Amino Acid Transport and Protein Synthesis in Muscle. Action of Insulin.* (29394)

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It has recently been demonstrated that insulin stimulates the transport of α-aminoisobutyric acid (AIB) into the cells of isolated, intact rat diaphragm muscle when protein synthesis is essentially abolished by addition of puromycin to the system *in vitro* (2,3). These observations suggested that the action of insulin on intracellular amino acid accumulation(4) is not secondary to an increase in protein synthesis as postulated by Manchester and Young(5). It remained to be determined, however, whether the transport of naturally occurring amino acids responds to insulin in like manner. In the present report, a clear stimulation of proline transport by addition of insulin to intact rat diaphragm *in vitro* under conditions wherein protein synthesis is blocked by puromycin is described.

Methods. Diaphragms were obtained from normal female rats (Charles River strain) weighing 60-75 g and fed Purina Lab Chow and water *ad libitum*. The animals were received from the supplier at approximately 50 g and were maintained in our laboratory for 3-5 days before sacrifice.

"Intact" hemidiaphragms were prepared as previously described(6,7). The tissue was briefly rinsed in chilled Krebs-Henseleit bicarbonate buffer, pH 7.4, blotted lightly on filter paper (Whatman No. 1), and placed in a 50 ml Erlenmeyer flask with 10 ml of Krebs-Henseleit bicarbonate buffer containing glucose (10 mM) and uniformly labeled C^{14} L-proline (0.1 mM; 0.05 μc/ml). The flasks were sealed with serum stoppers and incubated in a water bath at 37°C with constant shaking (112 oscillations/min). During the initial 5 minutes of incubation, the flasks were gassed with 5% CO_2 and 95% O_2. At the conclusion of the incubation period the hemidiaphragm was dissected from the rib cage and adhering tissue. The tissue was uniformly blotted on filter paper, weighed, and homogenized in 3 ml of an 8% trichloroacetic acid solution (TCA) containing 1 mg/ml C^{12} L-proline. Following centrifugation, a 0.5 ml aliquot of the supernatant and of the corresponding incubation medium were counted as previously described(7). Tissue water content was determined by drying to constant weight in a series of separate experiments(8). Extracellular space was estimated in separate experiments by incubating hemidiaphragms in the presence of inulin-1-C^{14} (2.25 mg/100 ml; 0.05 μc/ml) for each control and experimental condition. The results are expressed as the concentration ratio of C^{14} L-proline (cpm/ml intracellular water)/(cpm/ml incubation medium). When indicated, 0.1 unit/ml bovine insulin (Lilly, PJ-4609, 23.8 U/mg) and 500 μg/ml puromycin dihydrochloride were added to the incubation medium.

The TCA precipitate was washed twice with 3 ml of the 8% TCA solution followed by one ethanol-ether (1:1) and one ether (anhydrous) washing. The purified protein was then suspended in 1 ml of ether, transferred to a tared scintillation vial and dried to constant weight. After weighing, the protein was dissolved in 1 ml of p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide (Hydroxide of Hyamine 10 ×) in a 60°C water bath with vigorous shaking for 10-20 minutes. Fifteen ml of 4% Cab-O-Sil in a solution containing 5.0 g 2,5-diphenyloxazole (PPO) and 0.3 g 1,4-bis-2-(5-phenyloxazoyl)-benzene (POPOP) per liter of toluene were added to each vial and all samples counted by liquid scintillation spectrometry. Quenching was corrected for by internal standards. Specific activity of the diaphragm protein was expressed as counts per minute per milligram of protein.

Results. The incorporation of uniformly labeled C^{14} L-proline into diaphragm protein for the various *in vitro* treatments is sum-

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TABLE I. Effect of Insulin and Puromycin on Incorporation of C^{14}-L-proline* into Protein of Intact Rat Hemidiaphragm *in vitro*.

Additions		Time of incubation (min)		
Insulin (0.1 unit/ml)	Puromycin (500 μg/ml)	90	120	180
		Specific activity (cpm/mg protein)†		
—	—	134.6 ± 7.2 (6)	193.3 ± 6.1 (5)	338.0 ± 17.4 (6)
+	—	267.4 ±18.4 (6)	273.4 ±14.9 (6)	480.5 ± 17.7 (6)
—	+	2.1 ± .1 (6)	2.4 ± .1 (5)	3.4 ± .2 (6)
+	+	3.8 ± .2 (6)‡	2.8 ± .4 (6)§	5.4 ± .3 (6)‡

* 0.1 mM (0.05 μc/ml). † Mean ± S.E., No. of observations in parentheses. ‡ $P < 0.001$. § $P > 0.3$.

marized in Table I. Addition of insulin to diaphragm tissue *in vitro*, resulted in the expected stimulation of proline-C^{14} incorporation into protein at the 3 time intervals studied. Puromycin reduced proline incorporation to approximately 1% of that observed in control muscle in the presence or absence of insulin (Table I). In sharp contrast, the stimulation by insulin of proline-C^{14} accumulation in the intracellular water was clearly demonstrable when puromycin was added to the incubation medium in concentrations effecting a 99% reduction in amino acid incorporation into protein (Table II).

