Kleemann, W., and McConnell, H. M. (1976), *Biochim. Biophys. Acta 419,* 206.
Kuboyama, M., Yong, F. C., and King, T. E. (1972), *J. Biol. Chem. 247,* 6375.
Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.
op den Kamp, J. A. F., Kauerz, M. Th., and van Deenen, L. L. M. (1975), *Biochim. Biophys. Acta 406,* 169.
Peterson, S. W., and Deamer, D. W. (1977), *Arch. Biochem, Biophys. 179,* 218.
Phillips, M. C., Hauser, H., and Paltauf, F. (1972), *Chem. Phys. Lipid 8,* 127.
Robinson, N. C., and Capaldi, R. A. (1977), *Biochemistry 16,* 375.
Rouser, G., and Fleischer, S. (1967), *Methods Enzymol. 10,* 385.
Schneider, D. L., Kagawa, Y., and Racker, E. (1972), *J. Biol. Chem. 247,* 4074.
Williams, J. N. (1964), *Arch. Biochem. Biophys. 107,* 537.
Wrigglesworth, J. M., and Nicholls, P. (1975), *Biochem. Soc. Trans. 3,* 168.
Yonetani, T. (1961), *J. Biol. Chem. 236,* 1680.
Yu, C., Yu, L., and King, T. E. (1975), *J. Biol. Chem. 250,* 1383.

Amino Acid Dependent and Independent Insulin Stimulation of Cartilage Metabolism†

Stephen C. Beuttel,‡ George S. Eisenbarth, and Harold E. Lebovitz*

ABSTRACT: The effects of insulin on embryonic chicken cartilage in organ culture and the dependence of these effects on essential amino acids have been studied. In the presence of all essential amino acids, insulin: (1) increases 2-deoxy-D-glucose and α-aminoisobutyric acid uptake; (2) increases [5-^{3}H]uridine flux into uridine metabolites and the intracellular UTP pool; (3) expands the size of the intracellular UTP pool; (4) does not change the specific activity of the UTP pool; and (5) stimulates RNA, proteoglycan, and total protein synthesis. In lysine (or other essential amino acid)-deficient medium, the effects of insulin are different. While insulin stimulates incorporation of [5-^{3}H]uridine into RNA, it does so by increasing the specific activity of the UTP pool without increasing RNA synthesis. Insulin stimulates 2-deoxy-D-glucose and α-aminoisobutyric acid uptake but no longer stimulates proteoglycan, total protein, or RNA synthesis or expands the size of the UTP pool. These data indicate that there are amino acid dependent and independent effects of insulin on cartilage. Transport processes are amino acid independent, while synthetic processes are amino acid dependent.

Insulin actions on cartilage are of considerable interest because of the relationship between insulin and a variety of insulin-like serum factors (somatomedins and NSILAs)[1] that stimulate anabolic processes in cartilage (Lebovitz and Eisenbarth, 1975; Van Wyk et al., 1975). High concentrations of insulin stimulate the incorporation of radiolabeled precursers into DNA, RNA, and total proteins of costal cartilage removed from hypophysectomized rats (Salmon et al., 1968) and $^{35}SO_4$ incorporation into the proteins of embryonic chicken cartilage (Hall and Uthne, 1971). Some evidence suggests that insulin and the somatomedins interact with the same receptor on embryonic chicken cartilage (Hintz et al., 1972).

The dependence of RNA synthesis upon simultaneous protein synthesis is a significant regulatory mechanism in both prokaryotes and eukaryotes. Deprivation of an essential amino acid during incubation of eukaryotic cells markedly reduces protein synthesis by interfering with initiation of translation and causes stringent control of RNA synthesis (Juergen and Pogo, 1974).

The present study was undertaken to characterize the effects of insulin on transport processes and macromolecule synthesis in embryonic chicken chondrocytes in organ culture and to determine whether amino acid deprivation alters these effects.

Materials and Methods

Incubation Techniques. The incubation procedures were similar in all studies. Pelvic cartilages from 11 or 12 day chicken embryos were removed, cleaned, weighed, and distributed randomly into flasks containing 2 mL of incubation medium. The complete incubation medium was pH 7.45 50 mM Tris buffer which contained all essential amino acids, electrolytes, glucose, and penicillin as previously described (Drezner et al., 1975). In some experiments the essential amino acids lysine, arginine, phenylalanine, or valine were omitted from the incubation medium. All incubations were carried out in an atmosphere of 95% O_2–5% CO_2 (v/v) in a gyrorotary shaker bath at 37 °C.

