Bioresearch Communications
Volume 04, Issue 02, July 2018
Journal Homepage: www.bioresearchcommunications.com

Original Article

Epinephrine Inhibition of Pyruvate Carboxylation In Adipose Tissue

Rafiqur Rahman[1] and Robert L. Jungas[2]

[1]Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh, [2]Robert L. Jungas, Department of Cell Biology, University of Connecticut Health Center Farmington, Ct. 06032, USA

ABSTRACT: One µM epinephrine inhibited the incorporation of $H_{14}CO_3^-$ into protein by rat adipose tissue 50-60%, while it inhibited protein synthesis measured by the incorporation of tritiated water into protein by only 40%. The inhibition was blocked by either DL-propranol or by insulin. Epinephrine inhibited the incorporation of $H^{14}CO_3^-$ into acid-soluble products by 37%. A much greater inhibitory effect (76%) was observed on pyruvate carboxylation catalyzed by the infranatant fraction of homogenates prepared from adipocytes previously exposed to the hormone for 15 min. Freeze-thawing of the infranatant to rupture the mitochondrial membranes did not reduce the extent of inhibition, indicating that the hormonal effect was not on pyruvate transport. Addition of both ATP (5 mM) and acetyl CoA (0.25 mM) to the freeze-thawed mitochondrial suspensions increased pyruvate carboxylation forty-fold, and totally abolished the inhibitory effect of epinephrine treatment. It is suggested that the inhibitory action of epinephrine on bicarbonate fixation seen in intact cells and in mitochonrial suspensions results from the ability of the hormone to activate lipolysis, increase the level of uncoupling fatty acids in the cell, and therefore lower cellular ATP levels.

Keywords: bicarbonate, insulin, adipocytes, tritiated water, CO_2 fixation, protein synthesis, ATP

Article History
Received: 05 March 2018
Accepted: 08 May 2018

Scan the QR code to see the online version or,visit- www.bioresearchcommunications.com

Corresponding author
Rafiqur Rahman
University of Dhaka
Email: rrahman629@yahoo.com

Citation: Rahman R., Robert L.J., Uddin K. Epinephrine Inhibition of Pyruvate Carboxylation In Adipose Tissue. Biores Comm. V4-(2) 516-522.

INTRODUCTION

It has been well established that insulin activates the multi-enzyme system pyruvate dehydrogenase in rat adipose tissue mitochondria[1–5].This multi-enzyme system is more responsive to insulin activation when tissue segments are incubated in Krebs-Ringer bicarbonate (KRB) buffer than when incubated in Krebs-Ringer phosphate buffer[6]. The investigation of this enhanced responsiveness to insulin in KRB buffer led to the discovery that insulin doubled the rate of incorporation of labeled bicarbonate into rat adipose tissue protein[7]. However, the effect of insulin on the rate of protein synthesis was only one third as great as its effect on $H^{14}CO_3^-$ incorporation into protein. The pattern of labeling observed in the free amino acid pool when tissue was incubated with $H_{14}CO_3^-$ indicated that carboxylation occurred primarily at the pyruvate carboxylase step, but no activation of that mitochondrial enzyme could be demonstrated after treatment of either the tissue or the cells with the hormone.

Mitochondria isolated from tissues exposed to insulin retained the ability to fix carbon dioxide more rapidly, but this effect was lost in sonicated or freeze-thawed mitochondrial preparations[7]. Pyruvate uptake by intact mitochondria isolated from cells exposed to insulin could be shown to be increased. It was concluded from these observations that insulin's activation of $H_{14}CO_3^-$ metabolism could in part be explained by an effect of the hormone on the transport of pyruvate across the inner mitochondrial membrane. In the present paper

we describe the effects of epinephrine on these aspects of $H_{14}CO_3^-$ metabolism.

MATERIALS AND METHODS

Collagenase (Cl. Histolyticum), insulin (bovine pancreas), epinephrine bitartrate, dibutyrylcyclic-AMP (DBcAMP), DL-propranolol, isoproterenol, adenosine, acetyl-CoA, EDTA, and EGTA were all purchased from Sigma Chemical Company. Radiochemicals sodium bicarbonate, $H_{14}CO_3^-$, and tritiated water, ^3H_2O, were purchased from ICN. Bovine serum albumin, fraction V, "fatty acid free", (BSA) was purchased from Miles laboratories. The albumin was further defatted according to the method of Chen[8].

