The Assay of Adenosine-3′,5′-phosphate in Skeletal Muscle*

JEROME B. POSNER,† KARL E. HAMMERMEISTER,‡ GLORIA E. BRATVOLD, AND EDWIN G. KREBS

From the Department of Biochemistry, University of Washington School of Medicine, Seattle

Received February 28, 1964; revised May 8, 1964

A method for the assay of cyclic AMP in crude extracts of skeletal muscle is described. The muscle is extracted by boiling and the extracts are treated with trypsin to destroy interfering substances. The treated extracts or cyclic AMP standard solutions are then added to mixtures of Mg^{2+}, ATP, and phosphorylase *b* kinase. The degree of kinase activation by cyclic AMP is measured by the ability of the kinase to convert phosphorylase *b* to phosphorylase *a;* phosphorylase *a* production is measured in turn by its ability to release phosphate from glucose-1-phosphate in the presence of a glycogen primer and the absence of AMP. The relationship between the amount of phosphate produced and the concentration of cyclic AMP introduced in the first step is reproducible, so that the muscle extract can be compared with the standard cyclic AMP solutions. The validity of the assay of skeletal muscle extracts is checked by recovery from paper chromatography, by nucleotide-3′,5′-phosphodiesterase attack, and by recovery of known amounts of added cyclic AMP.

Adenosine-3′,5′-phosphate (cyclic AMP) appears to be an important substance in a variety of hormonal systems. Sutherland and Rall (1958), who isolated this nucleotide, showed that in particulate liver preparations epinephrine stimulates cyclic-AMP production and that cyclic AMP mediates glycogen-phosphorylase activation by epinephrine or glucagon. They concluded that increased cyclic-AMP production was responsible for the hyperglycemic effects of these hormones. Epinephrine was subsequently shown to stimulate cyclic-AMP production in particulate preparation of skeletal muscle, cardiac muscle, and brain (Klainer *et al.*, 1962), and thus it was inferred that cyclic AMP mediates epinephrine-produced glycogenolysis in these tissues. In other particulate and slice preparations cyclic-AMP production is stimulated by serotonin (Mansour *et al.*, 1960), vasopressin (Brown *et al.*, 1963), ACTH (Haynes, 1958), and thyrotropic hormone (Klainer *et al.*, 1962). Cyclic AMP has been said to increase steroid production in adrenal slices (Hayes *et al.*, 1959) and perfused adrenal (Hilton *et al.*, 1961), to promote water reabsorption in toad bladder (in a manner similar to vasopressin) (Orloff and Handler, 1962), and to stimulate relaxing-factor particles in skeletal muscle (Uchida and Mommaerts, 1963). The suggestions have been made that the inotropic effect of epinephrine on cardiac muscle is mediated by cyclic AMP through phosphorylase activation, or possibly by cyclic AMP independent of its effect on phosphorylase (Mayer *et al.*, 1963).

A demonstration that the above physiological effects of cyclic AMP are important in the intact animal has been lacking. Such demonstration would depend on correlating tissue levels of cyclic AMP with changes in the physiological state of the tissue being studied. The changes could be most easily shown by an assay for cyclic AMP applicable to crude tissue extracts. Unfortunately, the concentrations of cyclic AMP in tissues are extremely low, so that its measurement by ordinary chemical methods is not feasible. The enzymatic system of Sutherland and co-workers has been generally reliable in particulate preparations, but according to these investigators has failed to give accurate results in whole-tissue extracts (Butcher and Sutherland, 1962). These workers have applied their method successfully, however, after separation of the cyclic AMP using ion-exchange resins.

