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A New Assay for Adenosine 3′,5′-Cyclic Monophosphate in Tissue*

John R. Turtle† and David M. Kipnis‡

ABSTRACT: A new, highly sensitive assay for adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP) has been developed permitting measurement of the nucleotide in as little as 1 mg of tissue. 3′,5′-Cyclic AMP in tissue extracts is first separated from other nucleotides by thin layer chromatography. The isolated 3′,5′-cyclic AMP is then converted to 5′-AMP with 3′,5′-cyclic nucleotide phosphodiesterase. In the presence of γ-labeled [^{32}P]adenosine triphosphate and myokinase, the AMP is converted to [^{32}P]adenosine diphosphate which is then separated by thin layer chromatography. Using this method, the 3′,5′-cyclic AMP concentration has been measured in rat liver ($4.9 \pm 1.3 \times 10^{-9}$ mole/g), skeletal muscle ($1.05 \pm 0.2 \times 10^{-9}$ mole/g), and adipose tissue ($5.05 \pm 0.6 \times 10^{-10}$ mole/g). In isolated rat fat cells the 3′,5′-cyclic AMP concentration increased from 2.3 ± 0.3 to $9.5 \pm 0.5 \times 10^{-11}$ mole/10^6 cells in the presence of epinephrine.

Adenosine 3′,5′-cyclic monophosphate[1] is involved in many biological processes from activation of phosphorylase (Murad *et al.*, 1962), UDPG:glycogen α-4-glucosyltransferase (Sutherland and Rall, 1960), and tryptophan pyrrolase (Rosell-Perez and Larner, 1964) to stimulation of insulin secretion (Turtle *et al.*, 1967), lipolysis (Chytil and Skrivanova, 1963; Rizack, 1964), permeability of the toad bladder to water (Orloff and Handler, 1962), uterine protein synthesis (Creange and Roberts, 1965; Szego, 1965), and steroid hydroxylation (Butcher *et al.*, 1965). Two techniques have been described for measuring 3′,5′-cyclic AMP in tissues. One involves activation of the phosphorylase system by the cyclic nucleotide, as described by Posner *et al.* (1964), Øye *et al.* (1964), and Butcher *et al.* (1965), and the other is based on the enzymatic conversion of the cyclic nucleotide to 5′-AMP and subsequent measurement of the latter nucleotide by an enzymatic cycling procedure (Breckenridge, 1964). In both procedures, the assay of 3′,5′-cyclic AMP in tissue extracts has been limited by poor separation of the cyclic nucleotide from other nucleotides and limited sensitivity secondary to either interference from contaminant nucleotides in the enzymes used or nonspecific stimulation of glycogenolysis (Krebs *et al.*, 1959).

Because of these limitations, a minimum of 60–100

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[1] Abbreviations used: 3′,5′-cyclic AMP, adenosine 3′,5′-cyclic monophosphate; UDPG, uridine diphosphate glucose; ADP and ATP, adenosine di- and triphosphates; TCA, trichloroacetic acid.

mg of tissue is required for measurement of the cyclic nucleotide. We have developed a new, highly sensitive assay for 3′,5′-cyclic AMP permitting measurement of the nucleotide in as little as 1 mg of tissue. The procedure is summarized as follows: (I) isolation and separation of 3′,5′-cyclic AMP from other nucleotides by thin layer chromatography; (II) conversion of 3′,5′-cyclic AMP to 5′-AMP with 3′,5′-cyclic nucleotide phosphodiesterase; 3′,5′-AMP → 5′-AMP; (III) conversion of 5′-AMP in the presence of γ-labeled [^{32}P]ATP and myokinase to [^{32}P]ADP, adenosine∼P + adenosine∼P∼P∼^{32}P → 2adenosine∼P∼^{32}P; and (IV) separation and isolation of [^{32}P]ADP by thin layer chromatography.

Materials and Methods

Male Charles River rats weighing 150 g were used. Alkaline phosphatase (*Escherichia coli* type III), 3′,5′-cyclic AMP, ATP, ADP, cysteine, and Tris were purchased from Sigma Chemical Co. 3-Phosphoglycerate (tricyclohexylammonium salt), yeast phosphoglycerate kinase, muscle glyceraldehyde-3-P-dehydrogenase, and rabbit skeletal muscle myokinase were purchased from Boehringer & Mannheim Corp. 3′,5′-Cyclic [^{3}H]AMP (2.3 c/mmole) were obtained from Schwarz BioResearch and carrier-free orthophosphoric acid ^{32}P from New England Nuclear Corp. The purity of all nucleotides used was greater than 99% when chromatographed in at least two solvent systems. MN-cellulose powder 300 HR and MN-cellulose powder 300 DEAE (Macheley Magel & Co.) were purchased from Brinkmann Instruments, Inc.

