Effect of Metals upon the Conversion of Adenosine Triphosphate to Adenosine 3′, 5′-Monophosphate in Lipocytes* (32997)

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As has been reviewed (1–4), adenosine 3′,5′-monophosphate (c-AMP) participates in many reactions in various body tissues affected by hormones. Lipolytic hormones have been shown to increase c-AMP in adipose tissue and c-AMP increases lipolysis, whereas insulin markedly inhibits lipolysis and has been reported (5,6) to decrease c-AMP in adipose tissue. During the course of investigations into factors that influence c-AMP levels in lipocytes, a major effect of metallic ions was noted and these observations constitute the basis for this report.

Methods and Materials. Details of the methods used (for publication, *Metabolism*) are partially dependent upon observations by Rabinowitz *et al.* (7). Epididymal adipose tissue cells (lipocytes) from Wistar rats weighing approximately 250 gm were isolated by Rodbell's method (8). After washing the lipocytes three times in Tris · Cl-β-glycerophosphate buffer (each 0.077 *M*), pH 7.4, containing 4% albumin, they were centrifuged, the buffer withdrawn, and 0.15 ml of cells (average of 734,000) was added to 0.35 ml of incubation mixture composed of the following:- Tris · Cl-β-glycerophosphate buffer, 45 μmoles of each at pH 6.8; aminophylline, 3.0 μmoles; disodium ATP-8-^{14}C, 0.275 μmoles (2.7 μC/μmole); trisodium phosphoenolpyruvate, 3.0 μmole; 1.2 units of pyruvate kinase. The pH was 7.35–7.40. Metals were added with final molarities as follows: K(KCl), 4×10^{-3}; Mg ($MgSO_4$), 2.8×10^{-3}; Ca($CaCl_2$), 2.5×10^{-3}; Zn ($ZnCl_2$), 1×10^{-3}; F (NaF), 1×10^{-2}. The concentration of EGTA [ethyleneglycol-bis (β-aminoethyl ether) *N,N′*-tetraacetic acid] was 5×10^{-3} *M*, crystalline zinc insulin, 1000 μU/ml, and isopropylnorepinephrine, 1 μg/ml. In all instances the final volume of the incubation mixture was 0.5 ml. After incubation in a Dubnoff shaker at 37°C for 30 min the reaction was terminated by the addition of 0.5 ml of 1.0 *M* perchloric acid. After centrifugation, an aliquot of the solution was subjected to paper chromatography, using 1.15 *M* ammonium acetate–95% ethyl alcohol (26:74) as solvent. Nonlabeled c-AMP was added to the paper as a marker, and its migration was identified by ultraviolet light. Comparisons of *Rf* values were made with standard solutions. The c-AMP spots were excised, placed in scintillation fluid, and the ^{14}C counted in a Packard scintillation counter. The amount of c-AMP-^{14}C was calculated form the specific activity of ATP added initially.

The ATP-^{14}C was purchased from Schwarz BioResearch; crystalline bovine insulin, aminophylline, and EGTA from Sigma; pyruvate kinase and 2-P-enolpyruvate, from Calbiochem; ATP from P-L Biochemicals; isopropylnorepinephrine from Winthrop Laboratories. The inorganic salts were of standard ACS quality.

Results. As shown in Table I and Fig. 1, relatively little c-AMP-^{14}C was formed by lipocytes within 30 min when metal was not added, but the addition of Mg markedly increased it. Although Ca caused small increments, it significantly inhibited the amount of stimulation by Mg (Table II). When Zn was added, with or without Mg, the c-AMP-^{14}C levels were much lower than when the cells were incubated without added metal. Potassium addition did not alter the amount of c-AMP-^{14}C whether used alone, or with Mg, or with Mg + Ca (Tables I and II, Fig. 1). When isopropylnorepinephrine was incubated without added metal no effect was observed (Fig. 1), but it augmented the increments produced by Mg, and Mg + K (Tables I and II, Fig. 1). It did not influence significantly the effects caused by Ca, Zn, Mg + Ca, or Mg + Ca + K. It produced essentially no increment with K and Mg + Zn. The in-

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TABLE I. Effects of Metals and Hormones on c-AMP-^{14}C Production.
[Control[a] (no added metal): = 0.17 ± 0.13 nmoles of c-AMP-^{14}C]