Male rats were obtained from Charles River Breeding Laboratories, North Wilmington, Mass. and maintained as previously described[7]. Rats in the weight range 150–250g were selected for all experiments. Epididymal fat bodies were excised and the thinner portions divided longitudinally into two segments as before. Two segments, one from the left side, one from the right side of a different rat, were placed into each incubation vessel, yielding four groups of paired segments from each pair of rats. Tissues were incubated in Krebs-Ringer's bicarbonate (KRB) medium nominally containing 25 mM $H^{14}CO_3^-$,11 mM fructose, and in some cases, ^3H_2O. Flasks were gassed for 5 min with air:CO_2 (95:5), preincubated for 30 min at 37° to establish basal conditions and the tissues then transferred to fresh media for the experimental incubation period. Incubations were terminated and radioactivity in the acid-soluble and acid-insoluble products was measured essentially as described previously[7].

Fat cells were prepared according to Rodbell's procedure[9]. The isolated cells were suspended in Krebs-Ringer bicarbonate buffer containing 3% defatted and dialyzed BSA and 11 mM fructose. Cells were preincubated for 10 min in 25 ml Nalgene plastic erlenmeyers followed by the experimental incubation of 5-60 min. Following incubation, the fat cell suspensions were transferred to polycarbonate centrifuge tubes, and centrifuged at 1000 rpm for 1 min. This separated the cells as a fatty layer on top of the infranatant fluid which was siphoned off with a disposable pipette and discarded. The fat cells thus obtained were washed twice with buffered sucrose (0.30 M sucrose, tris 5 mM, EDTA 1 mM, pH 7.3). The adipocyte suspensions were transferred to glass centrifuge tubes and vortexed vigorously in an Arthur Thomas Super Mixer for 30 sec in order to break the cells, then centrifuged for 30 sec at 800 x g_{ave} in a clinical centrifuge. The infranatant fluid was recovered with disposable pipettes and stored in an ice-bath until used. Portions of these infranatants were frozen in a dry ice-alcohol bath, then thawed in running tap water in order to rupture the mitochondria. When needed, more purified mitochondrial suspensions were prepared according to Method II described previously[7].

RESULTS

The effect of 1 μM epinephrine on $H^{14}CO_3^-$ incorporation into protein by rat epididymal adipose tissue segments is shown in Table 1.

TABLE 1: Effect of epinephrine on $H^{14}CO_3^-$ incorporation into protein

Additions	cpm•g^{-1}•h^{-1}	Percent Inhibition
Control	7,300 ± 750	---
Epinephrine [1 μM]	3,500 ± 650	52
Epinephrine + propranolol [100μM]	5,840 ± 650	20
Epinephrine + phentolamine [200μM]	3,150 ± 360	59
Insulin [1 mUnit/ml]	13,700 ± 600	---
Insulin + epinephrine [1 μM]	5,840 ± 200	57

Adipose tissue segments were incubated in Krebs-Ringer bicarbonate (25 mM, 5 μCi/ml) containing 11 mM fructose for 1 hr. Figures are means ± SEM of 4 measurements.

Inhibition in the range of 50-60% was observed which was largely overcome by the addition of the β-blocker DL-propranolol (100 μM) while the α-blocker phentolamine (200 μM) had no effect. This suggests that the hormonal effect was mediated through β-adrenergic receptors, adenylate cyclase activation, and elevated cellular cAMP levels. Elevations in cAMP have been implicated in the inhibition of fat cell protein synthesis by others[10,11]. Accordingly,

antilipolytic agents known to lower intracellular cAMP levels in fat cells were tested. Adenosine (100 µM), nicotinic acid (100 µM), and prostaglandin E_1 (25 µM) while having very little effect in themselves, substantially inhibited the action of epinephrine (results not shown). Insulin (1 mU/ml) had a more complex effect. As shown previously[7], and confirmed in Table 1, insulin by itself approximately doubled labeled bicarbonate incorporation into protein. Epinephrine's inhibitory effect expressed as a percentage was the same in the presence of insulin; viewed another way, insulin was as effective as DL-propranolol in blocking the action of epinephrine.