Buffer. The buffer (hereafter referred to as the Tris–phosphate–Mg^{2+} buffer) used throughout for the enzymatic nucleotide conversion had the following composition: 5 mM Tris, 10 mM sodium phosphate, and 3.4 mM magnesium chloride (pH 8.0). Mg^{2+} was required for both the cyclic nucleotide phosphodiesterase (Sutherland and Rall, 1958) and the myokinase reactions (Bowen and Kerwin, 1953). Sodium phosphate was added to inhibit contaminating ATPase activity in the enzymes (Rabinowitz, 1962).

3′,5′-Cyclic Nucleotide Phosphodiesterase. This enzyme was prepared according to the method of Butcher and Sutherland (1962) from fresh calf hearts and stored in small aliquots at −65° as a solution capable of hydrolyzing a solution of 1 mM 3′,5′-cyclic AMP at the rate of 220 mmoles/l. per hr at pH 8.0 and 37°. Highly active material was obtained in the final isolation procedure by eluting protein from calcium phosphate gel first with 6% and then 10% ammonium sulfate. The enzyme was confined to the 6–10% fraction. This fraction was dialyzed against 10^{-3} M $MgSO_4$ and 10^{-3} M imidazole (pH 7.5) and concentrated against Pevikon C-870 (Fosfatbolaget, Stockholm). The concentrated enzyme solution was stable at −65° for prolonged periods (at least 6 months) and was diluted 1:10 with the Tris–phosphate–Mg^{2+} buffer immediately before use. This dilute solution lost 10% of its activity per month at 5°. At the concentrations used in the assay procedure, no detectable contamination with nucleotides was demonstrable.

[^{32}P]ATP Labeled Specifically in the γ-Phosphorus. [^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell (1964). The following materials were pipetted into a small stoppered centrifuge tube in the order listed: 50 μl of 1 M Tris (pH 8.0), 6 μl of 1 M $MgCl_2$, 102 μl of 0.1 N NaOH, 2 μl of 1 M cysteine, 3 μl of 1 M ATP, 10 μl of 100 mM 3-phosphoglycerate, 10 μl (0.1 mg) of muscle glyceraldehyde-3-P-dehydrogenase, and 1 μl (10 μg) of yeast phosphoglycerate kinase. To this was added 10 mc of carrier-free [^{32}P]H_3PO_4 in 1 ml of water. After incubating at 36° for 1 hr, 300 μl of 55% trichloracetic acid was added, and the mixture was shaken and centrifuged at 0° for 30 min at 18,000*g*. The supernatant was extracted three times with eight volumes of diethyl ether saturated with water. The aqueous phase was lyophilized to 200 μl and the [^{32}P]ATP was separated by thin layer chromatography using the system employed in the 3′,5′-cyclic AMP assay. The ATP spot was eluted in 2 ml of water and stored in small aliquots at −65° as a 10^{-5} M solution with a specific activity of 5–6 c/mmole. Greater than 99% of the ^{32}P was in the γ position when assayed by conversion to ADP with the glucose–hexokinase system (Lamprecht and Trautschold, 1965).

Myokinase. It is essential in the assay procedure that all enzymes be free of contaminating nucleotides. Myokinase obtained commercially from a variety of sources was contaminated to a variable degree with AMP in concentrations ranging from 10^{-4} to 10^{-7} M. No significant contamination with ATP or ADP was detected using the glucose–hexokinase reaction (Lamprecht and Trautschold, 1965) or the luciferin–luciferase reaction (Strehler, 1965). The contaminating AMP was hydrolyzed by digesting the commercial myokinase preparation with alkaline phosphatase. A suspension of myokinase (250 μl) in 3.2 M ammonium sulfate, containing 1.25 mg of myokinase, was centrifuged at 20,000*g* for 30 min and the supernatant was removed. To the precipitate was added 20 μl of 0.03 N NaOH, 10 μl of 1 M 2-amino-2-methyl-1,3-propanediol, and 15 μl of alkaline phosphatase (150 μg). The mixture was incubated for 1 hr at 37°; then 0.01 N HCl was added to bring the solution to pH 4.0. The tightly stoppered tube was boiled for 2 min to inactivate the alkaline phosphatase, and the precipitate was removed by centrifuging for 30 min at 20,000*g* and 0°. The supernatant myokinase solution was stored at 4° at a concentration of 5 mg/ml and used within 2 weeks.