This report describes a method for the assay of cyclic AMP in crude tissue extracts at concentrations as low as 5×10^{-8} M. The assay is based on the ability of cyclic AMP to increase the rate of activation of partially purified rabbit muscle phosphorylase *b* kinase by ATP (Krebs *et al.*, 1959). Activation of the kinase is indicated by an increased rate of phosphorylase *b* to *a* conversion. Separate reaction mixtures are employed for the kinase activation reaction, the phosphorylase *b* to *a* reaction, and for the measurement of phosphorylase *a* activity. This last determination is carried out in the direction of glycogen synthesis in the absence of AMP (Illingworth and Cori, 1953); the inorganic phosphate liberated is measured colorimetrically. The relationship between the amount of phosphate produced and the amount of cyclic AMP introduced in the first step of the assay is reproducible, so that unknowns can be compared with standard cyclic AMP solutions. This method of assay has been applied to crude extracts of skeletal muscle after administration of epinephrine *in vivo* (Posner *et al.*, 1962).

METHODS

Assay Method

Activation Reaction.—Phosphorylase *b* kinase is activated by Mg^{2+} and ATP in the presence of cyclic AMP. The reaction is allowed to proceed for 10 minutes and then is stopped by dilution. The "activation mixture" contains: 0.2 ml 0.125 M glycero-P, 0.125 M Tris buffer adjusted with HCl to *p*H 6.8; 0.2 ml 0.06 M $Mg(Ac)_2$, 0.018 M ATP adjusted with NaOH to *p*H 6.8; 0.2 ml cyclic AMP or treated crude muscle extract; and 0.2 ml H_2O or other additives. The mixture is warmed approximately 3 minutes in a 30° water bath and the reaction is then started by adding 0.2 ml of cold phosphorylase *b* kinase. The 30-supernatant fraction of nonactivated rabbit skeletal muscle kinase (Krebs *et al.*, 1964) is used and is diluted 1:5 in 0.015 M neutral cysteine just prior to the assay. The reaction proceeds at 30° for 10 minutes and is stopped by diluting an aliquot of the activation mixture 1:15 in 0.015 M neutral cysteine at 0°. Control reaction mixtures without cyclic AMP and without Mg^{2+}-ATP are included.

A full description of phosphorylase *b* kinase activa-

* This project was supported in part by grants from the Muscular Dystrophy Associations of America, Inc., and the National Institutes of Health, U. S. Public Health Service (A-859).

† Special Fellow (BT 801), National Institutes of Neurological Diseases and Blindness. Present address: Dept. of Neurology, New York Hospital, Cornell Medical Center.

‡ Present address: Barnes Hospital, St. Louis, Mo.

tion by Mg^{2+}-ATP both with and without added cyclic AMP is given elsewhere (Krebs *et al.*, 1964). The particular *p*H, salt concentration, activation time, and Mg^{2+}-ATP concentrations are not optimal for kinase activation but are chosen to give the widest possible difference between phosphorylase *b* kinase activation by Mg^{2+}-ATP alone and by Mg^{2+}-ATP in the presence of a low cyclic-AMP concentration. In addition, the buffer concentration is sufficient to prevent significant change in ionic strength or *p*H when crude muscle extract is added.

Conversion of Phosphorylase b to a.—Activated phosphorylase *b* kinase made in the activation reaction is used to convert phosphorylase *b* to phosphorylase *a*. Mg^{2+} and ATP are also required for this reaction (Krebs and Fischer, 1956), but the somewhat higher salt concentration, additional kinase dilution, and shorter period of incubation as compared to the activation reaction prevent significant activation in this step (Krebs *et al.*, 1959). The "conversion mixture" contains: 0.2 ml 0.125 M glycero-P, 0.125 M Tris buffer at *p*H 6.8; 0.1 ml 0.06 M $Mg(Ac)_2$ and 0.018 M ATP at *p*H 6.8; and 0.2 ml AMP-free phosphorylase *b* solution diluted in neutral 0.015 M cysteine to contain 40,000 units/ml (Cori *et al.*, 1943). The mixture is warmed to 30° for approximately 3 minutes and the reaction is started by adding 0.1 ml of the cold diluted activation mixture. A control reaction using 0.1 ml of cysteine solution instead of the kinase is included. The reaction is stopped after 5 minutes by diluting an aliquot of the conversion mixture 1:20 in 0.04 M glycero-P, 0.03 M cysteine adjusted to *p*H 6.8 with NaOH. The diluent is kept at 0°. This reaction is identical with that for measurement of phosphorylase *b* kinase activities (Krebs *et al.*, 1959) except that the reaction is started with phosphorylase *b* kinase rather than with Mg^{2+} and ATP. Starting kinase reactions with the latter components results in somewhat higher reaction rates but is considerably less convenient in this case.