In order to distinguish hormonal effects on net protein synthesis rates from their effects on $H_{14}CO_3^-$ incorporation into the amino acid precursors, studies were done with both $H_{14}CO_3^-$ bicarbonate and ^3H_2O present in the incubation medium. The use of tritiated water to measure protein synthesis rests on the assumption that the hydrogen atoms of many amino acids in the cellular pool will rapidly exchange with the hydrogens of water through the action of aminotransferases and other enzymes utilizing pyridoxal phosphate, and that this exchange will cease once the amino acids are incorporated into aminoacyl-tRNA or protein[7]. The data are presented in Table 2.

TABLE 2: Effect of epinephrine on $H^{14}CO_3^-$ and ^3H_2O incorporation into protein

Additions	^{14}C cpm•g^{-1}•h^{-1}	^3H cpm•g^{-1}•h^{-1}
Control	2,800 ± 200	4,700 ± 1200
Epinephrine [1 µM]	1,200 ± 100	2,800 ± 700
Percent inhibition	57	39

Adipose tissue segments were incubated in Krebs-Ringer bicarbonate (25 mM, 5 µCi/ml) containing 1 mCi/ml ^3H_2O and 2 mg/ml fructose for 1 hr. Figures are means ± SEM of 4 measurements.

The inhibition by epinephrine of tritium incorporation into TCA-insoluble products was only 39 percent compared to 57 percent for $H_{14}CO_3^-$ incorporation.

The larger inhibitory effect of epinephrine on $H_{14}CO_3^-$ incorporation into protein suggested that the hormone was inhibiting other metabolic steps of $H_{14}CO_3^-$metabolism in addition to inhibiting protein synthesis. We therefore examined the effect of the hormone on the incorporation of $H_{14}CO_3^-$ into acid soluble products. It was found utilizing the same conditions as in Table 1, that 1 µM epinephrine reduced the label incorporation from $H_{14}CO_3^-$ into TCA-soluble products from 38,500 ± 3,800 to 24,200 ± 2,500 cpm•g^{-1}•h^{-1}, an inhibition of 37%. The major labeled amino acids produced have been identified[7] and were: glutamate, aspartate, serine, alanine and proline. The primary reaction is assumed to be the mitochondrial carboxylation of pyruvate yielding oxaloacetate which is then converted into these amino acids. The inhibitory effect of epinephrine on $H_{14}CO_3^-$ incorporation into acid-soluble products was found to be much more pronounced when pyruvate carboxylation was examined in an 800 x g_{ave} infranatant obtained from an homogenate of adipocytes which had been exposed to epinephrine for 15 min. These results are presented in the first line of Table3.

TABLE 3: Pyruvate carboxylation by fat cell infranatants

Infranatant Treatment	Additions to Infranatant	Additions to Adipocytes		Percent Inhibition
		Control	Epinephrine	
		cpm/mg protein		
None	None	7,400 ± 600	1,800 ± 200	76
Freeze-Thawed	None	15,300 ± 700	1,900 ± 150	88
Freeze-Thawed	ATP, 5 mM	63,400 ± 7,900	34,800 ± 4,600	45
Freeze-Thawed	AcCoA, 0.25 mM	21,800 ± 2,400	5,900 ± 1,200	73
Freeze-Thawed	ATP + AcCoA	638,000 ± 62,000	599,000 ± 51,000	N.S.

Adipocytes were incubated 15 min at 37° in krebs-Ringer bicarbonate containing 11 mM fructose with or without epinephrine (1µM). Cells were then homogenized in buffered sucrose and the 800 x g_{ave} infranatant fluid was incubated 20 min at 30° with pyruvate (5mM) and $H^{14}CO_3$- (12.5 mM, 10 µCi/ml). After stopping the reaction with trichloroacetic acid, radioactivity in the acid-soluble fraction was measured and expressed per mg of infranatant protein. Figures are means ± SEM of 16 (no treatment) or 8 (freeze-thawed) paired measurements.