Thin Layer Chromatography Procedure. MN-cellulose 300 HR (15 g) was homogenized with 90 ml of water in a Virtis 45 homogenizer at low speed for 5 min and applied in a 250-μ layer to five 20 × 20 cm glass plates. The plates were dried at room temperature and then heated to 105° for 30 min before use. The solvent system used throughout was 1-butanol–acetone–glacial acetic acid–14.8 N ammonium hydroxide–water in a ratio of 90:30:20:1:60 (v/v). The purity of

TABLE I: R_F Values of Adenine Nucleotides.[a]

3′,5′-Cyclic AMP	0.30
AMP	0.15
ADP	0.07
ATP	0.03

[a] R_F values of nucleotides on 250-μ layers of MN-cellulose 300 HR at room temperature, developed in butanol–acetone–glacial acetic acid–14.8 N ammonium hydroxide–water (90:30:20:1:60, v/v) for 4 hr.

all nucleotides was checked in this system as well as on MN-cellulose 300 DEAE plates developed in isobutyric acid–0.5 M ammonium hydroxide (5:3, v/v).

Analytical Procedure

Preparation of Tissues. Rats were anesthetized with sodium pentobarbitone (4 mg/100 g) injected intraperitoneally and the liver, quadriceps muscle, or epididymal fat pad was exposed, frozen between aluminum clamps cooled in liquid nitrogen, and transferred to Dry Ice. Tissue fragments ranging from 1 to 50 mg were weighed at −20°, and then rapidly homogenized in 1 ml of cold 5.6% trichloracetic acid to which 5×10^{-13} M 3′,5′-cyclic [^{3}H]AMP had been added as a marker for determining recovery. The homogenates were centrifuged at 8000*g* for 30 min at 0° and the supernatant fractions were removed and extracted rapidly three times with eight volumes of diethyl ether saturated with water. The extracts were evaporated to dryness at room temperature in an air stream and dissolved in 100 μl of water.

Isolation of 3′,5′-Cyclic AMP. The extracts were applied 1 in. from the lower border of a 20 × 20 cm MN-cellulose 300 HR plate and dried in an air stream at room temperature. Six samples were applied to each plate with a marker of 10^{-8} mole of 3′,5′-cyclic AMP on each side. The development of the chromatogram was completed within 4 hr at room temperature. The 3′,5′-cyclic AMP marker was detected by ultraviolet light absorption. The corresponding areas of the tissue extract chromatograms were removed and the 3′,5′-cyclic AMP was eluted with three washes of 1, 0.5, and 0.5 ml of 50% ethanol. The ethanol extracts were pooled, evaporated to dryness in a stream of air at room temperature, and dissolved in 100 μl of the Tris–phosphate–Mg^{2+} buffer (pH 8.0). This solution (50 μl) was removed and counted by liquid scintillation in 10 ml of Bray's (1960) solution.

Conversion of 3′,5′-Cyclic AMP to [^{32}P]ADP. 3′,5′-Cyclic AMP standard solutions containing 5×10^{-13} to 2.5×10^{-11} mole in 10 μl or comparable aliquots of purified tissue extracts were pipetted in duplicate into 5 × 50 mm tubes. Buffer was added to bring the total volume to 20 μl. [^{32}P]ATP ($1–5 \times 10^{-11}$ mole) in a volume of 10 μl was added to each tube, and the reaction was started by the addition of 1 μl of cyclic nucleotide phosphodiesterase (a solution capable of hydrolyzing 3′,5′-cyclic AMP at the rate of 22 mmoles/l per hr) and 1 μl of myokinase (5 μg). The tubes were capped and incubated at 37° for 1 hr. At the end of the incubation, the tubes were placed in ice and 10 μl of cold 6 N perchloric acid containing 15 mM sodium EDTA and 5 mM sodium fluoride were added. These concentrations of EDTA and NaF were required to inactivate the myokinase (Noda, 1962) which otherwise maintained its activity in 2 N perchloric acid. After standing in ice for 30 min, 10 μl of an aqueous solution containing 10^{-12} mole of [^{3}H]-ADP was added, followed immediately by 30 μl of cold 2 N potassium bicarbonate. The $KClO_4$ was removed by centrifugation at 0°.