Phosphorylase a Measurement.—Phosphorylase activity is assayed by the method of Illingworth and Cori (1953) in the absence of AMP at a final 1:40 dilution of the conversion mixture. The phosphate released in 5 minutes is measured by the method of Fiske and Subbarow (1925) using a suitable colorimeter. In practice there has been no advantage in converting colorimeter readings into phosphorylase units and the latter into kinase units. The colorimeter readings (in this study, a Klett colorimeter was used) are simply plotted against known cyclic-AMP concentrations as in the typical standard curve shown in Figure 1.

Tissue Preparation

Extraction.—All tissue used in this study is frozen by immersion in isopentane precooled with liquid nitrogen to −160°. The frozen tissue is crushed in a precooled stainless steel pulverizer. The powdered tissue is then transferred to a cold tared glass tube. Three volumes (w/v) of extracting solution are added, and the tube is immediately immersed in a boiling-water bath for 5 minutes. The extracting solution is 0.025 M KH_2PO_4 adjusted to *p*H 7.8. The boiled sample is then cooled, centrifuged, and weighed. The supernatant fluid (boiled extract) is decanted.

Trypsin Attack.—To remove factors interfering with the assay, the boiled extract is treated with 10 μg/ml trypsin for 2 hours at 30°. The reaction is stopped by adding soybean-trypsin inhibitor to a final concentration of 100 μg/ml. The extract can then be assayed simply by adding 0.2 ml of this "treated extract" to the activation mixture. In an earlier study (Posner *et al.*, 1962) interfering factors in boiled-tissue extract were destroyed by treatment with NaOH. The present method utilizing trypsin was substituted because of the possibility of producing cyclic AMP from ATP with alkali as will be discussed in a later section.

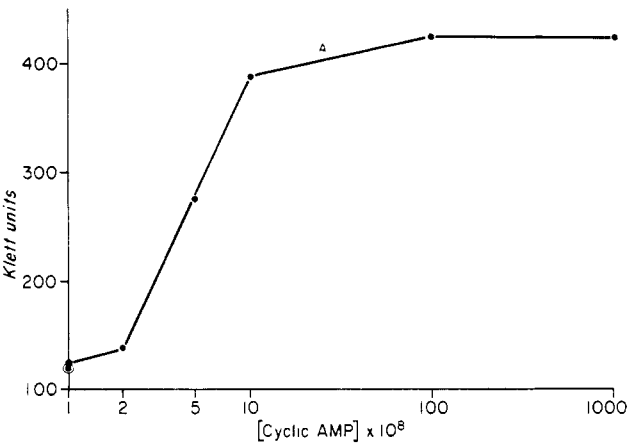

FIG. 1.—The standard cyclic-AMP curve. The abscissa is the concentration of cyclic AMP in the activation mixture, the encirlced point represents Mg^{2+} and ATP without added cyclic AMP.

TABLE I
TISSUE LEVELS OF CYCLIC AMP[a]

Animal	Samples	Tissue	Cyclic AMP (μmoles/kg wet tissue)
Rat	5	Gastrocnemius	0.66 ± 0.16[b]
Frog	6	Gastrocnemius	0.60 ± 0.24
Frog	6	Sartorius	0.67 ± 0.20

[a] The skeletal muscle samples were removed from anesthetized rats and pithed frogs and immediately immersed in cold isopentane. Analyses as described in the text. [b] Corresponds to 1.7×10^{-7} M in the extract and 3.3×10^{-8} M in the activation-reaction mixture.

