly, the kinase occurs in an inactive or inhibited form that can be

activated by several agents. Cyclic 3,5-AMP may directly or indirectly displace or neutralize an inhibitor or a group attached to the kinase, or may aid the transfer of phosphate to inactive phosphorylase in another unrecognized manner.

*2. Other possible action or effects.* In this section the terms "action" and "effect" will be used indiscriminately, since the primary or basic action of cyclic 3,5-AMP that promotes phosphorylase activation may produce other effects.

Berthet reported that the addition of cyclic 3,5-AMP to liver slices increased the formation of ketone bodies from acetate (6). Epinephrine and glucagon also have been reported by Haugaard and Haugaard (50) and by Berthet (6) to cause such an increase in ketone bodies when added to liver slices. These results by themselves could be interpreted as an effect of increased glycogenolysis stimulating the metabolism of acetate. However, it has also been reported that epinephrine and glucagon cause a decreased incorporation of acetate, pyruvate, *etc.*, into the fatty acid and cholesterol of liver slices (7, 50, 51). These reports of decreased incorporation of acetate into some compounds and increased incorporation into ketone bodies are difficult to correlate with an effect on glycogen breakdown alone. Therefore, the effect of cyclic 3,5-AMP on ketone body formation in liver slices is of considerable interest, since it may represent an action of the nucleotide other than an action leading to glycogenolysis.

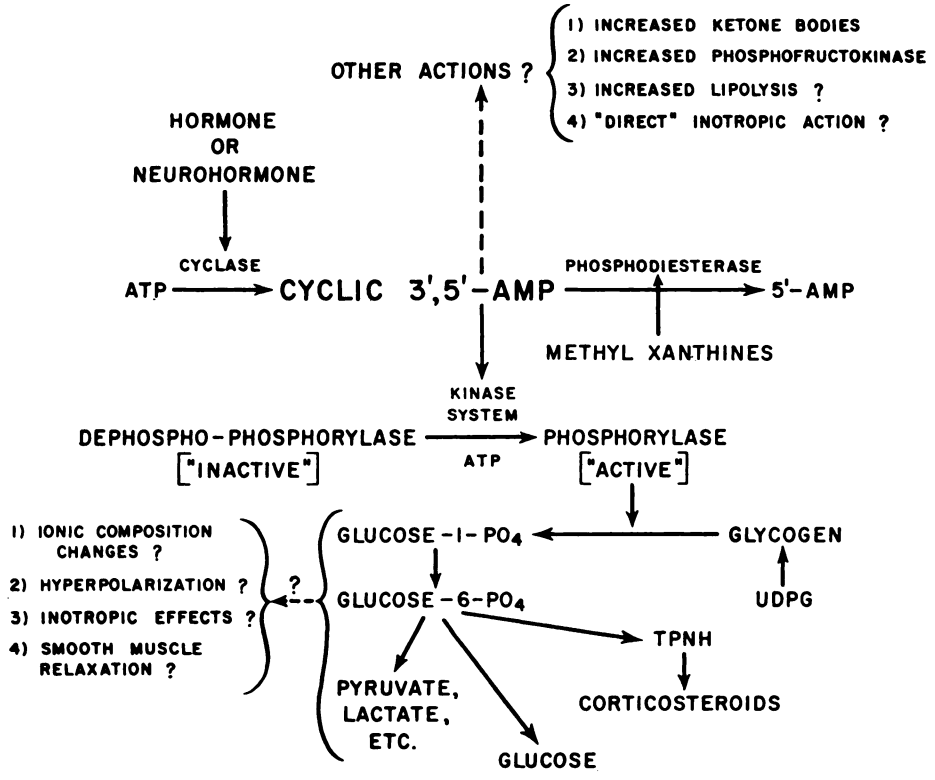


FIG. 2. Summary of some interrelationships involving adenosine-3',5'-phosphate

Epinephrine may increase glucose utilization in the fat pad (20) and in the rat diaphragm under certain conditions (116). Addition of 5-hydroxytryptamine to *Fasciola hepatica* causes an increased utilization of glucose (79). These changes in glucose utilization may be unrelated to cyclic 3,5-AMP action, but attention is called to a possible role of cyclic 3,5-AMP in glucose utilization of these tissues, since these agents are able to stimulate cyclic 3,5-AMP formation in these tissues. A similar speculative approach may be used when the lipolytic effects of epinephrine and adrenocorticotrophic hormone on adipose tissue are compared (47, 75, 128, 135).

Finally, the ability to form cyclic 3,5-AMP is possessed by many diverse animal tissues. In many cases hormonal control has not been demonstrated, and it is not clear that changes in glycogenolysis are involved in important control mechanisms in all cells, for example, in the avian red blood cell. These considerations also invite speculation regarding an action of the nucleotide other than that leading to glycogenolysis in animal cells.

(While this review was in preparation, Mansour and Menard reported that cyclic 3,5-AMP and 5-hydroxytryptamine increased the phosphofructokinase activity in homogenates of *F. hepatica* (79a). Although phosphofructokinase activity apparently limits the rate of lactate formation from hexose phosphates in homogenates of this organism, it is not yet clear whether increased activity of this enzyme in the intact organism would promote glucose utilization.)

## III. THE RELATION OF PHOSPHORYLASE ACTIVITY TO THE ACTION OF THE CATECHOLAMINES

### *A. Individual tissues*

*1. Liver. a. Slices.* Studies with rabbit liver slices led to the conclusion that epinephrine and glucagon stimulated the phosphorylase system (113). This conclusion was based on studies of substrate levels within the slices, and on determination of the enzyme activity which is limiting in the conversion of glycogen to glucose. Later studies showed that the active form of the enzyme itself (phosphorylase) was present in increased concentration in slices after incubation with epinephrine as compared to controls (117). Dog liver slices responded in a similar way with increased concentrations of active phosphorylase being present after incubation with epinephrine (101). Dog liver slices differed from rabbit liver slices in at least one respect. An immediate effect of epinephrine on slices from dogs was observed, while ordinarily slices from rabbits contain all the phosphorylase in the active form at zero time and epinephrine effects appear after an incubation period, *e.g.*, 10 minutes or more at 37°C.