Isolation of [^{32}P]ADP. The supernatant (50 μl) was applied 1 in. from the lower border of a 20 × 20

TABLE II: Recovery of 3′,5′-Cyclic [^{3}H]AMP from Adipose Tissue.[a]

	Sample (cpm)	
	A	B
3′,5′-Cyclic [^{3}H]AMP added	293	293
Extraction 1	272	270
Extraction 2	15	12
Extraction 3	1	3
Pooled ether washes	9	7

[a] Recovery of 5×10^{-13} mole of 3′,5′-cyclic [^{3}H]-AMP was added to adipose tissue. Extractions were performed with 1 ml of 5.6% TCA; each extract was washed with eight volumes of water-saturated diethyl ether and the ether extracts were pooled and dried. Extracts were counted in 10 ml of Bray's solution.

TABLE III: Recovery of 3′,5′-Cyclic [^{3}H]AMP from MN-cellulose 300 HR.[a]

3′,5′-Cyclic [^{3}H]AMP Added		Recovery after Extraction and Chromatography	
$\mu\mu$moles	Cpm	Cpm	% Recov
0.375	180	147	81.7
0.75	346	300	86.7
1.125	509	454	89.2
1.50	664	610	91.8

[a] Adipose tissue (50 mg) with added 3′,5′-cyclic [^{3}H]AMP was extracted and ether washed as described in Table II and then chromatographed. The 3′,5′-cyclic AMP was eluted in 1 ml of water and counted in 10 ml of Bray's solution.

TABLE IV: Conversion of 3′,5′-AMP to 5′-[^{3}H]AMP with 3′,5′-Cyclic Nucleotide Phosphodiesterase.[a]

3′,5′-Cyclic AMP		Recovery in 5′-AMP	
μμmoles	Cpm	Cpm	%
0.1	30	27	90
1.0	334	321	96.1
10	3,148	3,073	97.6
100	34,724	32,749	94.4

[a] Incubations were carried out in a volume of 35 μl of buffer containing 3′,5′-cyclic [^{3}H]AMP for 1 hr at 37°. The reaction was started by the addition of 1 μl of 3′,5′-cyclic nucleotide phosphodiesterase with activity capable of hydrolyzing 1 mM 3′,5′-cyclic AMP at the rate of 22 mmoles/l. per hr. 3′,5′-Cyclic [^{3}H]AMP from control incubations and 5′-[^{3}H]AMP from incubations with phosphodiesterase were eluted in 1 ml of water and counted in 10 ml of Bray's solution.

cm MN-cellulose 300 HR plate and dried in an air stream at room temperature. Six samples were applied to each plate with a marker of 10^{-8} mole of ADP on each side. The chromatogram was developed at room temperature, twice in the same direction. The area corresponding to the position of the ADP marker (detected by ultraviolet light absorption) was removed and the [^{32}P]ADP eluted with 1 ml of water. After centrifugation to remove the cellulose, 0.5 ml of the aqueous extract was counted by liquid scintillation in 10 ml of Bray's solution (Bray, 1960).

A standard curve of incorporation of ^{32}P into ADP was constructed; the 3′,5′-cyclic AMP content of the extracts were read from the curve and corrected for recovery of 3′,5′-cyclic [^{3}H]AMP.

TABLE V: Conversion of AMP to [^{32}P]ADP with Myokinase and [^{32}P]ATP.[a]

AMP (μμmoles)	[^{32}P]ATP μμmoles	[^{32}P]ATP Cpm	[^{32}P]ATP Converted to [^{32}P]ADP Cpm	[^{32}P]ATP Converted to [^{32}P]ADP μμmoles
0.1	25	300,000	648	0.053
1.0	25	300,000	7,116	0.58
5.0	25	300,000	29,568	2.48
10	25	300,000	61,520	5.05
25	25	300,000	147,752	12.1

[a] Incubations were carried out in a volume of 30 μl of buffer for 1 hr at 37°, containing AMP, [^{32}P]ATP, and purified myokinase (5 μg). The reaction was stopped with 10 μl of the perchloric acid reagent, then 10 μl of [^{3}H]ADP containing 1×10^{-12} mole of ADP and 30 μl of 2 N $KHCO_3$ were added. The mixture was centrifuged, the supernatant was applied to an MN-cellulose 300 HR plate and developed twice, then the area corresponding to ADP was eluted with 1 ml of water and counted in 10 ml of Bray's solution.