Sulfate Incorporation into Cartilage Proteoglycans. Carrier-free $Na_2{}^{35}SO_4$ (1.0 μCi) was added to each flask and the cartilages were incubated for 12 to 14 h. At the conclusion of the incubation, the cartilages were boiled for 8 min and soaked for an additional 2 h in 4 mL of saturated Na_2SO_4. The cartilages were rinsed, digested in Pirie's reagent, and $^{35}SO_4$ incorporation determined (Jeffay et al., 1960).

Uridine Incorporation into Cartilage RNA. [5-^{3}H]Uridine

† From the Duke University Medical Center, Departments of Medicine and Physiology, Division of Endocrinology, Durham, North Carolina 27710. *Received June 23, 1977.* Supported by Grants T1 AM 05074 and AM 01324 from the National Institue of Arthritis, Metabolic and Digestive Diseases, U.S. Public Health Service.

‡ Present address: U.S. Naval Hospital, Portsmouth, Virginia.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; AIB, α-aminoisobutyric acid; NSILAs, nonsuppressible insulin-like activities.

(1 μCi) was added to each flask and the cartilages were incubated for 12 h. The cartilages were homogenized in 1 mL of 5% trichloroacetic acid at 0 °C. The precipitate was washed twice with 5% Cl_3CCOOH at 0 °C and hydrolyzed at 100 °C for 90 min in 0.3 mL of formic acid. The [5-^{3}H]uridine in the hydrolysate was determined by liquid scintillation spectrometry using Bray's scintillation mixture. ^{3}H, 92–94%, in this Cl_3CCOOH-precipitable fraction is digestible in dilute alkali at 60 °C, and the percent digestible is the same in insulin-stimulated and control cartilages.

The Cl_3CCOOH supernatant also was counted for ^{3}H. This fraction contains tritiated uridine, UMP, UDP, UTP, and other uridine metabolites (including UDP monosaccharides) and is referred to as acid-soluble uridine radioactivity.

L-Leucine Incorporation into Cartilage Proteins. L-[4,5-^{3}H]Leucine (1 μCi) was added to the flasks and the cartilages were incubated for 12 h. The cartilages were removed, rinsed, and processed as described for uridine incorporation into RNA. This method of protein isolation gave identical results with studies using a modification of the methods of Maxwell and Siekevitz (Eisenbarth et al., 1973).

Thymidine Incorporation into DNA. Cartilages were preincubated in medium for 24 h following which they were removed, weighed, and distributed randomly into flasks with medium alone, or with medium containing added insulin. [*methyl*-^{3}H]Thymidine (0.5 μCi) was added to each flask and, after a 12-h incubation, the cartilages were removed and processed, as described for uridine incorporation into RNA.

UTP Pool Size and Specific Activity: Cartilages were incubated for 6 to 8 h in 50-mL flasks containing 10 cartilages, 20 mL, of the appropriate incubation medium alone or with added insulin and [5-^{3}H]uridine (10 μCi). At the termination of the incubation, one cartilage from each flask was removed and weighed and [5-^{3}H]uridine incorporation into RNA determined. The remaining cartilages were triturated in liquid nitrogen, had [^{14}C]UTP added (to correct for recovery), and were extracted twice with 5 mL of 2 N formic acid. Centrifugation was done at 15 000*g* for 10 min at 4 °C. The supernatants from both extractions were combined and 75 mg of acid-washed charcoal was added. The charcoal was separated by centrifugation and, after being washed, it was extracted three times with 2 mL of ethanol:water:NH_4OH (65:35:0.4). The extracts were combined, flash evaporated, and dissolved in 0.05 mL of deionized water. The solution was applied to the origin of a 1% polyethylenimine-cellulose plate and developed by a modification of the method of Randerath (Randerath and Randerath, 1967), with two-dimensional chromatography utilizing 1.0 M LiCl saturated with borate (pH 7.0) development followed by stepwise development in the second dimension with 0.50 M $(NH_4)_2SO_4$ to 4 cm followed by 0.70 M $(NH_4)_2SO_4$ to 12–16 cm. Plates with appropriate standards were run.

The UTP spot was identified with ultraviolet light, scraped, and extracted for 12 h with 1 mL of 0.1 N HCl. The solution was centrifuged at 15 000*g* at 4 °C for 10 min and the supernatant was transferred to a quartz cuvette. A full spectrum was obtained and proved UTP purity. The UTP concentration was calculated from a molar extinction coefficient at 262 nm in acid solution of 1×10^4. One hundred microliters was removed from each cuvette and the tritium and ^{14}C counts were determined by liquid scintillation spectrometry. From these data, the nanomoles of UTP isolated and the size and specific activity of UTP pool were determined.