Metals 1	c-AMP-^{14}C (nmoles) 2	Δ Metals and control 3	p 4	Δ Isopropylnorepinephrine + metals and metals alone 5	p 6	Δ Insulin + metals and metals alone 7	p 8
Mg	0.71 ± 0.27	0.54 ± 0.22[b]	<.0005	0.87 ± 0.52	<.0005	−0.09 ± 0.08	<.005
Ca	0.47 ± 0.16	0.24 ± 0.13	<.0005	0.04 ± 0.07	NS	0.03 ± 0.04	NS
Zn	0.05 ± 0.04	−0.07 ± 0.09	<.005	0.03 ± 0.03	NS	−0.01 ± 0.05	NS
K	0.15 ± 0.08	−0.08 ± 0.09	NS	0.07 ± 0.06	<.05	−0.04 ± 0.09	NS
Mg + Ca	0.40 ± 0.16	0.17 ± 0.22	<.005	0.23 ± 0.23	NS	0.08 ± 0.18	NS
Mg + Zn	0.01 ± 0.01	−0.11 ± 0.09	<.005	0.02 ± 0.03	<.05	−0.01 ± 0.01	NS
Mg + K	0.93 ± 0.17	0.70 ± 0.05	<.0005	0.99 ± 0.66	<.005	−0.11 ± 0.10	NS
Mg + Ca + K	0.39 ± 0.21	0.16 ± 0.31	<.025	0.06 ± 0.08	NS	0.03 ± 0.06	NS
Ca + K	0.38 ± 0.13	0.14 ± 0.24	<.025	0.00 ± 0.16	NS	0.00 ± 0.16	NS

[a] Twelve tests were conducted with the control and the Mg groups and 6 with the others. Column 1 shows the metals added to the incubation mixture. Column 2 demonstrates the nmoles of c-AMP-^{14}C formed per 0.15 ml of lipocytes/30 min, ± SD. Column 3 represents the effect of metals; Column 5, the effect of isopropylnorepinephrine + metals; Column 7, the effect of insulin + metals. Columns 4, 6, and 8 show the representative p values (NS = nonsignificant).

[b] The experiments were performed in duplicate or triplicate, but because of day-to-day variation in results, calculations were made of daily mean differences between the control and test groups. The p-values were derived by analysis of variance and the F test.