Tissue Assay.—All samples are run in triplicate and the average of the two closest points is chosen. These are rarely more than 15 Klett units apart. A standard curve covering the range of cyclic AMP concentrations from 1×10^{-8} M to 2×10^{-7} M (in the activation-reaction mixture) is run with each tissue sample; the point without added cyclic AMP, i.e., with Mg^{2+} and ATP alone, is also determined. Cyclic-AMP levels in the unknowns are read directly from the curve and corrected for dilution. Two additional controls are often run. In one, cyclic AMP is added to treated muscle extract to assess the degree of cyclic-AMP recovery, and in the other the trypsin, soybean-trypsin-inhibitor mixture is assayed to be sure that there is no free trypsin to activate phosphorylase *b* kinase (Krebs *et al.*, 1964).

RESULTS AND DISCUSSION

Tissue Levels

The assay was applied extensively to rat gastrocnemius muscle and to frog sartorius and gastrocnemius muscles. Anesthetized rats and pithed frogs were used to secure basal cyclic AMP levels in muscle. Tissue weighing 0.7–1.5 g was extracted in 3–4 volumes of KH_2PO_4 and assayed for cyclic AMP after trypsin treatment. Final values are expressed as μmoles/kg fresh muscle. Means and standard deviations for several samples of rat gastrocnemius and frog sartorius and gastrocnemius are shown in Table I. The effects of epinephrine and electrical stimulation on

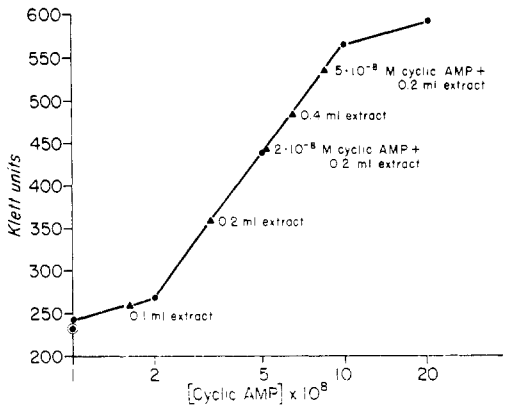

FIG. 2.—The assay of treated skeletal muscle extracts for cyclic AMP. Treated muscle extract is assayed with and without added cyclic AMP; the colorimeter readings (▲) are plotted on the simultaneously measured cyclic-AMP curve (●—●—●). The encircled point (◉) represents Mg^{2+} and ATP without added cyclic AMP. The following cyclic-AMP values were found: 1.0 ml extract = 1.6×10^{-8} M; 0.2 ml extract = 3.3×10^{-8} M; 0.4 ml extract = 6.4×10^{-8} M; 0.2 ml extract + 2×10^{-8} M added cyclic AMP = 5.2×10^{-8} M; 0.2 ml extract + 5×10^{-8} M added cyclic AMP = 8.5×10^{-8} M.

skeletal-muscle cyclic AMP will be the subject of a separate report.

Validity of Assay Method as Applied to Skeletal Muscle

Recovery Curve.—Figure 2 shows an assay of rat skeletal muscle extract measured at varying dilutions with and without added cyclic AMP. The colorimeter readings of the extract were plotted on a simultaneously measured standard cyclic-AMP curve and the following equivalent cyclic-AMP values were obtained: For 0.2 ml of extract (the usual quantity), the reading was equivalent to 3.3×10^{-8} M cyclic AMP. The extract diluted 1:2 gave a reading equivalent to 1.6×10^{-8} M. Doubling the amount of extract gave a reading equivalent to 6.5×10^{-8} M cyclic AMP. With 0.2 ml of extract plus added cyclic AMP at a level of 2×10^{-8} M, the reading was equivalent to 5.2×10^{-8} M. Addition of cyclic AMP at a level of 5×10^{-8} M gave a reading equivalent to 8.5×10^{-8} M. The data demonstrate both full recovery of cyclic AMP added to extract and that dilutions of muscle extract fall at appropriate points on the standard cyclic-AMP curve. It will be noted from the various figures and tables that while the shape of the "standard cyclic-AMP curve" remains essentially unchanged, the Klett values often vary from assay to assay. The reason for this is not clear but the major factor involved seems to be the degree to which Mg^{+2} and ATP alone activate the kinase. This not only varies from one kinase preparation to another but sometimes from one aliquot of a single kinase preparation to another taken at a later time.