Prior exposure of the cells to the hormone inhibited the carboxylation of pyruvate in the infranatants by 76 percent. Addition of 1 µM DBcAMP to the cells mimicked epinephrine action, while the further addition of the β-blocker DL-propranolol blocked the inhibitory effect of epinephrine completely (data not presented). In order to examine whether the inhibitory effect of epinephrine revealed in the cell extracts was achieved through inhibition of pyruvate transport across mitochondrial membranes, the effect was studied in preparations of ruptured mitochondria. As shown in the second line of Table 3, subjecting the 800 x g_{ave} infranatant to repeated freeze-thawing cycles doubled the rate of pyruvate carboxylation in the controls, and it increased the inhibitory effect of epinephrine to 88%. This suggested that the availability of the substrate pyruvate may have been limiting the rate of carboxylation by the intact mitochondria, but that pyruvate translocation was not a factor in the inhibition observed. Similar results were obtained when the mitochondria were ruptured by sonication rather than by freeze-thawing. A direct measurement of the rate of pyruvate transport into intact mitochondria according to the procedures described earlier[7], also failed to reveal any effect of epinephrine on the pyruvate uptake process (data not presented).

It appeared, therefore, that epinephrine was inhibiting either the carboxylation step itself or subsequent metabolic steps in the CO_2 fixation process. To make the extent of the incorporation of $H^{14}CO_3^-$ bicarbonate into acid soluble products by the ruptured mitochondrial suspensions a better reflection of their pyruvate carboxylase activity, the infranatants were supplemented with excess ATP. Adding 5 mM ATP caused a four-fold increase in bicarbonate fixation in the controls, but nearly a twenty-fold increase in the infranatants prepared from epinephrine-treated cells (line 3, Table 3). This result is consistent with there being a greater lack of ATP in the hormone-treated preparation. When 0.25 mM acetyl CoA, the allosteric activator of pyruvate carboxylase, was added to the infranatants it had an effect similar to that of ATP, though lesser in magnitude. By far the greatest effect was seen when both ATP and acetyl CoA were added. Bicarbonate fixation in the control preparations was increased 40-fold, and in the hormone-treated case by 300-fold. Indeed, the inhibitory effect of epinephrine was now completely abolished. Apparently, the action of epinephrine on bicarbonate fixation into acid-soluble substances results mainly from a change in the concentration of the substrates and activators of the carboxylase rather than from an effect on the catalytic properties of the enzyme.

DISCUSSION

The enzymatic reaction of bicarbonate with pyruvate is quantitatively the most important process involved in CO_2 fixation in most animal tissues. Because of the operation of the respiratory chain proton pumps, the mitochondrial matrix is a more alkaline compartment than the cytoplasm and, having therefore a much higher concentration of bicarbonate, is the obvious location for pyruvate carboxylase. It is well known that pyruvate carboxylase requires as a third substrate the magnesium-chelated form of ATP, and that carboxylation is promoted by the allosteric activator, acetyl CoA[12-14].

In the liver, the dominant function of pyruvate carboxylase lies in the formation of oxaloacetate to be used in the metabolic process of gluconeogenesis. Indeed, during gluconeogenesis from protein, some two-thirds of the four-carbon fragments eventually flowing to glucose are supplied by the pyruvate carboxylation reaction[15]. In adipose tissue, however, the major role of pyruvate carboxylase is in the metabolic process of lipogenesis. During lipogenesis from glucose, the flux through the mitochondrial pyruvate carboxylase is approximately equal to the flux through the cytoplasmic $NADP^+$–linked malate dehydrogenase[16,17]. The latter is a major source of the cytoplasmic NADPH utilized in fatty acid synthesis. Given these very different functions, it is to be expected that the regulation of the carboxylase by hormones will be quite different in liver compared to adipose tissue.

In contrast to the inhibitory effects reported here, epinephrine along with glucagon and DBcAMP have been shown to stimulate pyruvate carboxylation and gluconeogenesis in intact animal[18], perfused liver[19] and in hepatocytes[20, 21]. Mitochondria isolated from liver cells exposed to glucagon, epinephrine or adenosine 3',5`–cyclic monophosphate (cAMP) exhibited a 2– to 3–fold enhanced rate of pyruvate carboxylation[22]. Insulin by itself was reported to have no effect, but it diminished the stimulatory effects of glucagon and catecholamines. In those studies no direct effect of the agonists could be demonstrated on the activity of pyruvate carboxylase itself. The increased rate of carboxylation was attributed to hormonal stimulation of the pyruvate transporter in the inner mitochondrial membrane[23].