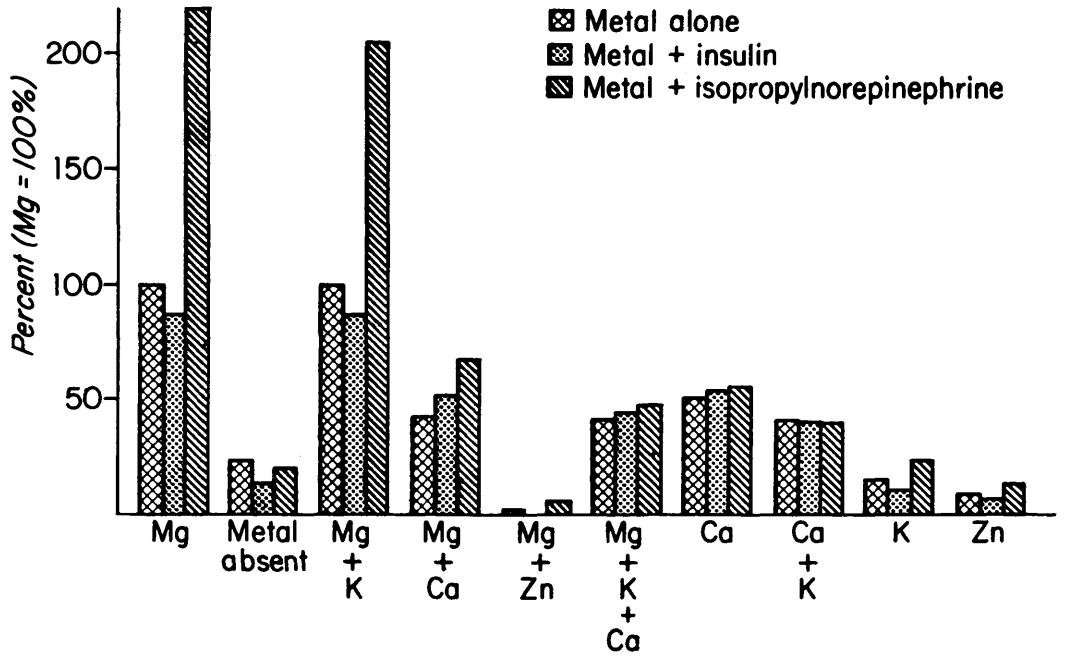

FIG. 1. The effect of Mg upon lipocyte c-AMP-^{14}C is given a value of 100%, and the relative effects of other metals and of insulin or isopropylnorepinephrine are shown. The primary data are shown in Table I.

TABLE II. Effect of Metals and Hormones on c-AMP-^{14}C Production. (nmoles/0.15 ml of lipocytes during 30 min)

	Δ Control and added metals	p
Mg (control):[a] 0.71 ± 0.27		
+ Ca	−0.53 ± 0.13[b]	<0.005
+ K	0.00 ± 0.07	NS
+ Zn	−0.48 ± 0.09	<0.0005
+ Ca + K	−0.54 ± 0.24	<0.0005
Mg + INE[c] (control):[a] 1.58 ± 0.65		
+ Ca	−1.49 ± 0.45[b]	<0.0005
+ K	−0.20 ± 0.72	NS
+ Zn	−1.01 ± 0.13	<0.0005
+ Ca + K	−1.67 ± 0.53	<0.0005
Mg + Insulin (control):[a] 0.62 ± 0.24		
+ Ca	−0.32 ± 0.26[b]	<0.0005
+ K	0.02 ± 0.16	NS
+ Zn	−0.44 ± 0.07	<0.0005
+ Ca + K	−0.38 ± 0.20	<0.0005

[a] $n = 12$; $n = 6$ in all other experiments.
[b] See footnote *b* for Table I.
[c] Isopropylnorepinephrine.

crement produced by Mg + isopropylnorepinephrine was decreased by Ca, Ca + K, and Zn (Table II), but it was not influenced by K. Experiments using a smaller concentration of Zn (1×10^{-5} *M*) caused a 50% reduction in the effects of Mg (results not presented in tables or figure). These concentrations of Zn were much larger than resulted from the addition of crystalline zinc insulin (ca. 3×10^{-10} *M*).

Insulin caused a very slight decrease in the effect of Mg (Table I), but it did not influence the results obtained with the other metals. The increments found with Mg + insulin were reduced by Ca, Ca + K, and Zn (Table II); K had no significant effect. The NaF produced no changes when no other metal was added. NaF + Ca caused essentially the same increments as Ca. The NaF caused a tenfold increase when used with Mg, and small increases with Mg + Ca (Table III).

The EGTA, a cation chelating compound with preferential affinity for Ca, significantly decreased the calcium effect, but increased the increments produced by Mg, and Mg + Ca (Table III). When lipocytes were preincubated with Ca for 10 min, washed 3 times with Tris-glycerophosphate buffer, and then incubated in the standard manner with Mg, no definite effect of the Mg was produced. With similarly prepared cells, Mg + EGTA produced significant increments, but much less than Mg + EGTA used with cells and preincubated with Ca.

Discussion. The results demonstrate that some of the metals, particularly Mg, Ca, and Zn exert a marked effect on the formation of c-AMP-^{14}C by lipocytes. The concentration of c-AMP depends largely upon the balance between adenyl cyclase and phosphodiesterase activity. With the methods used (for publication, *Metabolism*) in these experiments over 80% of the phosphodiesterase activity is inhibited by the concentration of aminophylline used. Thus, consideration must be given to the increases in c-AMP-^{14}C being due to stimulation of adenyl cyclase and/or decreases to its inhibition. The Mg has been reported to stimulate adenyl cyclase (9) and it increases lipolysis in adipose tissue homogenate (10) when c-AMP and ATP are added to the incubation mixture; results are similar

TABLE III. Effect of Mg, Ca, and F on c-AMP-^{14}C Production.[a]