Concentrations of epinephrine required for half maximal effects on glucose output by rabbit liver slices were about one part in 15 million (about $4 \times 10^{-7}$ M) and the relative activities of the sympathomimetic amines were as follows: epinephrine 100, *l*-norepinephrine 16, *d*-epinephrine 16, *d*-norepinephrine 2, and amphetamine 0.0 (117). The effect of maximally active concentrations of epinephrine were not additive to the effect of maximally active concentrations of glucagon.

Cornblath (32) studied the effects of epinephrine, ephedrine and glucagon on the reactivation of phosphorylase in rabbit liver slices. In this study preincubated liver slices were exposed to these agents for 10 minutes at 37°C, and then phosphorylase activity was measured in homogenates prepared from the slices. The sensitivity of the slices to epinephrine judged by enzyme assay was approximately the same as was noted when glucose output was determined. Ui *et al.* (129) have used a similar procedure for assay of glucagon.

Use of glucose production by liver slices for assay of glucagon has been described in detail by Vuylsteke and de Duve (133) with numerous references. The effect of catecholamines on liver, including liver slices, has been reviewed in detail by Ellis in this Journal (38), and excellent reviews of glucagon have been written by de Duve and Berthet (36) and by Berthet (8). Cahill *et al.* (19) have studied ionic and hormonal effects on phosphorylase and glycogen of rat liver slices. Epinephrine or glucagon rapidly and markedly increased the levels of active phosphorylase in preincubated rat liver slices. They noted that glycogen synthesis did not occur in slices when phosphorylase levels were high, for example, in the presence of epinephrine, observations in agreement with numerous older observations included in the reviews quoted above.

*b. Intact animals.* Attempts to demonstrate changes in phosphorylase in livers of intact animals following epinephrine injection have yielded positive results in some cases, and a negative result in one instance. In an early study, twelve pairs of rabbits were carefully matched, one of each pair serving as a control and the other receiving epinephrine subcutaneously 20 minutes before sacrifice. On an average, the increase in phosphorylase following epinephrine was 43% with a probability of about 1% that this difference was due to chance (117). In this series, however, there was a variability in the control level with two of the twelve pairs showing levels equal to the pairs injected with epinephrine. As noted earlier, in general in freshly removed rabbit liver the phosphorylase levels are at a maximum. The investigators felt that discharge of epinephrine or glucagon, or perhaps release of an endogenous agent (such as norepinephrine) on handling or cutting the liver might cause the variable and usually high levels noted in rabbit liver.

Cahill *et al.* (21) studied the effect of glucagon on liver phosphorylase levels of dog and found clear-cut increases in phosphorylase levels as glucagon produced glycogenolysis. Szilagyi and Szabo (125) likewise used dogs and studied the effect of epinephrine injection on levels of hepatic phosphorylase and glucose-6-phosphatase. They found the phosphorylase activity was increased after epinephrine, and, at the same time, no effect on glucose-6-phosphatase was observed.

Perske *et al.* (91) reported no changes in hepatic phosphorylase levels of the rat following injection of epinephrine. This report is interesting, and led Perske *et al.* to question the role of phosphorylase in glycogen breakdown in rat liver. However, several aspects of the experimental work and interpretation deserve careful examination before general acceptance is recommended. For example, breakdown of glycogen in the liver was not actually measured, and in his recent review (38) Ellis points out the need for attention to detail and dosage in order to produce glycogenolysis in rat liver. More important, however, is the possibility

that liver phosphorylase of rats and rabbits in general will be maximally activated by minimal handling; perhaps incision alone will suffice to activate phosphorylase. It would be of interest to see this work repeated with perfused livers where this difficulty may be minimized. Perske *et al.* observed that phosphorylase activity, measured in the direction of glycogen breakdown, was less than the glucose-6-phosphatase activity. Phosphoglucomutase was not measured, but has been reported by others to be considerably more active than phosphorylase (131). If phosphorylase is concerned primarily with glycogenolysis (see III-B), these measurements would support the view that phosphorylase activity may limit glycogenolysis.

In a later section, the effect of epinephrine on skeletal muscle phosphorylase of rats will be mentioned; increased levels of active phosphorylase followed injection of epinephrine. Therefore, at least one rat tissue is capable of responding to epinephrine with increased levels of active phosphorylase.

*c. Broken cell preparations.* It was known for some time that the concentration of phosphorylase in homogenates and crude extracts of liver would decline rapidly unless NaF was present (114). Subsequently, an "inactivating" enzyme inhibited by fluoride was purified several hundred-fold from liver extracts (137). When highly purified liver phosphorylase became available (123), it was observed that the enzymatic inactivation of phosphorylase was accompanied by the release of small amounts of inorganic phosphate (122). Both the fully active and maximally inactivated phosphorylase migrated in the ultracentrifuge at the same rate, both proteins having an apparent molecular weight of around 240,000 (137). The amount of inorganic phosphate appearing during the inactivation process was calculated to be two molecules per molecule of phosphorylase. The "inactivating" enzyme, apparently belonging to the category of protein phosphatases, was named phosphorylase phosphatase.

The product of the phosphatase reaction displays about 2 to 3% of the enzymatic activity of the original phosphorylase when assayed in the absence of adenosine-5′-phosphate and only about 10 to 15% of the original activity when assayed in the presence of adenosine-5′-phosphate. While deserving of the name "inactive phosphorylase," the product of the phosphorylase phosphatase reaction has been named dephospho-phosphorylase to call attention to the chemical nature of the change involved and to provide a name that could also be applied to the product of the muscle phosphorylase *a* to *b* conversion. It was hoped that this would avoid the confusion that arises from the fact that muscle phosphorylase *b* displays nearly the same enzymatic activity as the *a* form when assayed in the presence of sufficient quantities of adenosine-5′-phosphate. Since dephospho-phosphorylase still contains perhaps as many as six molecules of TCA-insoluble, esterified phosphate per molecule of protein, the prefix "dephospho-" should not be construed to indicate the complete absence of phosphate, in the same sense that "dehydro" does not indicate the complete absence of hydrogen atoms in a molecule.

Radioactive phosphorylase was recovered from slices of dog liver that had been incubated so as to contain a high proportion of dephospho-phosphorylase and

then had been exposed to epinephrine or glucagon and $P^{32}$ orthophosphate. More than 70% of the radioactivity could be recovered as orthophosphate after treatment of the labeled enzyme with phosphorylase phosphatase. Thus, it appeared that the reactivation process involved phosphorylation of the inactive enzyme, as would be predicted from the fact that inactivation involved dephosphorylation (101, 122, 137). The radioactivity in the reactivated phosphorylase appeared in the phosphate esterified to serine residues (136).