In our hands it was insulin rather than glucagon which stimulated the pyruvate transporter of fat-cell mitochondria[7]. As reported here, we found epinephrine to have no effect either on the transporter or on the activity of pyruvate carboxylase, though it clearly did inhibit pyruvate carboxylation in adipose tissue segments, adipocytes, or cell homogenate infranatants. A stimulation of pyruvate carboxylation by epinephrine as seen in the liver, would be inconsistent with the functions of the hormone in adipose tissue, namely, increasing lipolysis and decreasing lipogenesis. We have not been able to detect any effect of glucagon alone on bicarbonate incorporation either into TCA–soluble or TCA–insoluble products in adipose tissue. However, glucagon did counteract the stimulatory effects of insulin on bicarbonate incorporation into TCA–insoluble products (data not presented).

How then does epinephrine exert its inhibitory effect in adipose tissue? It has been reported repeatedly that epinephrine treatment of adipocytes decreases their ATP content[24-26]. This has been attributed to an uncoupling of oxidative phosphorylation caused by fatty acids elevated in response to the lipolytic hormone. All of the results reported here are consistent with epinephrine's inhibitory effect on bicarbonate fixation being a consequence of a reduction in the cellular ATP/ADP ratio[27, 28].

Jarett *et al.*[11] reported that although insulin caused no change in either basal or epinephrine-stimulated cAMP levels, it nevertheless overcame completely the inhibitory effect of epinephrine on amino acid incorporation into protein. This is similar to the effect of insulin reported here on epinephrine's inhibition of bicarbonate incorporation into protein.

A further comment may be in order on the use of tritiated water to measure rates of protein synthesis. When we used this method in an earlier paper, we stated that the method "has considerable promise", but that further work was needed to document the stoichiometry of the exchange of tritium from water into amino acid hydrogen atoms. Recent work by Previs and his colleagues[29,30] has now demonstrated that label from deuterated water exchanges virtually completely with both the alpha- and beta-hydrogens of alanine within 20 minutes in rats in vivo, which was the earliest time period examined. Thus the assumption that rapid hydrogen exchange occurs between water and amino acid hydrogen atoms during reactions involving pyridoxal phosphate, such as amino acid transamination, decarboxylation, racemization, β-elimination, β-condensation, and γ-elimination has been partially verified. Most such reactions may be largely confined to the liver, but a

sufficient number occur in adipose tissue to make the method useful in this tissue as well.

ACKNOWLEDGEMENTS

This work was supported by a Grant AM22142 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