Compounds tested	c-AMP-^{14}C (nmoles)	Δ
Control[b]	0.236 ± 0.056	0
NaF	0.236 ± 0.036	
Mg	0.712 ± 0.152	6.790[c]
Mg + NaF	7.502 ± 1.453	
Ca	0.405 ± 0.067	−0.043
Ca + NaF	0.362 ± 0.045	
Ca + Mg	0.328 ± 0.033	0.587[c]
Ca + Mg + NaF	0.915 ± 0.200	
Ca	0.405 ± 0.067	−0.364[c]
Ca + EGTA	0.041 ± 0.033	
Mg	0.712 ± 0.152	0.439
Mg + EGTA	1.151 ± 0.079	
Mg + Ca	0.328 ± 0.033	0.355[c]
Mg + Ca + EGTA	0.683 ± 0.036	

[a] See footnotes in Table I.
[b] $n = 5$ for each group, except for EGTA tests where $n = 3$.
[c] $p < 0.0005$, others nonsignificant.

when Ca is used instead of c-AMP. The degree of effectiveness of Mg in increasing c-AMP formation depends upon its concentration and its ratio to ATP (submitted for publication). The Mg affects many other enzyme actions. For example, it activates certain adenosine triphosphatases (11) and, indeed, all enzymes that catalyze the transfer of phosphorus from ATP, or from another phosphorylated compound to ADP (12). It stimulates phosphodiesterase activity (13). The Mg forms a 1:1 complex with ATP in a physiologic pH (12). Although rat adipose tissue has been reported (14) not to contain Mg, it seems probable that a small amount is present.

Although Ca increased the formation of c-AMP-^{14}C it decreased the increments produced by Mg; the reason for the inhibitory effect upon the Mg action has not been shown. Presumably, Ca becomes firmly bound to the cell membrane and as its concentration increases the intracellular Mg concentration tends to decrease (15). Although some net effects of Ca and Mg are similar there apparently are important differences in some of their mechanisms of action. The Ca activates some adenosine triphosphatases; in certain instances its activating effect is specific, as is the case with Mg (11). The Ca potentiates lipolysis in adipose tissue (10, 16). A decrease in Ca markedly decreases the lipolytic response to ACTH, but not to epinephrine (17); Mg or K can be used to restore the lipolytic response to ACTH.

Some experimental conditions suggest that K influences adenyl cyclase activity in adipose tissue and muscle. The K was reported to be the most active of a series of cations studied (18) in promoting lipolysis in rat epididymal fat pads. However, the concentration used (150 meq/liter) was more than three times that found (14) in normal adipose tissue, and much larger than the concentration used in this paper (4.0 meq/liter). Under certain conditions (19) the amount of adenyl cyclase activity has been reported to decrease upon removal of K from the incubation medium (Krebs–Ringer bicarbonate); there also was a decrease in hormone-stimulated lipolysis. Addition of ouabain to the incubation medium produced effects upon lipolysis and c-AMP concentrations which were similar to ones with K-lack (19); indeed, the ouabain effect probably resulted from its capacity to decrease intracellular potassium. Since in these reported experiments smaller quantities of K (2.95 m*M*) were used than in our studies, the effect of other ions upon K action must be considered since they used Krebs–Ringer bicarbonate buffer. High concentrations of K (145 meq/liter) have been shown (20) to increase the concentration of c-AMP in muscle. The K becomes strongly bound to mitochondria and the amount tends to be related to the concentration of ATP (21). Adenosine triphosphatase is one of the enzymes activated by K (22, 23).

That metals are important in the formation of c-AMP is illustrated by the frequent usage of EDTA to stop reactions that affect c-AMP levels. The mechanism by which Mg increases the rate of conversion of ATP-^{14}C to c-AMP-^{14}C is not established. Its activation of adenyl cyclase must be marked since it is known also to increase phosphodiesterase activity (13).

The pattern for isopropylnorepinephrine stimulation of adenyl cyclase is related to the presence of Mg; this hormone's augmentary effect is abolished by the presence of Ca. Under most of the experimental conditions insulin exerted no effect, but under other conditions it has decreased the levels of c-AMP (5,6). Since Ca also decreases the Mg effect despite the capacity of Ca to stimulate adenyl cyclase, the Ca pump may be considered to antagonize the intracellular fixation of Mg. The mechanism for the marked inhibition of adenyl cyclase by Zn is unknown. Moreover, the means by which NaF markedly increase the formation of c-AMP have not been elucidated.