An enzyme, dephospho-phosphorylase kinase, was then detected and was partially purified from extracts of liver which would transform dephospho-phosphorylase into phosphorylase in the presence of $Mg^{++}$ ions and the proper phosphorylating agent, ATP (101). In crude, relatively concentrated homogenates of liver, the transformation or reactivation process progressed more slowly than in more dilute homogenates, or than would be expected from the amount of kinase activity which could be recovered after a few purification procedures. Under these inhibited or restrained conditions, the addition of small amounts of either epinephrine or glucagon greatly increased the rate of formation of phosphorylase from dephospho-phosphorylase, the amount of the increase being related to the amount of hormones added (100). Under optimal conditions, the half-maximal effect was usually observed at near $1 \times 10^{-7}$ M epinephrine (around 0.02 $\mu$g per ml) and near $1 \times 10^{-8}$ M glucagon (9); *l*-norepinephrine was only about 10 to 15% as potent (9, 100) and *dl*-isopropylarterenol about twice as potent as epinephrine (99). At a concentration of 100 $\mu$g per ml, ergotamine tartrate completely suppressed the effect of up to 2 $\mu$g per ml of epinephrine, while not interfering with the effect of glucagon (9).

It was found that epinephrine and glucagon were unable to stimulate phosphorylase formation in broken cell preparations that had been centrifuged at low speeds (1200 to 2000 $\times$ *g*) even though the kinase reaction also proceeded slowly in such supernatant fractions. Recombination of a small amount of the low-speed pellet with the supernatant fraction restored the hormone effect. It was then found that incubation of the particulate material with $Mg^{++}$ ions, ATP, and the hormones for a short time followed by boiling and centrifugation yielded extracts which would stimulate phosphorylase formation in the low-speed supernatant fractions, while the hormones themselves would not (100). The active principle in these heated extracts was eventually isolated (119), and was identified as adenosine-3′,5′-phosphate (73, 118). As discussed in previous sections, the effect of epinephrine and glucagon was more directly concerned with the conversion of ATP to adenosine-3′,5′-phosphate, and the ability to influence phosphorylase concentration was thus a property of the cyclic nucleotide.

The stimulatory influence of adenosine-3′,5′-phosphate on the dephospho-phosphorylase kinase reaction depends both upon the inhibited or partially active state of the kinase in crude preparations and upon the response of some system to the cyclic nucleotide acting to overcome or relieve the restraint. While the opposition of phosphorylase phosphatase is a very important restraint upon the accumulation of the product of the kinase reaction, the effect of adenosine-3′,5′-phosphate is undiminished in aged preparations exhibiting little or no phos-

phorylase phosphatase activity and is even augmented in the presence of NaF (93), an inhibitor of the phosphatase. The reader is referred to a previous section (see II, F) for further discussion of the relationship of cyclic 3,5-AMP and dephospho-phosphorylase kinase activity.

Broken cell preparations of dog liver, from whole homogenates to 100,000 × *g* supernatant fractions, respond to graded concentrations of cyclic 3,5-AMP of the order of $10^{-7}$ M with graded increments of phosphorylase formation (94, 100). Using 11,000 × *g* supernatant fractions of dog liver homogenates, supplemented with partially purified dephospho-phosphorylase, an assay system was devised to estimate the content of cyclic 3,5-AMP in various biological materials and in experimental samples. The various non-cyclic adenine mononucleotides, as well as adenosine-2′,3′-phosphate, had no detectable effect in this system (94). The inosine and uridine analogues of adenosine-3′,5′-phosphate, prepared and kindly supplied by Dr. D. Lipkin and Dr. H. G. Khorana, respectively, were relatively inactive (see II, F). It will be of great interest to determine the effect of other analogues, prepared as potential mimetic or blocking agents on broken cell systems, as well as on intact cell preparations in which cyclic 3,5-AMP is inactive or acts only at high concentrations.

*2. Skeletal muscle.* Working primarily with skeletal muscle, the Cori's carried out their classical work with phosphorylase over a period of years, which led to crystallization of the enzyme from rabbit muscle extracts and detailed studies of the properties of the enzyme (25–30, 48, 49, 60). The enzyme catalyzed the readily reversible reaction: glycogen + inorganic phosphate $\rightleftarrows$ glycogen-glucose residue + glucose-1-phosphate, with the equilibrium of the reaction in favor of glycogen synthesis. Phosphorylase *a* could be converted to phosphorylase *b* which was inactive when assayed in the absence of adenosine-5′-phosphate, but fully active in the presence of adenosine-5′-phosphate; the molecular weight of phosphorylase *b* was approximately one-half that of the *a* form. (In these two respects the inactive phosphorylase (dephospho-phosphorylase) from liver differs from that from muscle, *i.e.*, the inactive form from liver shows little or no activity when assayed with adenosine-5′-phosphate, and the molecular weights of the active and inactive forms are the same as discussed in the previous section.) It was assumed that phosphorylase catalyzed both the synthesis and degradation of glycogen *in vivo* as it did *in vitro*.

The concept of the roles of phosphorylase *a* and *b* in relation to the function of skeletal muscle has been revised in recent years. In 1945, it was concluded (27) that resting muscle of rabbits contains mainly phosphorylase *a*, while during strong contractions, phosphorylase *a* is converted to the *b* form. Later, however, it was shown that epinephrine increased the relative amount of phosphorylase *a* in rat diaphragm (115). These results with rat diaphragms did not bear directly on the problem of resting *versus* exercised muscle, but were puzzling since both epinephrine and muscular contractions led to glycogen breakdown and lactic acid production. Krebs and Fischer (64) restudied the problem of phosphorylase activity in rabbit muscle and found that essentially all the phosphorylase of resting rabbit muscle was in the *b* form. At the same time, Fischer

and Krebs reported that phosphorylase *b* can be converted to phosphorylase *a* in muscle extracts in the presence of ATP and a divalent ion (42). In subsequent reports they have pursued the studies of the interconversions of rabbit muscle phosphorylase and conclude that ATP donates phosphate to the *b* form, the phosphate being esterified to serine residues in the enzyme (42a, 66). The conversion of phosphorylase *a* to *b* represents a phosphatase action (47a), and the overall process would be basically the same as that occurring in the interconversions of the enzyme from liver and heart. Krebs, Graves and Fischer (65, 65a) have reported that calcium ions may increase the rate of formation of active phosphorylase in extracts of rabbit muscle.