Determination of Intracellular and Extracellular Water Spaces in Cartilage. Sorbitol space (extracellular water space) of the cartilage was determined by incubating cartilages in medium containing 0.2 μCi [U-^{14}C]sorbitol for 4 h. The cartilages were homogenized in 5% Cl_3CCOOH (0 °C) and centrifuged at 15 000*g*. Radioactive sorbitol in the Cl_3CCOOH supernatant and the incubation medium were determined and the size of the cartilage extracellular space was calculated to be 76 ± 1.5% by weight. Insulin (10 000 μU/mL) had no significant effect on the sorbitol space (79 ± 2.6%).

The total water content of the cartilage was determined as follows: cartilages were incubated for 4 h in medium and were then transferred to small aluminum foil sheets which had been brought to a constant weight in a 100 °C oven. The wet cartilage weight was determined and the cartilages were placed in a 100 °C oven until they came to constant weight. The intracellular cartilage water space was calculated as the total water space minus the extracellular water space and was 11.2 ± 0.6% by weight. Insulin (10 000 μU/mL) had no significant effect on the intracellular water space (9.9 ± 1.1%).

2-Deoxy-D-glucose (2DG) and α-Aminoisobutyrate (AIB) Uptake. For assessing the glucose transport and phosphorylation system, 0.5 μCi of [U-^{14}C]-2-deoxy-D-glucose was added to the incubation medium and cartilages were incubated for 4 h. The cartilages were homogenized in 5% Cl_3CCOOH (0 °C). Following centrifugation, an aliquot of the supernatant was measured for ^{14}C. Net uptake of 2-deoxy-D-glucose was calculated by subtracting the quantity of 2DG in the extracellular water space of the cartilage from the total cartilage 2DG uptake and dividing it by the intracellular water volume.

For assessing amino acid transport, the uptake of [U-^{14}C]-α-aminoisobutyrate (AIB) was measured. Cartilages were incubated for 4 h in incubation medium to which 0.2 μCi [U-^{14}C]AIB per flask had been added. The cartilages were homogenized in 5% Cl_3CCOOH (0 °C). ^{14}C in the supernatant was measured by liquid scintillation spectrometry.

Chemicals and Hormones. Pork insulin (glucagon free) was generously provided by the Eli Lilly Co. (Indianapolis, Ind.). Carrier-free $Na_2{}^{35}SO_4$ was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). L-[4,5-^{3}H]Leucine (52 Ci/mmol), [U-^{14}C]-α-AIB (12.2 Ci/mmol), [U-^{14}C]sorbitol (200 Ci/mol), and [U-^{14}C]-2-deoxy-D-glucose (45 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.); and [5-^{3}H]uridine (24.3 Ci/mmol), [*methyl*-^{3}H]-thymidine (6.0 Ci/mmol), and [U-^{14}C]uridine 5′-triphosphate (75 mCi/mmol) were from Schwarz/Mann (Orangeburg, N.Y.).

Statistics. Significance of difference between means was determined by the Student's *t* test ($p < 0.05$ defined as significant).

Results

Effects of Insulin on Protein Synthesis. The effect of insulin on $^{35}SO_4$ incorporation into embryonic chicken cartilage proteoglycans is shown in Figure 1a. Concentrations as low as 200 μU/mL significantly stimulate $^{35}SO_4$ incorporation. A log dose–response relationship exists throughout the range of insulin concentrations from 100 to 10 000 μU/mL. Evidence that the changes in $^{35}SO_4$ incorporation observed represent changes in proteoglycan synthesis are: (1) $^{35}SO_4$ enters cartilage by passive diffusion and the sulfate pool size is stable; (2) $^{34}SO_4$, once incorporated, is slowly released (washout experiments demonstrate a 10–20% loss of incorporated $^{35}SO_4$ during the total experimental time period and insulin treatment does not alter this rate of degradation); (3) chromatography of the precipitated cartilage proteins reveals that the $^{35}SO_4$ is associated with proteins of molecular weight corresponding to proteoglycans; and (4) insulin effects on amino acid incor-

TABLE I: Insulin Effects on UTP Pool Size and Specific Activity of Cartilage Incubated in Complete Medium or Lysine-Deficient Medium.[a]

Insulin (μU/mL)	UTP isolated (nmol)	Recovery (%)	UTP pool (nmol/40 cartilages)	^{3}H in UTP (cpm/40 cartilages)	UTP sp act. (cpm/nmol)	^{3}H in RNA (cpm/mg of cartilage)
			Expt 1: Complete Medium			
0	3.2	15.4	20.8	35 750	11 171	258 ± 23
10 000	4.0	15.3	26.2	51 130	12 783	466 ± 20
% stimulation by insulin			26	43	14	81
			Expt 2: Lysine-Deficient Medium			
0	6.3	31	20.3	59 640	9 467	273 ± 18
10 000	7.1	34	20.8	100 590	14 168	477 ± 19
% stimulation by insulin			3	69	50	63

[a] UTP was isolated as described in Materials and Methods from groups of 40 cartilages incubated with [5-^{3}H]uridine with or without insulin. [U-^{14}C]UTP was added at completion of incubation to monitor recovery of UTP during the purification. Amount of UTP was determined spectrophotometrically. In the same experiment, [5-^{3}H]uridine incorporation into RNA was determined (mean ± SE of 5 cartilages).