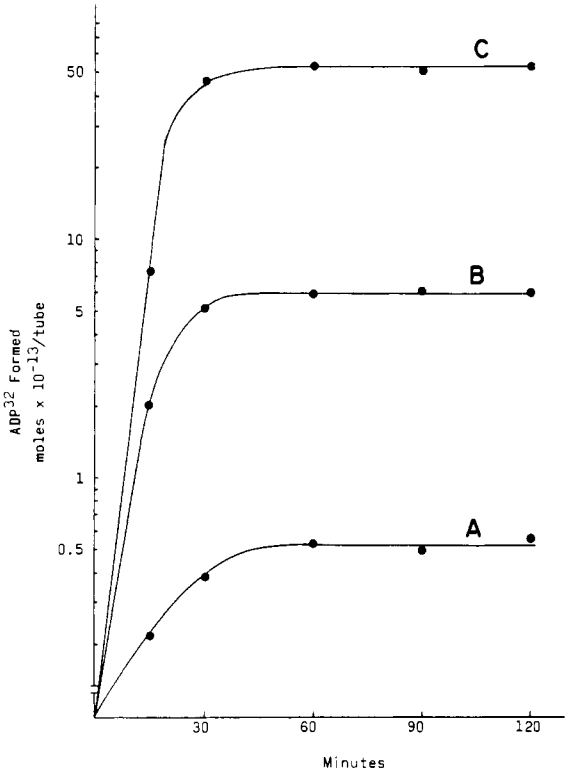

FIGURE 1: Conversion of AMP to [^{32}P]ADP in the presence of γ-labeled [^{32}P]ATP and myokinase. Incubations were carried out at 37° in 30 μl of Tris–phosphate–Mg^{2+} buffer (pH 8.0), containing 2.5×10^{-11} mole of [^{32}P]ATP, 10^{-13} (curve A), 10^{-12} (curve B), and 10^{-11} (curve C) mole of 5′-AMP, and 5 μg of myokinase.

Results and Discussion

3′,5′-Cyclic AMP separated clearly from other adenine nucleotides on cellulose-coated thin layer chromatography plates with butanol–acetone–glacial acetic acid–ammonium hydroxide–water as the developing solvent (Table I). The minimum concentration of 3′,5′-cyclic AMP detected by ultraviolet light was 10^{-9} mole, approximately 50–100 times the concentration of the cyclic nucleotide in the tissue extracts. For this reason, it was necessary to use a marker of 10^{-8} mole of 3′,5′-cyclic AMP developed adjacent to the tissue extract chromatograms.

Recovery of 3′,5′-Cyclic AMP from Tissues. 3′,5′-Cyclic [^{3}H]AMP (5×10^{-13} mole) was added to the tissue extract fluid (5.6% TCA) before the tissues were homogenized. Greater than 92% of the cyclic nucleotide was recovered with the first trichloroacetic acid extrac-

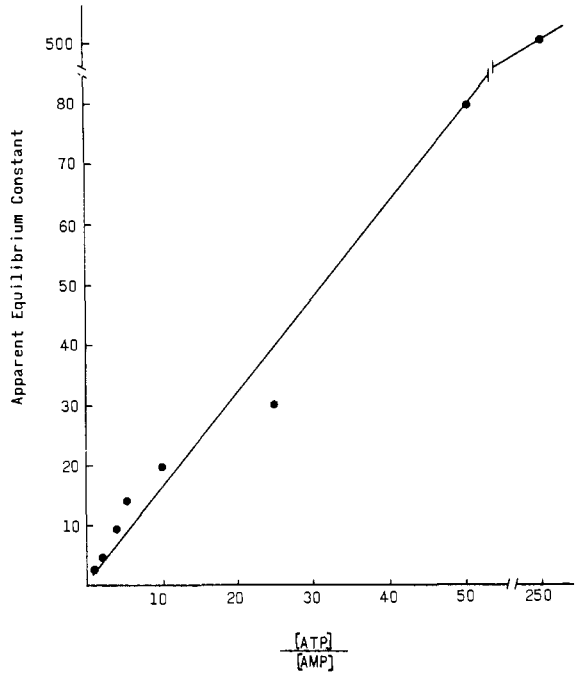

FIGURE 2: Effect of the [ATP]:[AMP] ratio on the apparent equilibrium constant of the myokinase reaction. Incubations were carried out for 1 hr at 37° in 30 μl of Tris–phosphate–Mg^{2+} buffer (pH 8.0), containing 2.5×10^{-11} mole of [^{32}P]ATP, 5×10^{-13}–2.5×10^{-11} mole of 5′-AMP, and 5 μg of myokinase. The [Mg^{2+}]:[ATP] ratio was held constant at 4100.