In 1956, Dr. Carl Cori reported results from a restudy of the problem of phosphorylase activity in resting and active muscle (24). Using both frogs and rats, he reported confirmation of the observations of Krebs and Fischer (64), *i.e.*, the active form, phosphorylase *a*, is relatively low during rest and increases rapidly during contraction. With fatigue, the *a* form decreases. Epinephrine injection into rats caused a marked increase in the phosphorylase *a* content of skeletal muscles. The increase occurred within one minute after intravenous injection; subcutaneous injection caused a sustained increase of the phosphorylase *a* content of muscle. Epinephrine also caused an increase in the active phosphorylase content of isolated frog muscle. These findings were also reported by Cori and Illingworth (31).

As a result of these more recent investigations, the following concept has gained acceptance. Resting skeletal muscle contains largely inactive phosphorylase, *i.e.*, phosphorylase *b* or dephospho-phosphorylase. On stimulation the active phosphorylase content increases rapidly. The factors involved in this rapid activation of phosphorylase have not been elucidated. The active phosphorylase of skeletal muscle decreases on prolonged stimulation leading to fatigue. Addition of epinephrine to muscle *in vivo* and *in vitro* produces an increased content of active phosphorylase.

Ellis has studied the biochemical effects of epinephrine in relation to its effects on muscle and has reviewed this area recently in this Journal (39). Readers are referred to this article for references and interpretations of work in this area. In brief summary, activation of glycogenolysis may be important for the muscular effects of epinephrine, but the picture is not at all clear. Perhaps hexosephosphates are implicated in the control of contractility, as was proposed by Ellis previously (37). Muscular contraction itself is complex and the sequence of events occurring in contraction has not been clarified; therefore, one might anticipate some difficulty in attempting to relate the complex events of glycogenolysis to the unknown sequence of events occurring during contraction. In any event, Ellis has shown that the relative potencies of the sympathomimetic amines in producing inotropic effects in rat diaphragm were correlated with the relative potencies in producing glycogenolysis (41).

*3. Heart.* It has been known for some time that the administration of epinephrine *in vivo* or *in vitro* to preparations from various species may be observed to result in a decrease in cardiac glycogen. Ellis has discussed this area in a

recent review (38). In this publication, Ellis also refers to some unpublished observations from his laboratory to the effect that epinephrine reduced glycogen and increased the phosphorylase activity of rabbit ventricle slices. Hess and Haugaard observed that epinephrine and aminophylline increased the phosphorylase *a* content of perfused rat hearts (59). Similar observations were reported by Mayer and Moran (81, 82), using epinephrine, norepinephrine and isopropylarterenol on open-chest dogs, and by Belford and Feinlieb (4, 5), using epinephrine on isolated rat auricles.

Although no published data were found that directly compared glycogenolysis and phosphorylase activity in cardiac tissue, it seems safe to presume that the increased glycogenolysis elicited by epinephrine is the result of an increase in active phosphorylase, as is the case in liver and muscle. The analogy to liver and skeletal muscle appears to go much further. Dog cardiac muscle was observed to contain two forms of phosphorylase, the *a* form (active in absence of 5-AMP) and the *b* form (inactive in absence of 5-AMP and active only in the presence of 5-AMP) (102), analogous to skeletal muscle phosphorylase. While the phosphorylase preparations were not pure enough to determine whether a gross molecular weight change occurred, the transformation of the *a* form to the *b* form was accompanied by a release of orthophosphate when the reaction was catalyzed by partially purified phosphorylase phosphatase prepared from extracts of dog heart or liver. The phosphorylase *b* could be transformed back to the *a* form in the presence of $Mg^{++}$ and ATP by the action of dephospho-phosphorylase kinase obtainable from extracts of dog heart or liver (102). Furthermore, the heart dephospho-phosphorylase kinase, operating in crude extracts was stimulated by low concentrations of adenosine-3′, 5′-phosphate (84). The final segment in the analogy is that the formation of cyclic 3,5-AMP by particulate preparations of dog cardiac muscle can be observed to increase in the presence of epinephrine (94), as discussed in a previous section (see II, D-3). The sequence of events leading from epinephrine to increased glycogenolysis would seem to be identical to that in liver and skeletal muscle involving the increased formation of active phosphorylase and mediated by cyclic 3,5-AMP.

There have been numerous attempts to relate the metabolic effects of epinephrine to contractile effects. The possibility that the intracellular concentration of hexose monophosphates may be important in the regulation of muscular contraction has been mentioned in the preceding section. In this view, for example, the contractile responses of the heart to epinephrine would be a result of an increased concentration of hexose monophosphate, which is one of the observed consequences of increased glycogen breakdown. Without discussing the merits of this hypothesis, there are a number of recent studies that tend to relate the inotropic effects of epinephrine and certain other agents to the level of active phosphorylase in cardiac tissue. Kukovetz *et al.* (67), extending their earlier work with perfused rat hearts (59), observed a close correlation between increased force of contraction and increased content of phosphorylase *a* in response to a number of sympathomimetic amines at a number of dose levels. For both the contractile and phosphorylase effects, the order of potency was *dl*-iso-

proterenol, *l*-norepinephrine, and epinephrine. The less potent agents, epinine, synephrine and phenylephrine, even at high doses, were capable of eliciting only moderate effects on contractile force and small, but significant, increases in phosphorylase *a*. Metanephrine, methoxamine and mephentermine displayed no positive inotropic actions in these preparations and no phosphorylase changes were noted. More recent work from the same laboratory (Haugaard *et al.*, 52) indicates that if the breakdown of ATP to 5-AMP is prevented during the extraction of phosphorylase, or if 5-AMP is removed from the extracts, the apparently high control estimation of phosphorylase *a* is markedly reduced and the increase of phosphorylase *a* under the influence of epinephrine is greatly magnified.