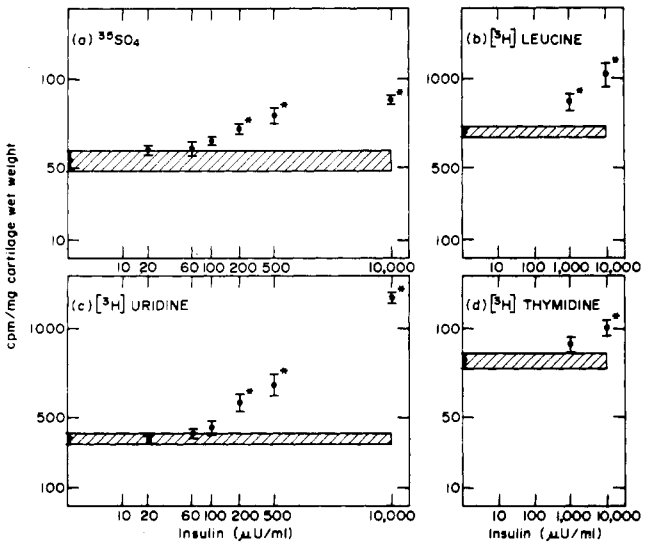

FIGURE 1: Effect of insulin (log-dose response) on $^{35}SO_4$ incorporation into proteoglycan, L-[4,5-^{3}H]leucine into protein, [5-^{3}H]uridine into RNA, and [*methyl*-^{3}H]thymidine into DNA in embryonic chicken cartilage incubated in vitro in medium containing all essential amino acids (see Materials and Methods). Each point represents the mean ± SE of five cartilages (each cartilage 9–15 mg). Insulin stimulates all four processes (* = $p < 0.05$). Cross-hatched area represents mean ± SE of incorporation without added insulin.

poration into protein correlate with its effects on $^{35}SO_4$ incorporation (Eisenbarth et al., 1973, 1974; Ebert and Prockop, 1967).

Insulin stimulation of leucine incorporation into total cartilage proteins is shown in Figure 1b. At both insulin concentrations there was significant stimulation and the stimulation appeared to be dose related.

Effect of Insulin on RNA Synthesis. Insulin markedly stimulates the incorporation of [5-^{3}H]uridine into the RNA of embryonic chicken cartilage (Figure 1c). A log dose–response increase in radiolabeled uridine incorporation occurs from 200 μU/mL to 10 000 μU/mL. In the particular study shown, 203% stimulation above control occurred at 10 000 μU/mL. Most other experiments yielded a 75 to 125% stimulation at 10 000 μU/mL; no further stimulation was seen with higher concentrations.

To ascertain whether the increased radiolabel in RNA was secondary to insulin inhibition of RNA breakdown, degradation experiments were performed. Only 10% of prelabeled cartilage RNA [5-^{3}H]uridine was lost during a 10-h incubation, and insulin did not significantly alter the amount of [5-^{3}H]RNA breakdown. Thus insulin does not significantly alter cartilage RNA degradation.

When radiolabeled precursors are utilized in experiments such as those above, changes in transport or phosphorylation could alter the specific activity of the intracellular precursor pools. Thus, an effect of insulin on transport or phosphorylation could change the rate of incorporation of labeled uridine into RNA without altering the synthesis of RNA. To determine whether insulin stimulates RNA synthesis, the specific activity of the intracellular UTP pool was measured. A representative experiment is shown in Table I, experiment 1. Insulin stimulation resulted in an 81% increase in the incorporation of [5-^{3}H]uridine into RNA and a 14% rise in the UTP specific activity when compared with control cartilages. The small rise in specific activity is insufficient to account for the much larger rise in incorporation of [5-^{3}H]uridine into RNA. Therefore, insulin does stimulate RNA synthesis. In addition, the influx of radiolabeled uridine into the UTP pool and the size of the UTP pool increase during insulin stimulation. Combining the results of four additional experiments with the one presented in Table I, experiment 1, insulin stimulation increased the flux of [5-^{3}H]uridine into UTP 83 ± 28% as compared with control cartilages. There was an equivalent rise in the size of the UTP pool (73 ± 26%) and this maintained the specific activity of the UTP pool at a value close to that of control cartilages (9 ± 19%).