Puromycin blockade of protein synthesis alone increased the accumulation of proline in the intracellular water (Table II).

Discussion. The assumption that the radioactivity recovered in the cell water was largely in the form of proline is supported by the finding of Kipnis *et al*(9) and of Akedo and Christensen(4) that more than 90% of the radioactivity added to rat diaphragm preparations as C^{14}-proline is recoverable as such, after periods of incubation similar to the ones employed in this study. It may be concluded, therefore, that the transport of proline, like that of AIB, is stimulated by insulin whether or not protein synthesis is drastically reduced by addition of puromycin to the medium. It should be noted, however, that at 90 and 180 minutes of incubation, when proline incubation into protein was reduced to 1% of control by addition of puromycin, insulin effected a statistically significant increase ($P < .001$) in the specific activity of the protein. Whether any biological significance can be attributed to this small increment in amino acid incorporation is uncertain at the present time. Nevertheless, the data warrant the conclusion that insulin appears to have an action on amino acid transport which is independent of its action on protein synthesis.

The inhibition of protein synthesis by puromycin resulted in a near doubling of the intracellular accumulation of proline. This phenomenon, which has also been observed with valine (unpublished observations), can best be interpreted by a reduced removal of the amino acid from the intracellular pool in the face of an undiminished influx from the extracellular compartment.

Summary. Intact hemidiaphragms from normal female rats (60-75 g) were utilized to study the effect of insulin on C^{14} L-proline

TABLE II. Effect of Insulin and Puromycin on Accumulation of C^{14}-L-Proline* in Intracellular Water of Intact Rat Hemidiaphragm *in vitro*.

Additions		Time of incubation (min)		
Insulin (0.1 unit/ml)	Puromycin (500 μg/ml)	90	120	180
		Concentration ratio†		
—	—	1.13 ± .02 (6)	1.77 ± .02 (5)	1.97 ± .05 (6)
+	—	2.47 ± .10 (6)	2.85 ± .17 (6)	2.67 ± .10 (6)
—	+	1.89 ± .07 (6)	2.73 ± .12 (5)	2.69 ± .11 (6)
+	+	4.27 ± .19 (6)	4.97 ± .11 (6)	4.30 ± .13 (6)

* 0.1 mM (0.05 μc/ml). † Mean ± S.E., No. of observations in parentheses.

transport as well as its incorporation into protein as a function of protein synthesis. Bovine insulin (0.1 unit/ml) significantly increased the intracellular accumulation of proline when protein synthesis was maximally reduced by addition of puromycin to the tissue. It is concluded that the stimulation of amino acid transport into muscle cells by insulin is independent of the increase in protein synthesis effected by the hormone.

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Transketolase Activity in Experimental Thiamine Deficiency and Hepatic Necrosis.* (29395)

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Measurement of red blood cell transketolase activity has been widely used to evaluate thiamine status(1). However, there is often no relationship between enzyme and vitamin levels in thiamine deficiency. Absent correlation is most marked in the presence of liver disease(2). The reason for this lack of agreement is unknown since no data are available on the effects of hepatic injury on circulating and tissue transketolase and thiamine levels. We investigated the influence of experimental liver necroses with and without dietary-induced thiamine deficiency on these parameters.

Materials and methods. One hundred and twenty weanling Sprague-Dawley rats were fed a complete diet containing all of the B-complex vitamins. After 10 days on this regimen, one-half of the animals were switched to a thiamine deficient diet for 21 days; litter mate controls were maintained on the complete diet. After 21 days on a thiamine deficient diet, animals were given 200 μg of thiamine HCl intramuscularly each day for 5 to 21 days. Acute liver injury was induced in thiamine deficient and repleted rats by a single oral dose of 0.2 ml of carbon tetrachloride (CCl_4) per 100 g rat weight. At 48 hour intervals from the start of the thiamine deficient diet, representative animals were anesthetized and 5 to 6 ml of blood drawn from the aorta and collected in citrated and dry tubes. The liver was then removed, a section obtained for histology, and the remainder flushed with saline and rapidly frozen. Transketolase activity was measured in red blood cell homogenates, serum, and 10% liver homogenates, using the method of Brin *et al*, with and without *in vitro* addition of thiamine pyrophosphate(3). For this purpose, red blood cells were washed twice with saline; particular care was taken in washing because of the known hemolytic tendency of red cells from CCl_4-intoxicated animals. Results were expressed in micrograms of ribose utilized per milliliter per hour. Thiamine levels were determined in whole blood and liver with a protozoan, *Ochromonas danica* (4). Tissue, enzyme, and vitamin levels were

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