Mayer and Moran have recently published an extensive study of the relation of phosphorylase changes to changes in the heart rate and force of contraction, using dog open-chest preparations (82). These workers concluded that the adrenergic "receptor" for phosphorylase activation is similar to that for augmentation of contractile force in that they observed that epinephrine and *l*-norepinephrine were equipotent and about one-seventh as potent on a molar basis as *dl*-isopropylarterenol in eliciting both responses. In addition, 3,4-dichloroisopropylarterenol (DCI) was observed to prevent both types of effects in response either to injected neurohormone or to nerve stimulation, while phenoxybenzamine did not prevent either effect. Initial doses of ephedrine caused an increase both in phosphorylase and in contractile force, while after repeated doses, both effects disappeared. While the results of these workers qualitatively supported a close association of phosphorylase activation and increased contractile force, there were enough quantitative differences in the two responses to limit possible interpretations. For example, agents that caused only moderate increases in contractile force, including theophylline and low doses of the catecholamines, appeared to cause little or no increase in phosphorylase *a* concentration. In addition, these workers were unable to correlate increases in heart rate with increased phosphorylase *a*.

When agents other than sympathomimetic amines are used, it does not seem to follow that whenever contractile force is increased, phosphorylase *a* content will rise and *vice versa*. For example, Mayer and Moran (82) observed that increased contractile force induced by ouabain was not accompanied by a phosphorylase change. Belford and Feinlieb (4) reported that with concentrations of calcium ions and strophanthin-K producing positive inotropic effects in isolated guinea pig auricles, the phosphorylase *a* content rose, while with higher doses of these agents which depressed the contractile force, the phosphorylase level was also elevated. However, in dog hearts the effect of $Ca^{++}$ ions upon phosphorylase *a* concentration was less striking (82). A possible explanation for the effect of calcium on phosphorylase *a* content is supplied by the observations of Krebs, Graves and Fischer (65, 65a). These workers reported that under certain conditions, calcium ions markedly activate dephospho-phosphorylase kinase of rabbit skeletal muscle, although not in the sense of a cofactor. Under these conditions, it would seem that calcium mimics the

effect of cyclic 3,5-AMP. While not seen with dog liver dephospho-phosphorylase kinase (93), this effect of calcium has not been investigated in preparations of the enzyme from cardiac tissue.

While future work may accumulate evidence that cardiac inotropic effects of sympathomimetic amines are *not* the *result* of effects on cardiac glycogenolysis, but instead are separate, although coordinated effects, it may be more difficult to exclude cyclic 3,5-AMP from a role as a mediator of both effects. First, if in the studies cited above, an increase in phosphorylase *a* is considered presumptive evidence for an increased formation of cyclic 3,5-AMP, then the adrenergic receptor responsible for cyclic nucleotide formation and that responsible for inotropic effects must have very similar properties. These properties include the relative potencies of isoproterenol, epinephrine and norepinephrine (67) and blockade by DCI (81, 82). The direct observation of the relative potency of sympathomimetic amines in stimulating the formation of the cyclic nucleotide by particulate preparations of dog ventricle substantiates this conclusion. In this broken cell system, *l*-isoproterenol was about ten times as effective as either epinephrine or *l*-norepinephrine, which were about equipotent (89, 96). The same order of relative potency can be deduced from the studies of Cotten, Moran and Stopp (33), Moran and Perkins (87), and Cotten and Pincus (34), in which the increase in cardiac force of contraction of open-chest dog preparations was measured in response to intravenous administration of the agent. Finally, the notion that the two receptors may be one and the same and that cyclic 3,5-AMP may mediate both effects receives added support from consideration of the cardiac actions of the methyl xanthines, particularly caffeine and theophylline. Hess and Haugaard observed not only the well-known stimulating effects of aminophylline when perfused into rat hearts, but also an increase in phosphorylase *a* (59). It will be remembered that cardiac muscle contains a powerful phosphodiesterase that acts to inactivate cyclic 3,5-AMP (118) (see II, C). Theophylline and, to a lesser extent, caffeine have been observed to be potent inhibitors of both crude and partially purified preparations of the enzyme (16). Thus, the methyl xanthines are able to increase the accumulation of cyclic 3,5-AMP in broken cell systems (94), and might be expected to do so *in vivo*, even though Mayer and Moran were unable to observe significant increase in phosphorylase *a* after theophylline in dog hearts (82).

A recent report by Vincent and Ellis (132) may well serve to broaden our concepts concerning the correlation of the factors controlling cardiac glycogenolysis with neurohormonal regulation of cardiac function. In addition to noting in perfused guinea pig hearts that epinephrine brought about increased glycogen loss, while increasing rate and amplitude of contraction, and that DCI prevented all these effects, these workers reported that the simultaneous infusion of acetylcholine and epinephrine resulted in no increased glycogen loss and a marked reduction of the effects of epinephrine on rate and amplitude. In this connection it has been observed that acetylcholine and carbachol apparently reduce the formation of cyclic 3,5-AMP by dog and rabbit ventricle particles (89). This effect amounted to about 20% depression of cyclic nucleotide forma-

tion in the presence or absence of added epinephrine and thus was small compared to the stimulatory effect of catecholamines.

*4. Smooth muscle.* In his recent reviews (38, 39) Ellis has surveyed and discussed the evidence that in the smooth muscle of a number of organs, glycogenolysis is a result of exposure to epinephrine. The evidence cited was both direct by determination of glycogen depletion and indirect by detection of increased lactate output in muscular organs which were either inhibited (or relaxed) or stimulated by epinephrine. Ellis also reported that epinephrine increased phosphorylase activity in strips of rabbit uterus *in vitro* (38). An increase in phosphorylase activity (phosphorylase *a* concentration) after exposure to epinephrine in strips of rabbit uterus, but not of rat or guinea pig uterus, could be confirmed (11).

Recently, Axelsson, Bueding and Bülbring reported that epinephrine increased the phosphorylase activity in strips of guinea pig *taenia coli* (3). In this study membrane potentials with extracellular electrodes were recorded from concurrently tested strips; at the time at which the increased phosphorylase activity was seen, there occurred a hyperpolarization. Spike discharge was abolished by epinephrine. Occasionally they observed depolarization after epinephrine; in these cases phosphorylase activity did not change, or increased at a later time. Prolonged exposure of the strips to glucose-free medium gradually abolished the hyperpolarizing action of epinephrine as the glycogen content fell to low levels (phosphorylase activity was not reported) until eventually epinephrine caused only depolarization and initiated spike discharge. These very interesting observations suggest that epinephrine can elicit two different and opposing electrical responses. Membrane hyperpolarization which is associated with relaxation (13) would appear to be also associated with increased glycogen breakdown, again mediated by increased phosphorylase activity. In the absence of increased phosphorylase activity, or in the relative absence of glycogen to be broken down, epinephrine would appear to cause membrane depolarization, which is associated with contraction (13), apparently relatively unopposed or not masked by the hyperpolarization phenomenon.