TABLE VI: Recovery of [^{32}P]ADP from MN-cellulose 300 HR.[a]

3′,5′-Cyclic AMP Converted to [^{32}P]ADP ($\mu\mu$moles)	[^{3}H]ADP Added		Recovery of [^{3}H]ADP	
	$\mu\mu$moles	Cpm	Cpm	%
0.5	1.0	1,605	799	49.8
1.0	1.0	1,605	835	52.0
5	1.0	1,605	707	44.0
10	1.0	1,605	558	34.8
25	1.0	1,605	424	26.5

[a] Incubations were carried out in a volume of 30 μl of buffer containing 2.5×10^{-11} M [^{32}P]ATP, 3′,5′-cyclic AMP, 1 μl of cyclic nucleotide phosphodiesterase, and 5 μg of myokinase. The reaction was stopped with 10 μl of the perchloric acid reagent after 1 hr at 37°, then 10 μl of [^{3}H]ADP containing 1×10^{-12} mole and 30 μl of 2 N potassium bicarbonate were added. The mixture was centrifuged and the supernatant was applied to an MN-cellulose 300 HR plate and developed twice; then the area corresponding to ADP was eluted with 1 ml of water and counted in 10 ml of Bray's solution.

tion; the remaining 4–5% was found in the second extraction (Table II). In view of the completeness of recovery, a single trichloroacetic acid extraction was carried out for the isolation of 3′,5′-AMP from tissues, thereby avoiding further dilution of the nucleotide. The subsequent removal of trichloroacetic acid during the ether washing procedure resulted in the loss of 2–3% of 3′,5′-cyclic [^{3}H]AMP. Isolation of the 3′,5′-cyclic AMP by chromatography of the tissue extract gave a recovery of 81–92% of the 3′,5′-cyclic [^{3}H]AMP up to the point of enzymatic nucleotide conversion (Table III). Comparable recoveries were obtained from skeletal muscle and liver.

Conversion of 3′,5′-Cyclic AMP to 5′-AMP. When incubated for 1 hr at 37° in the Tris–phosphate–Mg^{2+} buffer containing 1 μl of cyclic nucleotide phosphodiesterase solution, 90–97% of 3′,5′-cyclic [^{3}H]AMP in amounts ranging from 10^{-13} to 10^{-10} mole in 35 μl of buffer was converted to 5′-AMP (Table IV). In other experiments, it was observed that similar conversions were obtained after only 15-min incubation.

Conversion of AMP to ADP. The conversion of AMP to [^{32}P]ADP in the presence of [^{32}P]ATP and myokinase reached equilibrium after 30–60-min incubation under the conditions of the assay procedure: 30 μl of Tris–phosphate–Mg^{2+} buffer, containing 10^{-13} to 2.5×10^{-11} mole of 5′-AMP, 2.5×10^{-11} mole of [^{32}P]ATP, and 3.4 mM $MgCl_2$ (Figure 1). It is of interest that over this range of 5′-AMP concentrations, the amount of [^{32}P]ATP converted was equal to approximately 50% of the 5′-AMP level (Table V). The constancy of this ratio of conversion indicates that the apparent equilibrium constant of the myokinase reaction under the conditions of these experiments varies at different 5′-AMP levels (Figure 2), decreasing from 500 at the lowest nucleotide level (1×10^{-13} M) to approximately 2.8 at a nucleotide level of 2.5×10^{-11} M. Although comparable studies of the equilibrium characteristics of the myokinase reaction have not been previously reported, it has been noted that the equilibrium constant increases (in the direction of ADP) as the [ATP]:[AMP] and [Mg^{2+}]:[ATP] concentration ratios increase (Bowen and Kerwin, 1956). Under the conditions of this assay procedure, the [Mg^{2+}]:[ATP] ratio is 3000–4000 and the [ATP]:[AMP] ratio varies from 1 to 250. At a [Mg^{2+}]:[ATP] and [ATP]:[AMP] concentration ratio of unity, the equilibrium constant is approximately 0.44–0.48 (Eggleston and Hems, 1952); a value of 0.48 has been obtained under these conditions in our laboratory.