Effects of Insulin on [methyl-^{3}H]Thymidine Incorporation. Insulin significantly stimulated the incorporation of [*methyl*-^{3}H]thymidine into cartilage DNA (Figure 1d). Because of the low levels of intracellular TTP, we were unable to determine the specific activity of this nucleotide pool. Therefore, the precise mechanism responsible for the effect of insulin on [*methyl*-^{3}H]thymidine incorporation into DNA is unknown.

Lysine Dependence of Insulin Action. We next sought to determine if insulin effects on cartilage were dependent on essential amino acids. The results of studies in which lysine was excluded from the incubation medium are illustrated in Figure 2. Insulin stimulation of sulfate and leucine incorporation into proteoglycan and total proteins, respectively, were abolished. Salmon (1960) has shown similar inhibition of protein synthesis in coastal cartilage from hypophysectomized rats with amino acid deficient medium and this result was not unexpected. Surprisingly, the stimulation of [5-^{3}H]uridine incorporation into RNA was unaffected.

To determine whether the increased [5-^{3}H]uridine incor-

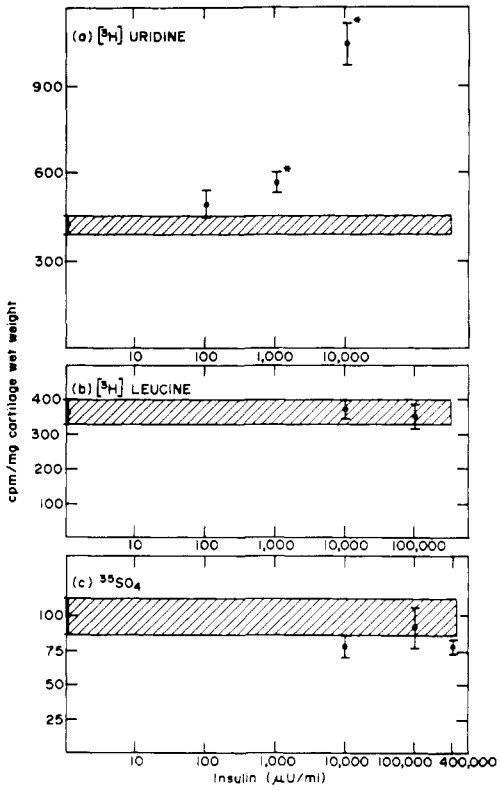

FIGURE 2: Lack of effect of insulin on $^{35}SO_4$ incorporation into proteoglycan and L-[4,5-^{3}H]leucine incorporation into protein in embryonic chicken cartilage incubated in lysine deficient medium. [5-^{3}H]Uridine incorporation is still stimulated. Each point represents the mean ± SE of five cartilages incubated in vitro (* = $p < 0.05$). Cross-hatched area represents mean ± SE of incorporation without added insulin.

TABLE II: Stimulation of α-Aminoisobutyrate (AIB) Transport by Insulin.[a]

Expt	Insulin (μU/mL)	[^{14}C]AIB (cpm/mg)	% stimulation
1	0	222 ± 7.4	
	10 000	296 ± 13	34
2	0	182 ± 4.8	
	10 000	218 ± 4	20

[a] Results are expressed as cpm/mg wet weight of cartilage (mean ± SE of 6 cartilages). Expt 1 used complete incubation medium. Expt 2 used lysine-deficient medium. Stimulation was significant at $p < 0.001$ for each experiment.

TABLE III: Stimulation of 2-Deoxy-D-glucose Transport by Insulin.[a]

Expt	Insulin (μU/mL)	Net Transport (pmol/g of cartilage)	% stimulation
1	0	42 ± 3	
	1 000	48 ± 1	14
	10 000	53 ± 2	26
2	0	41 ± 2	
	10 000	52 ± 1	27

[a] Results expressed as pmol of 2-deoxy-D-glucose transported/g of cartilage wet weight (mean ± SE of 8 cartilages). Expt 1 used complete incubation medium. Expt 2 used lysine-deficient medium. Stimulation was significant at $p < 0.01$ (Expt 1) and $p < 0.001$ (Expt 2).

poration into RNA represented increased RNA synthesis, the specific activity of the UTP pool was measured in cartilages incubated with or without insulin in lysine deficient medium. A representative experiment is shown in Table I, experiment 2. In five such experiments insulin increased the mean incorporation of radiolabel into RNA, 76 ± 31%, compared with control cartilages. At the same time, insulin caused a 115 ± 31% rise in the UTP pool specific activity. Thus, the effects of insulin on incorporation of [5-^{3}H]uridine into cartilage RNA, in lysine deficient medium, are secondary to the change in the specific activity of the UTP pool. Insulin retains its ability to stimulate the influx of labeled uridine into the UTP pool (114 ± 23%), but no longer expands the cold pool (−8 ± 23%).