The results of Axelsson *et al.* might be interpreted to lend support to the theory of Mohme-Lundholm (85) and Lundholm (76) which proposes that smooth muscle relaxation by epinephrine is caused by the increased production of lactate, a consequence of increased glycogenolysis. Ellis has discussed this theory and has proposed alternatively that increased tissue levels of hexose phosphates resulting from glycogenolysis may be involved in the processes of hyperpolarization and smooth muscle relaxation (39).

Both theories have difficulty when an explanation is sought for the increased formation of lactate (presumably due to increased glycogenolysis) by smooth muscles that are contracted by epinephrine, *e.g.*, arterial strips (112). Experiments with such tissues similar to those on *taenia coli* (3), studying the correlation of membrane potential and phosphorylase activity (and/or glycogenolysis) would be of great interest and help in estimating the role of glycogenolysis in contractile effects. Future work on the formation of cyclic 3,5-AMP by prepara-

tions from various smooth muscles might also be helpful. As discussed in a previous section (see II, D-4), although broken cell preparations of dog and guinea pig intestinal smooth muscle were able to form detectable amounts of the cyclic nucleotide, epinephrine was not observed to influence the formation in a few preliminary experiments (99).

Leonard (72) has studied the effect of various hormones, including catecholamines, on the phosphorylase activity of the rat uterus. His observations are rather difficult to correlate with other observations regarding the effect of catecholamines on phosphorylase, because of the time factor. His earliest reported observations with catecholamines were one hour after injection of these agents.

### *B. Relation of phosphorylase activity to glycogen synthesis*

For years it has been assumed that phosphorylase catalyzes the net formation of glycogen *in vivo*, as well as catalyzing the degradation of glycogen *in vivo*. This appeared to be a reasonable assumption, since phosphorylase was the only enzyme in animals which was known to catalyze the synthesis of glycogen *in vitro*, and the equilibrium of the reaction favored glycogen synthesis rather than degradation. The ratios of inorganic phosphate to hexose phosphates found in tissue did appear unfavorably high; however, because of possible flaws of measurement, or compartmentalization of reactions, this unfavorable ratio was not viewed as a serious handicap to the assumption. Indeed, there are many examples of separation of enzyme reactions by cell particles.

If one assumed that phosphorylase *in vivo* catalyzed a "freely reversible" reaction, it was difficult to theorize that an agent such as epinephrine could produce its effect, even on glycogen alone, by increasing the concentration of this catalyst. Some other factor should change, such as the amount of inorganic phosphate. This reasoning became necessary because it is known that epinephrine favors glycogen breakdown and not glycogen synthesis, an area reviewed by Ellis (38) and by de Duve and Berthet (36). It seems likely that this reasoning was a factor in the rather indirect approach to the problem of epinephrine action where liver slices were used, and intermediates in the slices were measured (113). For some years the physiological significance of the effect of catecholamines on phosphorylase activation has been debated, since it was not clear theoretically that increased phosphorylase activity would increase glycogenolysis. Nevertheless, as mentioned in III, A-1-a, all studies showed that increased phosphorylase activity was associated with increased glycogenolysis.

Several recent developments have brought about a revision in our concept of the mechanism of glycogen synthesis *in vivo*. Most important of all was the report by Leloir and Cardini (71) that glycogen synthesis could be catalyzed by an enzyme from liver that used uridine diphosphate glucose as the source of the glucose residues in glycogen. Shortly thereafter Villar-Palasi and Larner (130) described the presence of a similar enzyme in skeletal muscle and diaphragm. Breckenridge and Crawford (12) have demonstrated glycogen synthesis by such an enzyme from brain. Therefore, another enzyme does exist in animal

tissue which is capable of synthesizing glycogen; furthermore the reaction catalyzed is essentially irreversible.

In addition, more studies have been made of the ratio of inorganic phosphate to hexose-phosphates in tissues. Larner *et al.* (70) have studied the ratio of inorganic phosphate to glucose-1-phosphate in rat diaphragms that are actively synthesizing glycogen from glucose. They have reported ratios of about 300:1, which is a ratio far removed from the phosphorylase equilibrium, *i.e.*, a ratio such that glycogen breakdown is strongly favored.

Finally, two types of glycogen storage disease have been reported recently in which phosphorylase is essentially absent or low, even though the glycogen content of the tissue is high. In the McArdle type, muscle phosphorylase has been reported absent or almost absent (69, 86, 108, 109). In a recent study of hepatic glycogen disease, Hers (58) has found that about one-third of the cases studied have no demonstrable enzyme disorder, except for a low concentration of liver phosphorylase.

This recent evidence, therefore, has led to a new concept regarding the synthesis and breakdown of glycogen *in vivo*. In this new concept two enzymes are immediately involved; one catalyzing the synthesis of glycogen in a reaction which is essentially irreversible, the other, phosphorylase, catalyzing the breakdown of glycogen primarily because its environment includes relatively high levels of inorganic phosphate.[2]

### *C. Other hormones*

*1. Glucagon.* In addition to the two reviews of glucagon already cited (8, 36), readers are referred to review articles by Foa *et al.* (43), Best *et al.* (10), Makman *et al.* (77), by participants in a Ciba Foundation colloquium (22), and to an earlier review by Burger (14).

An effect of glucagon on phosphorylase activation has been observed only in liver tissue.[3] Broken cell preparations from skeletal muscle did not respond to addition of glucagon with increased formation of cyclic 3,5-AMP, although they did respond to catecholamines (94). Haynes and Berthet (54) found that glucagon did not increase adrenal phosphorylase or the accumulation of cyclic 3,5-AMP in adrenal slices (53). These observations do not appear to provide an explanation for the increased concentrations of glycogen found in liver some hours or one day after glucagon injection.

When protected from proteolytic attack (127, 133), glucagon is more effective than epinephrine in the activation of liver phosphorylase, as judged by molarity

[2] The authors do not wish to imply that these two enzymes alone are responsible for the synthesis and breakdown of glycogen. In fact, knowledge of the new pathway *via* UPDG might well encourage more speculation regarding other pathways of synthesis and breakdown.