Summary. Investigations with isolated lipocytes were conducted concerning the effects of Mg, Ca, Zn, and K upon the formation of c-AMP-^{14}C from ATP-^{14}C. The Mg markedly increased the production of c-AMP-^{14}C. The Ca stimulated a smaller increment, but reduced that caused by Mg. When the cells were preincubated with Ca, and then

repeatedly washed with Tris-glycerophosphate buffer, no effect was produced by Mg unless EGTA was also used, suggesting a stronger affinity of the lipocytes for Ca than for Mg. A similar level of c-AMP-^{14}C was found whether Ca was used alone, or in each combination tested with Mg, isopropylnorepinephrine, K, or insulin. The Zn markedly reduced the increments of c-AMP-^{14}C when used alone or with Mg. The K alone had no effect and influenced the results with Ca and/or Mg relatively little. The NaF, used alone or with Ca, produced no changes, but caused tenfold increments with Mg. Isopropylnorepinephrine had no effect alone, or with Ca or Zn, but it produced marked additional increments when used with Mg. Insulin reduced very slightly the Mg action but did not affect the action of the other metals.

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1. Sutherland, E. W., Øye, I., and Butcher, R. W., Recent Progr. Hormone Res. **21**, 623 (1965).

2. Sutherland, E. W. and Robison, G. A., Pharmacol. Rev. **18**, 145 (1966).

3. Butcher, R. W., Pharmacol. Rev. **18**, 237 (1966).

4. Butcher, R. W. and Sutherland, E. W., Ann. N. Y. Acad. Sci. **139**, 849 (1967).

5. Butcher, R. W., Ho, R. J., Meng, H. C., and Sutherland, E. W., J. Biol. Chem. **240**, 4515 (1965).

6. Jungas, R. L. and Ball, E. G., Proc. Nat. Acad. Sci. U. S. **56**, 757 (1966).

7. Rabinowitz, M., Desalles, L., Meisler, J., and Lorand, L., Biochim. Biophys. Acta **97**, 29 (1965).

8. Rodbell, M., J. Biol. Chem. **239**, 375 (1964).

9. Sutherland, E. W. and Rall, T. W., J. Am. Chem. Soc. **79**, 3608 (1957).

10. Rizack, M. A., J. Biol. Chem. **239**, 392 (1964).

11. Rostgaard, J. and Behnke, O., "Intracellular Transport," Warren, K. B., ed., Vol. 5, p.103. Academic Press, N. Y., 1966.

12. Wacker, W. E. C. and Vallee, B. L., "Mineral Metabolism," Comar, C. L. and Bronner, F., eds, Vol. 2A, p.483. Academic Press, New York 1964.

13. Drummond, G. I. and Perrott-Yee, S., J. Biol. Chem. **236**, 1126 (1961).

14. Martin, H. E., Tranquada, R. E., Beigelman, P. M., and Jones, R., Metab., Clin. Exptl. **11**, 993 (1962).

15. Williams, R. J. P. and Wacker, W. E. C., J. Am. Med. Assoc. **201**, 18 (1967).

16. Bleicher, S. J., Farber, L., Lewis, A., and Goldner, M. G., Metab., Clin. Exptl. **15**, 742 (1966).

17. Lopez, E., White, J. E., and Engel, F. L., J. Biol. Chem. **234**, 2254 (1959).

18. Bleicher, S. J., Lewis, A., Farber, L., and Goldner, M. G., Proc. Soc. Exptl. Biol. Med. **121**, 980 (1966).

19. Ho, R. J., Jeanrenaud, B., Posternak, Th., and Renold, A. E., Biochim. Biophys. Acta **144**, 74 (1967).

20. Lundholm, L., Rall, T., and Vamos, N., Acta Physiol. Scand. **70**, 127 (1967).

21. Ling, G. N., "A Physical Theory of the Living State: the Association-Induction Hypothesis," Ginn (Blaisdell), New York, 1962.

22. Skou, J. C., Physiol. Rev. **45**, 596 (1965).

23. Kernan, R. P., "Cell K," p.121. Butterworths, London and Washington, D. C., 1965.

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