Paper Chromatography.—Paper chromatography of rat skeletal muscle extract was done to test validity of the assay system. On Whatman No. 1 filter paper, 500 μl of crude rat skeletal muscle extract was placed in a 10-cm-long streak. On an adjacent spot, 10 μl of 10^{-2} M cyclic AMP served as a visible control. The paper was treated by ascending chromatography for 16 hours in 95% ethanol (7 parts) and 1 M NH_4Ac, *p*H 7.5 (3 parts). After the paper had dried, it was cut into several 5-cm-wide strips the length of the original streak, and these were eluted with water. The elution fluids were assayed along with the original extract. Only the streak at R_F 0.45 (the R_F of the visible cyclic-AMP spot in this system) had cyclic-AMP activity in this assay. This streak gave 100% recovery when compared with the assay of the original boiled extract. Rat skeletal muscle extract was also chromatographed in a column of Dowex-1 (formate) by the method of Hurlbert *et al.* (1954). The material from the column in the predicted cyclic-AMP zone had 55% of the activity of the original extract, a recovery somewhat lower than that achieved by Sutherland and Rall (1958).

Phosphodiesterase Attack.—To further assess the validity of the assay, boiled extracts were treated with nucleotide-3′,5′-phosphodiesterase[1] using the method of Butcher and Sutherland (1962). These results are summarized in Table II. A control was run to show

TABLE II
DIESTERASE ATTACK OF BOILED SKELETAL MUSCLE EXTRACT[a]

Experiment	Substance Assayed	Cyclic AMP Levels Before Diesterase (M × 10^8)	Cyclic AMP Levels After Diesterase (M × 10^8)
1	Trypsin-treated boiled rat muscle extract	1.5	0
	Trypsin-treated boiled rat muscle extract + 5×10^{-8} M cyclic AMP	6.0	0
2	Trypsin-treated boiled frog muscle extract	2.0	0
	Trypsin-treated boiled frog muscle extract + 5×10^{-8} M cyclic AMP	7.0	0

[a] The activation of phosphorylase *b* kinase is expressed in terms of molarity of cyclic AMP in the "activation mixture." Diesterase treatments carried out by the method of Butcher and Sutherland (1962).

that the diesterase itself, when appropriately diluted, had no significant effect on the cyclic-AMP assay system. Boiled-muscle extracts with and without added cyclic AMP were incubated with the diesterase for 3 hours at 30°. (In separate experiments it was shown that known cyclic AMP was completely stable under these conditions in the absence of diesterase.) These extracts, along with similarly diluted extracts not diesterase-treated, were then assayed for cyclic AMP. There was complete loss of cyclic-AMP activity in the treated boiled extracts which were incubated with diesterase. Cyclic AMP which was added to these extracts was also totally destroyed.

Other Factors Affecting the Cyclic-AMP Assay

Other factors are known to activate phosphorylase *b* kinase (Krebs *et al.*, 1959) by mechanisms not involving ATP or cyclic AMP. In addition several factors other than cyclic AMP affect activation of the kinase by ATP. Examination of potentially interfering substances was undertaken, therefore, to evaluate any effect which these substances might have on the cyclic-AMP assay and to estimate the degree of interference which their presence might introduce into the system.

Glycogen.—Glycogen has been shown to activate phosphorylase *b* kinase under certain conditions. It is effective when introduced either into the phosphorylase *b* to *a* reaction mixture or when introduced into the activation mixture with Mg^{2+} and ATP (Krebs

[1] Nucleotide-3′,5′-phosphodiesterase was kindly supplied by Dr. R. E. Butcher.

et al., 1964). In this latter action it mimics cyclic AMP and could potentially interfere in the assay if tissue levels were sufficiently high. It was determined that the smallest detectable glycogen effect occurred at 0.1% glycogen in the activation mixture. With the usual method of preparing tissue samples, a tissue-glycogen level of 2% would be required before the substance could interfere. Such levels were not encountered in normal skeletal or heart muscle but occur in certain other tissues.