[3] Farah and Tuttle have reported recently that glucagon produced a positive chronotropic and inotropic effect on isolated hearts of dog, cat, guinea pig, and rat. Glucagon also produced a relaxation and inhibition of spontaneous activity in the isolated rabbit and rat intestine similar to that produced by epinephrine (41A).

of solution required for a given effect (9). As mentioned previously, the mechanism of action of glucagon in liver appears basically the same as that of epinephrine in terms of final results, but differs in sensitivity of response and susceptibility to blockade by ergotamine. Perhaps these two differences may be related to an extremely high affinity of the receptor for glucagon, so that ergotamine is unable to displace glucagon.

The final metabolic product resulting from the action of glucagon on liver is glucose. The effects on lipid metabolism noted with epinephrine on liver slices are also observed with glucagon; here another action of cyclic 3,5-AMP may be the common factor rather than phosphorylase activation. No studies have been made regarding the possibility that cyclic 3,5-AMP may be able to exit from liver or other tissues and appear in the blood.

*2. ACTH.* A very interesting relation of ACTH to adrenal cortical phosphorylase activation has been discovered and elucidated by Haynes and Berthet (54), Haynes (53), and Haynes *et al.* (55). Previously, Reich and Lehninger (103) had shown that addition of fumarate increased the production of corticosteroids in adrenal homogenates. Sweat and Lipscomb (124) had found that reduced triphosphopyridine nucleotide (TPNH) stimulated at least one step in the production of the corticosteroids. Haynes, studying the effect of ACTH on adrenal slices and homogenates, found that TPN, together with fumarate, increased the production of corticosteroids in homogenates more than fumarate alone. Since Kelly *et al.* (61) had demonstrated a very high level of glucose-6-phosphate dehydrogenase activity in adrenal cortex, Haynes considered the possibility that the levels of glucose-6-phosphate in the cells might control steroid production by controlling the supply of reduced TPNH that is formed as glucose-6-phosphate is oxidized. Homogenates from adrenal slices, preincubated with ACTH, produced more steroids than controls when incubated with glycogen; addition of hexose phosphates increased the production of steroids, but the increase was not greater in ACTH-treated preparations (54). These observations indicated that phosphorylase activity was greater in homogenates prepared from slices preincubated with ACTH. Haynes then observed that active phosphorylase in adrenal tissue slices was rapidly and specifically increased after addition of ACTH (54). Later it was found that ACTH caused an accumulation of cyclic 3,5-AMP in adrenal tissue slices (53) and that cyclic 3,5-AMP caused an increased concentration of phosphorylase in adrenal slices. In collaboration with Koritz and Peron (55), it was shown that addition of cyclic 3,5-AMP to slices of rat adrenal produced a marked increase in the production of steroids.

*3. 5-Hydroxytryptamine.* Mansour and Lago (79) have shown that addition of 5-hydroxytryptamine (serotonin) to the parasitic trematode, *Fasciola hepatica*, produced an increased breakdown of glycogen, increased uptake of glucose, and increased production of lactic acid along with an increased motility. These effects were not observed when catecholamines were added. Mansour subsequently found that the active phosphorylase content of these parasites was increased following the addition of 5-hydroxytryptamine (78). As mentioned in a previous section (II, E), 5-hydroxytryptamine increased the formation of

cyclic 3,5-AMP in particulate preparations from these worms, while the catecholamines did not.

## IV. CYCLIC 3,5-AMP FORMATION AND RECEPTOR THEORY

The word receptor is derived from a Latin source meaning to receive, and receptor has been used in this limited sense. In physiology and pharmacology the term receptor has been used in a more active sense: the receptor not only receives an agent, but also frequently evokes some response when in combination with the active agent, and perhaps is active in its absence. Generally, the term is used when the underlying events are obscure and is rarely applied to such substances as cholinesterase or hemoglobin, when the active agents under discussion are physostigmine and carbon monoxide; in pharmacology the term has found its greatest use in the study of autonomic agents.

Receptors for the sympathomimetic amines have been classified on the basis of sensitivity of response to the various amines, and the blockade of these responses by certain adrenergic blocking agents, primarily ergot alkaloids and dichloroisopropylarterenol. The alpha receptors are more sensitive to epinephrine and norepinephrine than to isopropylarterenol, *i.e.*, will respond to a lower concentration of epinephrine or norepinephrine, while the beta receptors are more sensitive to isopropylarterenol. In general, the alpha receptors are blocked by ergotamine or dibenzylchlorethylamine (Dibenamine), while the beta receptors are blocked by dichloroisopropylarterenol. Furchgott (46) has proposed that four receptors may be present; alpha receptors for contraction of smooth muscle; beta receptors for relaxation of smooth muscle, other than intestines, and also for increases in rate and strength of cardiac contraction; gamma receptors for glycogenolysis, and delta receptors for inhibition of intestinal smooth muscle. Some results have been interpreted by investigators (15) as being compatible with a single receptor theory, but careful restudy (45) and review (46) by Furchgott has led to the conclusion of Dale (35), Ahlquist (1), Lands (68), and others that more than one receptor exists.

According to the classification of Ahlquist or Furchgott, the beta receptors in the presence of sympathomimetic amines promote relaxation of certain smooth muscles and promote increased rate and force of contraction in heart. Therefore, the overall response of a tissue may differ tremendously when a beta receptor interacts with an active agent, *e.g.*, isopropylarterenol.

Although the above classifications of receptors do not allow us to predict the nature of the tissue response, we might ask the following question. Does the classification of receptors lead one to predict that the basic or primary functions of the different receptors are different? To rephrase the question, do these different receptor substances have different enzymatic activities, or other activities which are basically or primarily different? We might assume (as a working hypothesis) that if two or four different receptors exist, their basic or primary functions are different. This seems to be a reasonable assumption and perhaps in the future such an assumption will be substantiated. Furchgott (46) has discussed the implication of placing receptors for glycogenolysis in a separate

class (gamma). This separation "implies that glycogenolysis is not responsible, either directly or indirectly, for the effects of catecholamines on the contractile activity of heart and smooth muscle." The author was aware of the possibility that future work might alter his current evaluation and the statement is included here as an example of a logical approach in which different primary functions were assigned to different receptors.