The cyclic nucleotide phosphodiesterase preparation used in these studies does exhibit ATPase activity sufficient to hydrolyze about 10% of the [^{32}P]ATP during a 1-hr incubation. Since the [^{32}P]ATP is labeled exclusively in the γ position, hydrolysis will not result in labeling of any ADP. When this assay procedure

TABLE VII: 3′,5′-Cyclic AMP Content of Tissues.[a]

	moles/g wet wt[b]
Liver	$4.9 \pm 1.3 \times 10^{-9}$
Skeletal muscle	$1.05 \pm 0.2 \times 10^{-9}$
Adipose tissue	$5.05 \pm 0.6 \times 10^{-10}$
Isolated Fat Cells (moles/10^6 cells[c])	
Control	$2.3 \pm 0.3 \times 10^{-11}$
Epinephrine (0.05 μg/ml)	$9.5 \pm 0.5 \times 10^{-11}$

[a] Tissues from pentobarbitone-anesthetized rats were immediately frozen in liquid nitrogen as described in the text. Aliquots of 40–50 mg of liver and skeletal muscle and 60–70 mg of epididymal fat were extracted. Isolated fat cells were prepared by the procedure of Rodbell (1964) and incubated for 30 min at 37° in the presence of 2% bovine serum albumin and 3 mM glucose. [b] Mean of six to eight determinations plus or minus the standard error of the mean. [c] Mean of four determinations plus or minus the standard error of the mean.

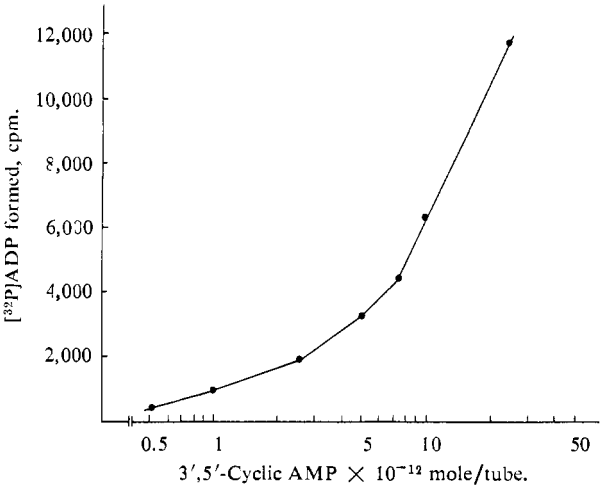

FIGURE 3: Standard curve of the concentration of 3′,5′-cyclic AMP against the total counts per minute incorporated into [32P]ADP. Incubations were carried out for 1 hr at 37° in 30 μl of Tris–phosphate–Mg^{2+} buffer (pH 8.0), containing 2.5×10^{-11} mole of [32P] ATP, 5×10^{-13}–2.5×10^{-11} mole of 3′,5′-cyclic AMP, 1 μl of cyclic nucleotide phosphodiesterase, and 5 μg of myokinase.

was developed [3H]ATP (labeled on the adenine base) was initially used and its conversion to [3H]ADP determined as a measure of the 5′-AMP concentration. However, the ATPase activity in the phosphodiesterase preparation could not be completely and consistently inhibited by sodium phosphate and produced a high and variable blank incorporation into ADP. ATPase activity was not detected in any of the myokinase preparations used in this study.

Immediately before chromatography of the reaction mixture 10 μl of a solution of [3H]ADP containing 10^{-12} mole of ADP was added to correct for recovery of [32P]ADP from the cellulose plate. Within the range of concentrations of 3′,5′-cyclic AMP assayed (5×10^{-13} to 2.5×10^{-11} mole) the recovery of [3H]ADP added ranged from 26.5 to 52.0% (Table VI).