REFERENCES

1. Jungas RL (1970) Effect of insulin on fatty acid synthesis from pyruvate, lactate, or endogenous sources in adipose tissue: *evidence for the hormonal regulation of pyruvate dehydrogenase*, Endocrinology. **86**, 1369-1375
2. Jungas RL (1971) Hormonal regulation of *pyruvate dehydrogenase*, Metabolism. **20,** 43-53
3. Weiss L, Loffler G, Schirmann A, Wieland O (1971). Control of pyruvate dehydrogenase interconversion in *adipose tissue by insulin*, FEBS Lett. **15,** 229-231
4. Coore HG, Denton RM, Martin BR, Randle PJ (1971) Regulation of adipose tissue pyruvate dehydrogenase by *insulin and other hormones*, Biochem J. **125**, 115-127
5. Mukherjee C, Jungas RL (1975) Activation of pyruvate dehydrogenase in adipose tissue by insulin: *evidence for an effect of insulin on pyruvate dehydrogenase phosphate phosphatase*, Biochem J. **148**, 229-235
6. Taylor SI, Mukherjee C, Jungas RL (1973) Studies on the mechanism of activation of adipose tissue pyruvate dehydrogenase by insulin, J Biol Chem. **248**, 73-81
7. Rahman R, O'Rourke F, Jungas RL (1983) Effects of insulin on CO_2 fixation in adipose tissue: *evidence for regulation of pyruvate transport*, J Biol Chem. **258,** 483-490
8. Chen RF (1967) Removal of fatty acids from serum albumin by charcoal treatment, J Biol Chem. **242,** 173-181
9. Rodbell M (1964) Metabolism of isolated fat cells, I. *Effects of hormones on glucose metabolism and lipolysis*, J Biol Chem. **239,** 375-380
10. Minemura T, Lacy WW, Crofford OB (1970) Regulation of the transport and metabolism of amino acids in isolated fat cells*: effect of insulin and a possible role for adenosine 3',5'-monophosphate*, J Biol Chem. **245,** 3872-3881
11. Jarett L, Steiner AL, Smith RM, Kipnis (1972) DM The involvement of cyclic AMP in the hormonal regulation of protein synthesis in rat adipocytes, Endocrinology. **90,** 1277-1284
12. Utter MF, Keech DB, Scrutton MC(1964) A possible role for acetyl CoA in the control of gluconeogenesis, Adv Enzyme Regul. **2,** 49-68
13. Patel MS, Hanson RW (1970) Carboxylation of pyruvate by *isolated rat adipose tissue mitochondria*, J Biol Chem. **245,** 1302-1310
14. Martin BR (1971) Denton RM. Metabolism of pyruvate and malate *by isolated fat-cell mitochondria*, Biochem J. **125,** 105-113
15. Jungas RL, Halperin ML, Brosnan JT (1992) Quantitative analysis of amino acid oxidation and related gluconeogenesis in humans, Physiol Rev. **72,** 419-448
16. Rognstad R, Katz J (1966) The balance of pyridine nucleotides and ATP in adipose tissue, Proc Natl Acad Sci USA; **55**, 1148-1156
17. Flatt JP (1970) Conversion of carbohydrate to fat in adipose tissue*: an energy-yielding and, therefore,* self-limiting process, J Lipid Res. **11,** 131-143
18. Adam PAJ, Haynes RC (1969) Control of hepatic mitochondrial CO_2 fixation by glucagon, epinephrine, and cortisol, J Biol Chem. **244,** 6444-6450
19. Exton JH, Park CR (1968) Control of gluconeogenesis in liver, II. Effects of glucagon, catecholamines, and adenosine 3',5'-*monophosphate on gluconeogenesis in the perfused rat liver*, J Biol Chem. **243,** 4189-4196
20. Johnson MEM, Das NM, Butcher FR, Fain JN (1972) The regulation of gluconeogenesis in isolaated rat liver cells by *glucagon, insulin, dibutyryl cyclic adenosine monophosphate*, and fatty acids, J Biol Chem. **247,** 3229-3235
21. Chisholm AB, Allan EH, Titheradge MA (1983) Regulation of mitochondrial pyruvate carboxylation in *isolated hepatocytes by acute insulin treatment*, Biochem J. **214,** 451-458
22. Garrison JC, Haynes RC (1975) The hormonal control of gluconeogenesis by regulation of the mitochondrial pyruvate carboxylation in *isolated rat liver cells*, J Biol Chem. **250,** 2769-2777
23. Halestrap AP, Scott RD, Thomas AP (1980) *Mitochondrial pyruvate transport and its*

hormonal regulation, Int J Biochem. **11,** 97-105
24. Hepp D, Challoner DR, Williams RH (1968) Studies on the action of insulin in isolated adipose tissue cells, I. Stimulation of incorporation of ^{32}*P-labeled inorganic phosphate into mononucleotides in the absence of glucose,* J Biol Chem. **243,** 4020-4026
25. Bihler I, Jeanrenaud B (1970) ATP content of isolated fat cells. *Effects of insulin, ouabain, and lipolytic agents,* Biochim Biophys Acta. **202,** 496-506
26. Angel A, Desai KS, Halperin ML (1971) Reduction in adipocyte ATP by lipolytic agents: *relation to intracellulr free fatty acid accumulation,* J Lipid Res. **12,** 203-213
27. Rognstad R, Katz J (1969) The effect of 2,4-dinitrophenol on adipose-tissue metabolism, *Biochem* J. **111,** 431-444
28. Kopecký J, Flachs P, Bardová K, Brauner P, Prožak T, Šponarová J (2002) *Modulation of lipid metabolism by energy status of adipocytes,* Ann N Y Acad Sci. **967,** 88-101
29. Previs SF, Fatica R, Chandramouli V, Alexander JC, Brunengraber H, Landau BR (2004) Quantifying rates of protein synthesis in humans by use of $^{2}H_2O$: *application to patients with end-stage renal disease,* Am J Physiol Endocrinol Metab. **286,** E665-E672
30. Dufner DA, Bederman IR, Brunengraber DZ, Rachdaoui N, Ismail-Beigi F, Siegfried BA, Kimball SR, Previs SF (2005) Using $^{2}H_2O$ to study the influence of feeding on protein synthesis: *effect of isotope equilibration in vivo vs. in cell culture,* Am J Physiol Endocrinol Metab. **288,** E1277-E1283