Lysine deficiency therefore affects [5-^{3}H]uridine incorporation into cartilage in at least two ways: Expansion of the intracellular UTP pool is blocked and there is no stimulation of RNA synthesis.

The studies presented thus far demonstrate that insulin stimulation has both lysine dependent (L-[4,5-^{3}H]leucine and $^{35}SO_4$ incorporation, expansion of the UTP pool size and stimulation of RNA synthesis) and lysine independent processes ([5-^{3}H]uridine flux into the UTP pool). The data suggested that insulin stimulation of transport processes might be lysine independent whereas stimulation of synthetic processes is lysine dependent. It should be noted that leaving lysine out of the incubation medium has little effect on basal (unstimulated) synthetic rates (10–20% decrease).

Effects of Insulin on Amino Acid Transport. α-Aminoisobutyric acid (AIB) was used to estimate amino acid transport because it is not metabolized. As shown in Table II, insulin stimulates AIB transport. In cartilage incubated in lysine deficient medium, insulin similarly stimulates AIB transport.

Effects of Insulin on the Transport of 2-Deoxy-D-glucose. The effect of insulin on glucose transport was assessed utilizing the nonmetabolized analogue, 2-deoxy-D-glucose. In these incubations, the concentration of medium glucose was reduced to 1.4 mM to decrease competition for the transport sites. Insulin stimulates the accumulation of 2-deoxy-D-glucose (Table III). Removal of lysine from the incubation medium does not affect the insulin-stimulated accumulation of this glucose analogue.

Effects on Insulin Stimulation of Other Amino Acid Deficiencies. After we found a qualitative effect of lysine on insulin stimulation of cartilage metabolism, the next question we asked was whether this effect was unique to lysine or shared by other essential amino acids.

In the absence of either lysine, valine, phenylalanine, or arginine, insulin does not stimulate L-[4,5-^{3}H]leucine incorporation into cartilage proteins (Table IV). In the absence of any of these essential amino acids, insulin stimulates [U-^{14}C]AIB uptake (Table V). Furthermore, Table VI illustrates that insulin stimulates [5-^{3}H]uridine uptake into the trichloroacetic acid soluble cartilage pool in the absence of these essential amino acids (see Materials and Methods). Such stimulation probably reflects increased transport or phosphorylation of [5-^{3}H]uridine. The correlation between increased [5-^{3}H]uridine incorporation into RNA and into Cl_3CCOOH-soluble counts in the absence of any of the four amino acids is excellent (linear regression coefficient = 0.86, $p < 0.01$, slope = 0.95). In the presence of all essential amino acids in the medium this correlation is no longer present (125% vs. 53% stimulation). Thus, insulin stimulates RNA synthesis and [5-^{3}H]uridine uptake in cartilage incubated in medium containing all the essential amino acids while it only stimulates

TABLE IV: Insulin Stimulation of L-[4,5-^{3}H]leucine Incorporation into Cartilage Proteins in Vitro.[a]

Insulin (μU/mL)	All essential amino acids	Lysine deficient	Phe deficient	Val deficient	ARG deficient
0	767 ± 63	574 ± 30	583 ± 36	441 ± 25	481 ± 11
10 000	1394 ± 51	478 ± 45	644 ± 33	351 ± 19	398 ± 34

[a] Results expressed as cpm/mg of cartilage wet weight (mean ± SE 5 cartilages). Only in incubations with all essential amino acids present was there significant ($p < 0.05$) stimulation of L-[4,5-^{3}H]leucine incorporation.

TABLE V: Insulin Stimulation of [U-^{14}C]AIB Transport.[a]

Insulin (μU/mL)	All essential amino acids	Lys deficient	Phe deficient	Val deficient	Arg deficient
0	922 ± 42	996 ± 31	933 ± 28	1018 ± 55	1008 ± 30
10 000	1224 ± 43	1229 ± 68	1080 ± 36	1170 ± 41	1264 ± 48

[a] Results are expressed as cpm/mg of cartilage wet weight (mean ± SE of 5 cartilages). In the presence of insulin, AIB transport is significantly increased ($p < 0.05$) in all groups.