While it seems reasonable to assume that different receptors have different primary functions, we may find it helpful on occasions to consider that this working hypothesis may not be necessary. Several studies have shown that the properties of a specific catalyst in a single animal may vary considerably from tissue to tissue even though in each tissue an identical reaction was catalyzed. For example, using antigen-antibody techniques, Schlamowitz showed that alkaline phosphatase from dog intestine could be differentiated from the alkaline phosphatase of dog liver and kidney (106); this approach has been extended by Schlamowitz and Bodansky (107), and has been used to differentiate phosphorylases from various tissues of a single animal (56). Phosphorylases from different tissues also show physical and kinetic differences. Thus, while phosphorylase is not the receptor in the glycogenolytic action of the sympathomimetic amines, it provides an example of a complex catalyst which varies considerably in properties from tissue to tissue while being a catalyst for an identical reaction in these tissues.

Although the exact nature of the receptor for glycogenolysis has not been elucidated, it appears that combination of catecholamines with this receptor influences the accumulation of cyclic 3,5-AMP in a number of tissue preparations. If the primary function of the receptor substance is to influence the accumulation of cyclic 3,5-AMP, implications become apparent. Table 1 summarizes some of the tissues in which formation of cyclic 3,5-AMP has been demonstrated. Tissues that are known to accumulate more of the nucleotide in

TABLE 1

*Tissues in which formation of cyclic 3,5-AMP has been demonstrated*

| Formation Influenced by Hormone | | Formation Not Yet Shown To Be Influenced by Hormone |
|---|---|---|
| Tissue | Hormone | Tissue |
| Liver | Glucagon | Kidney |
| Liver | Catecholamines | Spleen |
| Heart | Catecholamines | Intestine |
| Skeletal muscle | Catecholamines | Uterus |
| Brain | Catecholamines | Testis |
| Fat | Catecholamines | Lung |
| Adrenal cortex | ACTH | Aorta and femoral artery |
| Blood cells (pigeon) | Catecholamines | Blood cells (chicken) |
| *Fasciola hepatica* (whole animal) | 5-Hydroxytryptamine | Minnow (whole animal) |
| | | Fly larvae (whole animal) |
| | | *Lumbricus terrestris* |

the presence of some hormone are listed separately along with the hormone which is active in that tissue. Liver preparations are the only preparations to date which responded to catecholamines and also to glucagon. Several tissues responded to catecholamines and not to glucagon. The activity of epinephrine relative to norepinephrine was higher with liver preparations from dog than with heart preparations from dog. Adrenal slices responded only to ACTH, and preparations from *Fasciola hepatica* responded to 5-hydroxytryptamine and not to the catecholamines. This receptor substance, the apparent primary function of which is to influence the accumulation of cyclic 3,5-AMP, could not be classified as a single receptor substance in terms of sensitivity of response either to the catecholamines, or to any recognized chemical agent. In other words within the limitations of our knowledge, it is possible to recognize a number of "receptors" for cyclic 3,5-AMP formation (based on sensitivity to hormones) the primary function of which is identical. Therefore, studies on the formation of cyclic 3,5-AMP provide an example where prediction that the basic or primary functions of different "receptors" are different would not have been accurate.

It is apparent that an increased accumulation of the cyclic nucleotide can produce different responses in different tissues. Glucose is released from the liver into the blood, but skeletal muscle lacks glucose-6-phosphatase and releases lactic acid instead of glucose. The adrenal gland responds with an increased production of steroids. The series of events leading from cyclic 3,5-AMP to these responses has been fairly well clarified in these three tissues, which provide examples of how an initial event can lead to a series of events which elicit different responses.

The discussion in this section is oriented to indicate the flexibility available within the receptor concept. The concept of receptors for sympathomimetic amines is useful, and it seems possible or likely that the adrenergic receptors do have different primary functions. At the same time, awareness of the flexibility of the concept may offer encouragement in a search for one or two primary or basic actions of epinephrine.

## V. CONCLUDING REMARKS

Special emphasis has been placed on cyclic 3,5-adenylate, the increased accumulation of which represents an early event detected in the action of the catecholamines. The cyclic nucleotide promotes the accumulation of active phosphorylase in tissues and as a result, glycogenolysis is increased. The response of the tissue to increased glycogenolysis varies from tissue to tissue, the liver releasing glucose while the adrenal releases steroids. Results to date are compatible with the possibility that the cardiac inotropic effects of catecholamines and methyl xanthines are mediated via cyclic 3,5-AMP. The possible role of cyclic 3,5-AMP as a mediator in other responses to the catecholamines cannot be evaluated at present, since such studies are absent or in a preliminary stage. Studies of the action of the cyclic nucleotide itself appear to be limited by the rate of its entry into cells, and probably by its rapid inactivation during

or after entry into cells. Liver slices, adrenal slices, and adrenal quarters have responded to high concentrations of cyclic 3,5-AMP in the incubation media. It is hoped that numerous analogues of cyclic 3,5-AMP will become available, especially analogues substituted with lipid-soluble groups.

Scattered bits of information invite speculation regarding possible effects of the cyclic nucleotide which are separable from the stimulation of glycogenolysis. The most tangible information in this respect is the report by Berthet (6) that cyclic 3,5-AMP increases the incorporation of acetate into ketone bodies formed by liver slices.

The enzyme system catalyzing the formation of cyclic 3,5-AMP is widely distributed in the animal kindgom; it has been found in all tissues of all animals[4] examined in four phyla, with the possible exception of blood cells from dogs. At the present time, four hormones or neurohormones are known to influence the rate of formation of cyclic 3,5-AMP in various tissues. Specificity of the hormone response is striking, with liver being the only tissue where the effect of two types of hormones has been demonstrated. It is anticipated that more overlap of activity will be observed in the future, *e.g.*, with adipose tissue. In addition, it seems possible that certain other hormones may act by stimulating or depressing the formation of the cyclic nucleotide.

A large number of areas related to the formation and action of cyclic 3,5-AMP are virtually unexplored; some of these have been indicated in previous sections. Study of hormonal control mechanisms led to the discovery of the cyclic nucleotide in animal tissues, and since that time attention has been directed primarily toward its role in hormonal action. Undoubtedly, research will continue in this direction, but possible relationship of cyclic 3,5-AMP to the effects of other chemical agents, including the various inorganic ions, may receive increased consideration.

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[4] A material similar to or identical with cyclic 3,5-AMP has been found in urine of dog and man (17).

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