Heparin.—Heparin is an activator of phosphorylase *b* kinase, again activating either at the level of the phosphorylase *b* to *a* reaction or by increasing the velocity of kinase activation by ATP during preincubation (Krebs *et al.*, 1964). A concentration of 0.05 mg/ml in the activation mixture was found to be equivalent to $> 1.0 \times 10^{-7}$ M cyclic AMP and 0.1 mg/ml to 1×10^{-6} M cyclic AMP. There is little information available on the concentration of heparin in skeletal muscle and the possibility that it might interfere in the assay must be borne in mind. However, there was no indication of heparin interference since phosphodiesterase destroyed all of the kinase-activating activity in the treated boiled extracts.

Calcium.—Ca^{2+} in the presence of a protein factor (kinase-activating factor) is a potent phosphorylase *b* kinase activator (Meyer *et al.*, 1964). Since the 30-supernatant phosphorylase *b* kinase fraction used in the cyclic AMP assay contains the factor, the effect of Ca^{2+} added to the activation mixture was determined. There was no kinase activation at 1×10^{-5} M or below but concentrations greater than this did activate kinase. Ca^{2+} at 1×10^{-4} M gave activation equivalent to 2×10^{-8} M cyclic AMP while 1×10^{-3} M Ca^{2+} gave kinase activation equivalent to 5×10^{-8} M cyclic AMP.

Calcium concentration in skeletal muscle is approximately 1.6×10^{-3} M (Gilbert and Fenn, 1957). A 1:20 dilution of muscle would give a Ca^{2+} concentration of 8×10^{-5} M in the activation mixture if all were extracted from the muscle. This Ca^{2+} concentration might be expected to activate kinase sufficiently to give a colorimeter reading equivalent to somewhat less than 2×10^{-8} M cyclic AMP. That this is not the case is attested to not only by the recovery curves (Fig. 2) and the complete destruction of kinase activation by phosphodiesterase (Table II), but also by the fact that muscle extracts gave comparable kinase activation with 10^{-3} M EDTA in the activation mixture. It is probable that only a small part of the muscle Ca^{2+} was extracted.

Unknown Activating and Inhibitory Substances.—Unidentified heat-stable factors which potentiate or inhibit the activation of phosphorylase *b* kinase by ATP have been found in boiled extracts of various tissues. In skeletal muscle these factors can be destroyed by trypsin, and this constitutes the basis for using this protease as described in the method presented here. In the first experiment of Table III a boiled rat muscle shows the same apparent cyclic-AMP level with or without addition of 5×10^{-8} M cyclic AMP. After treatment with trypsin, added cyclic AMP is quantitatively recovered. Since the untreated extract completely inhibited the response due to added cyclic AMP, it is probable that the apparent initial level of 4.2×10^{-8} M cyclic AMP was due to a kinase activator other than cyclic AMP. Experiment 2 (Table III) shows that trypsin treatment destroys an activating substance in boiled frog muscle extract, and the third experiment shows that the diesterase of Butcher and Sutherland (*vide supra*) is ineffective in destroying the activating substance. In this last experiment a muscle extract was stored for several weeks at 0° before boiling; this treatment greatly reduces the amount of inhibitory factor present. In separate experiments it was ascertained that trypsin preparations had no effect on cyclic AMP itself.

No chemical characterization of the activating material(s) beyond that given above has been attempted. The inhibitory substance(s) is completely destroyed by heating at 100° in 0.1 N NaOH but is only partially destroyed by boiling in 1 N HCl for 60 minutes. It is destroyed by trypsin (Table III) and is precipitable with trichloroacetic acid (Gonzalez, 1962). It thus appears to be a heat-stable protein or a large polypeptide.

TABLE III
ACTIVATOR AND INHIBITOR SUBSTANCES IN SKELETAL MUSCLE[a]

Experiment	Substance Assayed	Real or Apparent Cyclic AMP (M × 10^8)
1	Boiled rat muscle extract	4.2
	Boiled rat muscle extract + 5×10^{-8} M cyclic AMP	4.0
	Trypsin-treated boiled rat muscle extract	3.6
	Trypsin-treated boiled rat muscle extract + 5×10^{-8} M cyclic AMP	8.7
2	Boiled frog muscle extract	10.0
	Trypsin-treated boiled frog muscle extract	2.0
3	Boiled frog muscle extract (stored) + 5×10^{-8} M cyclic AMP	8.0
	Boiled frog muscle extract (stored) + 5×10^{-8} M cyclic AMP. Mixture treated with diesterase	4.0

[a] The activation of phosphorylase *b* kinase is expressed in terms of molarity of cyclic AMP in the "activation mixture."