Estimation of 3′,5′-Cyclic AMP Concentration in Tissues. The total cpm incorporated into [32P]ADP in the reaction mixtures of both standard solutions and tissue samples was calculated by dividing the counts per minute recovered in [32P]ADP by the per cent recovery of the added [3H]ADP. A standard curve was drawn plotting the 3′,5′-cyclic AMP content of the reaction mixture (containing 5×10^{-13} to 2.5×10^{-11} mole of 3′,5′-cyclic AMP) against the total counts per minute incorporated into [32P]ADP (Figure 3). The 3′,5′-cyclic AMP content of the reaction mixtures containing the tissue samples was then read from the standard curve. The tissue 3′,5′-cyclic AMP concentration was calculated using the following formula

$$\text{3′,5′-cyclic AMP/g of tissue} = \frac{\text{3′,5′-cyclic AMP content of the reaction mixture}}{\text{tissue weight (g)} \times \%\text{ recovery of 3′,5′-cyclic [3H]AMP}}$$

The 3′,5′-cyclic AMP concentration was assayed in the livers, skeletal muscle, and epididymal fat pads obtained from six to eight rats (Table VII). The results obtained are similar to the levels reported previously for these tissues by Posner *et al.* (1964), Butcher *et al.* (1965), and Sutherland and Rall (1958). The 3′,5′-cyclic AMP concentration in isolated fat cells prepared by the method of Rodbell (1964) was $2.3 \pm 0.3 \times 10^{-11}$ mole/10^6 cells. Studies in our laboratory have shown that the DNA content of rat epididymal adipose tissue is 0.20 ± 0.01 mg of DNA/g and that the DNA content per cell is $7.9 \pm 0.2 \times 10^{-12}$ g. When the 3′,5′-cyclic AMP content of the epididymal fat pad is expressed in terms of the concentration per 10^6 cells a value of 2.01×10^{-11} mole/10^6 cells is obtained, identical with the 3′,5′-cyclic AMP concentration as measured in the isolated fat cell preparation. In the presence of 0.05 μg of epinephrine/ml, the 3′,5′-cyclic AMP concentration in isolated fat cells increased over 400% from 2.3 ± 0.3 to $9.5 \pm 0.5 \times 10^{-11}$ mole/10^6 cells.

Acknowledgments

The authors gratefully acknowledge the expert technical assistance of Mrs. Ingeborg Pommer.

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Some Chemical and Physical Properties of Human Pituitary Follicle-Stimulating Hormone*

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ABSTRACT: A procedure has been developed for the purification of human follicle-stimulating hormone (FSH) which employs ammonium sulfate and pH fractionation, chromatography on sulfoethyl Sephadex C-50 and carboxymethyl Sephadex C-50, and gel filtration on Sephadex G-100. The purified human FSH has been characterized by disk and free-boundary electrophoresis, ultracentrifugation, terminal group analysis, and amino acid and carbohydrate content. The isoelectric point is located at pH 5.6. Terminal groups were found to be absent by the fluorodinitrobenzene and carboxypeptidase procedures. The hormone was found to contain 3.9% hexose, 2.4% hexosamine, 0.4% fucose, and 1.4% sialic acid. These results have been compared with those of ovine FSH.

Studies on the purification of human pituitary follicle-stimulating hormone (FSH)[1] have recently been reported (Roos and Gemzell, 1964, 1965; Amir *et al.*, 1966; Parlow *et al.*, 1965). These studies have all remarked on the instability of the hormone. The factors responsible for the observed losses of biological potency are not, however, clear at this time. Virtually nothing has been reported on the chemistry of human FSH, information which could possibly shed light on the instability of the hormone. In addition, the most successful of the above cited studies (Roos and Gemzell, 1964, 1965) leaves much to be desired because of the low yield of hormone obtained as well as the reliance on technically difficult methods of purification (*i.e.*, preparative polyacrylamide gel electrophoresis). We have endeavored to develop a simple purification procedure which would result in a good yield of highly purified FSH. The purification procedure and some of the properties of the product are described in this paper.

* From the Hormone Research Laboratory, University of California Medical Center, San Francisco, California 94122. *Received August 28, 1967.* This work was supported in part by U. S. Public Health Service Grant A-6097, from the National Institutes of Arthritis and Metabolic Diseases. One of us (H. P.) is a career development awardee, Institute of General Medical Sciences, U. S. Public Health Service. Standard gonadotropin preparations were a gift of the Endocrinology Study Section.

[1] Abbreviations used: FSH, follicle-stimulating hormone; ICSH, interstitial cell stimulating hormone; GH, human growth hormone; FDNB, fluorodinitrobenzene.