TABLE VI: Insulin Stimulation of [5-^{3}H]Uridine Incorporation into RNA and Trichloroacetic Acid Soluble Cartilage Fraction.[a]

Insulin (μU/mL)	All essential amino acids	Lys deficient	Phe deficient	Val deficient	Arg deficient
		(a) Acid-Soluble ^{3}H Counts			
0	6218 ± 468	6070 ± 277	6470 ± 449	5106 ± 172	5962 ± 611
10 000	9543 ± 691	8338 ± 174	9205 ± 1018	8465 ± 454	8374 ± 479
% stimulation	53	37	42	66	40
		(b) ^{3}H in RNA			
0	749 ± 80	692 ± 15	735 ± 67	549 ± 24	657 ± 75
10 000	1684 ± 102	940 ± 32	1160 ± 142	932 ± 63	960 ± 74
% stimulation	125	36	58	70	46

[a] Results expressed as the mean ± SE of five cartilages (cpm/mg of cartilage wet weight [cartilage 9-15 mg]). Stimulation is statistically significant for all groups. Because of the tremendous concentrative ability of this tissue for [5-^{3}H]uridine (of approximately 300 000 cpm added to 2000 μL of medium for each cartilage, 60 000 cpm are incorporated into the acid-soluble pool per cartilage) less than 3% of acid-soluble counts represent extracellular [5-^{3}H]uridine in equilibrium with the medium.

[5-^{3}H]uridine uptake in medium which is deficient in an essential amino acid.

Discussion

The present study indicates that different responses to insulin occur in vitro depending on the presence or absence of essential amino acids. In our study, insulin stimulated [5-^{3}H]uridine incorporation into RNA represented synthesis of RNA in the presence of all essential amino acids. In lysine deficient medium (or presumably any essential amino acid deficient medium), similar stimulation of [5-^{3}H]uridine incorporation was the result of changes in UTP specific activity. These observations add complexity to modeling insulin action with in vitro studies.

Insulin stimulation of cartilage macromolecule synthesis (RNA, total protein, and proteoglycan) in vitro requires medium containing all the essential amino acids. In contrast, transport processes (2-deoxy-D-glucose and AIB uptake), as well as [5-^{3}H]uridine flux into uridine metabolites (and specifically into the UTP pool) are stimulated by insulin in lysine-deficient medium. Other essential amino acids besides lysine similarly influence insulin actions on cartilage. Assuming insulin action is mediated via a single membrane receptor, these observations suggest a refinement in insulin action with an amino acid dependent and independent "effector" system.

Insulin effects on radiolabeled uridine uptake and incorporation into RNA have been studied in several other organ and cell culture systems. Mammary gland in organ culture responds to insulin stimulation with an increase in radiolabeled uridine uptake, no alteration in UTP specific activity and an increase in radiolabeled uridine incorporation into RNA. The effect of essential amino acid deficiency is not known but antibiotics which inhibit protein synthesis block the effect of insulin on labeled uridine uptake and incorporation into RNA (Rillema, 1975). In chick embryo fibroblasts in culture, insulin has no effect on uridine uptake as assessed by estimates of expansion of the UMP pool, but does stimulate incorporation into ribosomal RNA and tRNA. Cyclohexamide blocks the insulin stimulation of radiolabeled incorporation into RNA (Baseman et al., 1974). Cultured bone cells stimulated with insulin show an increase in [^{14}C]uridine uptake, presumably no effect on the intracellular UTP pool size and an increase in [^{14}C]uridine incorporation into RNA. Essential amino acid deficiency and treatment of the cultures with inhibitors of protein synthesis had no effect on insulin stimulation of radiolabeled uridine uptake and incorporation into RNA (Peck and Messinger, 1970). It appears that no uniform action of insulin on uridine uptake and incorporation into RNA can be extrapolated from the above studies and our data. Perhaps the effects of insulin on uridine uptake and RNA synthesis are tissue specific.

The site of the amino acid dependence of insulin stimulated macromolecule synthesis in chicken embryonic cartilage is unknown. Studies relating the effects of essential amino acid

deficiencies on cellular protein synthesis in eukaryotes show that deficiency causes a very rapid decrease in protein synthesis, a significant reduction in charged tRNA, diminished ribosome content, a dissolution of polysomes into monosomes and marked impairment of the initiation of protein synthesis (Juergen and Pogo, 1974; Vaughan et al., 1971; Allen et al., 1969; Vaughan and Hansen, 1973). The dependence of RNA synthesis upon simultaneous protein synthesis is a significant regulatory mechanism in both prokaryotes and eukaryotes. This stringent control has been characterized in simple eukaryotes such as yeast and appears to occur primarily at the transcription of rRNA (Schulman et al., 1977). The mechanism of this control involves the binding of RNA polymerases to the nuclear DNA template. On the basis of our present data, it is reasonable to speculate that the mechanism of the amino acid dependence of insulin stimulation of macromolecule synthesis may involve similar mechanisms to those described for other eukaryotes. Further studies are necessary to substantiate this thesis.

References

Allen, R. E., Raines, R. L., and Regen, D. M. (1969), *Biochim. Biophys. Acta 190,* 323.

Baseman, J. B., Paolini, D., and Amos, H. (1974), *J. Cell Biol. 60,* 54.