Other Substances Tested.—A variety of nucleotides including AMP, ADP, CMP, CTP, UMP, UTP, GMP, GTP, IMP, ITP, as well as adenine and adenosine, were tested at 1×10^{-3} M and were found to be without effect on the assay. Other substances tested and found to be without effect were epinephrine (10^{-3} M), glucose (10^{-3} M), glucose-1-P (10^{-3} and 10^{-4} M), glucose-6-P (10^{-3} and 10^{-4} M), inositol (10^{-3} and 10^{-4} M), glutathione (10^{-3} M), lactic acid (10^{-3} M), acetylcholine (10^{-4} M), UDPG (1.25×10^{-3} M), DPN (10^{-4} M), L-anserine (10^{-3} M), L-carnosine (10^{-3} M), DL-carnitine (10^{-3} M), creatinine (10^{-4}), creatine (10^{-4} M), and creatine-P (10^{-4} M).

Methylxanthines.—Methylxanthines have been shown to inhibit 3′,5′-nucleotide phosphodiesterase, an enzyme which converts cyclic AMP to AMP (Butcher and Sutherland, 1962). Since the 30-supernatant fraction of phosphorylase *b* kinase used in the cyclic-AMP assay contains detectable amounts of phosphodiesterase, addition of caffeine or theophylline was tried in an attempt to increase the sensitivity of the assay. The results are shown in Figure 3. Caffeine at 2×10^{-2} M had no significant effect on the assay. Theophylline at 2×10^{-3} M gave small but definite increases in phosphorylase *b* kinase activation at all levels of cyclic AMP except 1×10^{-5} M (a supermaximal concentration). Theophylline had no effect on Mg^{2+} and ATP activation of phosphorylase *b* kinase in the absence of cyclic AMP.

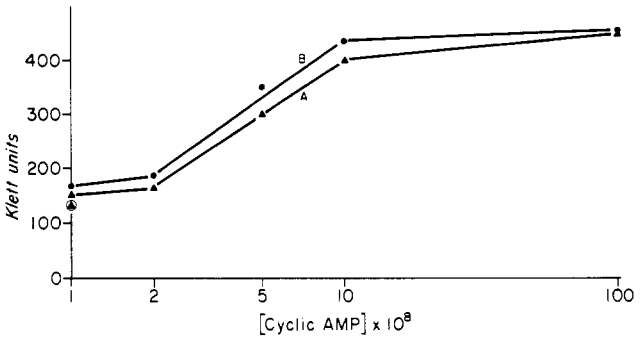

FIG. 3.—The effect of methylxanthines on the cyclic-AMP-assay system. Curve A is a standard cyclic-AMP curve run without addition of methylxanthines. Curve B is a simultaneously run curve with 2×10^{-3} M theophylline present. Another assay in the presence of caffeine, 0.02 M, is not shown since the values were identical with the control points. The encircled point represents no added cyclic AMP.

Nonenzymatic Production of Cyclic AMP

In early experiments the frozen muscle powder was extracted with H_2O and then boiled in 0.1 N NaOH to destroy substances interfering with the assay. The possibility presented itself that this procedure might result in the nonenzymatic production of traces of cyclic AMP from ATP in the presence of a divalent metal ion (e.g., Mg^{2+} or Ca^{2+}) extracted from the muscle. It was known that heating ATP with $Ba(OH)_2$ would produce cyclic AMP (Cook *et al.*, 1957). Accordingly experiments were run in which the nonenzymatic production of cyclic AMP was studied. Mixtures of Mg^{2+} and ATP were boiled either in 0.1 N NaOH or at *p*H 7 and assayed for cyclic AMP; a typical experiment is shown in Table IV. There was detectable formation of cyclic AMP even when neutral mixtures were boiled and more marked production of this nucleotide in alkali. It appeared prudent to abandon the use of NaOH in preparing extracts for assay.[2] Since it was still desirable to be able to extract the powder by boiling, experiments were carried out to see whether nonenzymatic cyclic-AMP production from ATP and Mg^{2+} could be prevented in the presence of a buffer that would chelate the metal. KH_2PO_4, 0.025 M, was found to block cyclic production (Table IV) and was chosen as the extracting medium to use in the assay.

TABLE IV
NONENZYMATIC PRODUCTION OF CYCLIC AMP[a]

Medium	Cyclic AMP Present (M × 10^7)
No addition (*p*H 7)	1.0
0.1 N NaOH	5.0
0.012 M KH_2PO_4 (*p*H 7.8)	0.5
0.025 M KH_2PO_4 (*p*H 7.8)	0
0.038 M KH_2PO_4 (*p*H 7.8)	0
0.050 M KH_2PO_4 (*p*H 7.8)	0

[a] ATP (0.004 M) and Mg^{2+} (0.015 M) were boiled for 5 minutes in the various media and then cooled in ice. Aliquots (0.2 ml) were assayed for cyclic AMP. Values are reported in terms of the concentration of cyclic AMP in the ATP solution.

[2] In practice there may actually be no significant nonenzymatic cyclic-AMP production in skeletal muscle extracts treated with NaOH, since the mean cyclic-AMP values for rat muscle in the present study using the trypsin method do not differ greatly from previously published values (Posner *et al.*, 1962).

REFERENCES

Brown, E., Clarke, D. L., Roux, V., and Sherman, C. H. (1963), *J. Biol. Chem. 238*, PC852.
Butcher, R. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237*, 1244.
Cook, W. H., Lipkin, D., and Markham, R. (1957), *J. Am. Chem. Soc. 79*, 3607.
Cori, C. F., Cori, G. T., and Green, A. A. (1943), *J. Biol. Chem. 151*, 39.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Gilbert, D. L., and Fenn, W. O. (1957), *J. Gen. Physiol. 40*, 393.
Gonzalez, C. G. (1962), thesis, University of Washington.
Haynes, R. C., Jr. (1958), *J. Biol. Chem. 233*, 1200.
Haynes, R. C., Jr., Koritz, S. B., and Peron, F. G. (1959), *J. Biol. Chem. 234*, 1421.
Hilton, J. G., Kruesi, O. R., Nedeljkovic, R. I., and Scian, L. F. (1961), *Endocrinology 68*, 908.
Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.
Illingworth, B., and Cori, G. T. (1953), *Biochem. Prepn. 3*, 1.
Klainer, L. M., Chi, Y.-M., Freidberg, S. L., Rall, T. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237*, 1239.
Krebs, E. G., and Fischer, E. H. (1956), *Biochim. Biophys. Acta 20*, 150.
Krebs, E. G., Graves, D. J., and Fischer, E. H. (1959), *J. Biol. Chem. 234*, 2867.
Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964), *Biochemistry 3*, 1022 (this issue; second preceding paper).
Mansour, T. E., Sutherland, E. W., Rall, T. W., and Bueding, E. (1960), *J. Biol Chem. 235*, 466.
Meyer, S. E., Cotton, M. DeV., and Moran, N. C. (1963), *J. Pharmacol. 139*, 275.
Mayer, W. L., Fischer, E. H., and Krebs, E. G. (1964), *Biochemistry 3*, 1033 (this issue, preceding paper).
Orloff, J., and Handler, J. S. (1962), *J. Clin. Invest. 41*, 702.
Posner, J. B., Stern, R., and Krebs, E. G. (1962), *Biochem. Biophys. Res. Commun. 9*, 293.
Sutherland, E. W., and Rall, T. W. (1958), *J. Biol. Chem. 232*, 1077.
Uchida, K., and Mommaerts, W. F. H. M. (1963), *Biochem Biophys. Res. Commun. 10*, 1.