Drezner, M. K., Eisenbarth, G. S., Neelon, F. N., and Lebovitz, H. E. (1975), *Biochim. Biophys. Acta 381,* 384.

Ebert, P. S., and Prockop, D. J. (1967), *Biochim. Biophys. Acta 136,* 45.

Eisenbarth, G. S., Beuttel, S. C., and Lebovitz, H. E. (1973), *Biochim. Biophys. Acta 331,* 397.

Eisenbarth, G. S., Drezner, M. K., and Lebovitz, H. E. (1975), *J. Pharmacol. Exp. Ther. 192,* 630.

Hall, K., and Uthne, K. (1971), *Acta Med. Scand. 190,* 137.

Hintz, R. L., Clemmons, D. R., Underwood, L. E., and Van Wyk, J. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2351.

Jeffay, H., Funso, O. O., and Jewell, W. R. (1960), *Anal. Chem. 32,* 306.

Juergen, K., and Pogo, A. (1974), *J. Biol. Chem. 249,* 568.

Lebovitz, H. E., and Eisenbarth, G. S. (1975), *Vitam. Horm. (N.Y.) 33,* 575.

Peck, W. A., and Messinger, K. (1970), *J. Biol. Chem. 245,* 2722.

Randerath, K., and Randerath, E. (1967), *Methods Enzymol. 12,* 323.

Rillema, J. A. (1975), *Am. J. Physiol. 228,* 1531.

Salmon, W. D. (1960), *J. Lab. Clin. Med. 56,* 673.

Salmon, W. D., DuVall, M. R., and Thompson, E. Y. (1968), *Endocrinology 82,* 493.

Schulman, R. W., Sripati, C. E., and Warner, J. R. (1977), *J. Biol. Chem. 252,* 1344.

Van Wyk, J. J., Underwood, L. E., Baseman, J. B., Hintz, R. L., Clemmons, D. R., and Marshall, R. N. (1975), *Adv. Metab. Disord. 8,* 127.

Vaughan, M. H. and Hansen, B. S. (1973), *J. Biol. Chem. 248,* 7087.

Vaughan, M. H., Pawlowski, P. J., and Forchhammer, J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2057.

Accumulation of Immunoglobulin Messenger Ribonucleic Acid in Immunized Mouse Spleen†

Tasuku Honjo,* Akira Shimizu, Masa-aki Tsuda, Shunji Natori, Tohru Kataoka, Chieko Dohmoto, and Yoshitake Mano

ABSTRACT: We have measured the concentration of mRNAs coding for immunoglobulins, κ and λ type light chains and γ_1 type heavy chain, in mouse spleen cells activated by bacterial lipopolysaccharide or sheep red blood cells. These mRNAs were quantitated by hybridization to radioactive DNA complementary to highly purified immunoglobulin mRNAs from mouse myelomas. In the lipopolysaccharide-stimulated spleen cells, only light chain mRNA accumulated, whereas γ_1 type heavy chain mRNA remained unvaried. The light chain mRNA concentration also increased in purified bone-marrow-derived lymphocytes. The lipopolysaccharide-induced light chain mRNA was similar to light chain mRNAs purified from myelomas. The accumulation and disappearance of light chain mRNA in bone-marrow-derived lymphocytes coincide with the kinetics of synthesis of immunoglobulin M which is the major species induced by lipopolysaccharide. In sheep red blood cell stimulated spleen, the specific accumulation of κ type light chain and γ_1 type heavy chain mRNAs parallels immunoglobulin G synthesis. These results seem to indicate that the increment of immunoglobulin mRNA concentration in bone-marrow-derived lymphocytes is important for induction of immunoglobulin synthesis.

In order to elucidate the molecular mechanisms of the immune response, it is essential to know which step(s) of antibody synthesis is stimulated upon activation of immunocompetent lymphocytes. A vital question is whether induction of antibody synthesis is due to activation of Ig[1] gene transcription or of translational steps of preexisting Ig mRNA. Purification of

† From Department of Physiological Chemistry and Nutrition, Faculty of Medicine and Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo, Japan. *Received April 25, 1977; revised manuscript received September 1, 1977.* This investigation was supported by grants from Jane Coffin Child Memorial Fund for Medical Research, from Matsunaga Science Foundation, and from Ministry of Education, Science and Culture of Japan.

[1] Abbreviations used are: Ig, immunoglobulin; L_κ and L_λ chains, κ and λ type light chains; H_{γ_1} chain, γ_1 type heavy chain; LPS, lipopolysaccharide; SRBC, sheep red blood cells; C_rt, the product of the concentration of RNA and the time of incubation; T and B lymphocytes, thymus-derived and bone-marrow-derived lymphocytes; poly(A), poly(adenylic acid).