The Effect of Insulin on Free Amino Acid Pools and Protein Synthesis in Rat Skeletal Muscle *in vitro*

By R. C. HIDER,* E. B. FERN AND D. R. LONDON

Department of Chemical Pathology, St Thomas's Hospital, London S.E.1., U.K.

(Received 7 June 1971)

1. The effects of insulin *in vitro* on tissue pools and incorporation into protein of glycine and leucine in the extensor digitorum longus muscle of the rat are reported. 2. It was found that insulin decreased the lag period before the establishment of a linear rate of incorporation of radioactive glycine into protein. 3. The hormone increased the size of the free intracellular glycine pool. No such effect was found for leucine. The accumulation of radioactive glycine in the intracellular fluid compartment was increased. The content of radioactive leucine in the intracellular compartment was decreased. 4. Insulin decreased the specific radioactivity of both glycine and leucine in the extracellular fluid. 5. The hormone also decreased protein catabolism. 6. The effect on protein synthesis was not caused by an increase in the specific radioactivity of the extracellular pool but was possibly related to increased amino acid concentrations in this pool, which could in turn have affected the aggregation of ribosomes.

The conclusion that insulin can stimulate protein synthesis is based on many reports that the hormone can enhance the incorporation of labelled amino acid into protein. However, the amount of label incorporated is determined by the specific radioactivity of the immediate protein precursor, aminoacyl-tRNA. The effect of insulin on incorporation could result from an increase in the specific radioactivity of this pool, which, in turn, could be affected by alterations in the specific radioactivity of the precursor pool of free amino acids. Indeed, the hormone has been shown to increase the specific radioactivity of both tyrosyl- and leucyl-tRNA in heart muscle (Davey & Manchester, 1969). A further factor complicating the interpretation of studies on protein synthesis is the finding that insulin also inhibits protein breakdown in adipose tissue (Christophe & Wodon, 1963) and in liver (Mondon & Mortimore, 1967; Mortimore & Mondon, 1970).

For muscle there is evidence that protein is synthesized either directly from extracellular amino acids or from a small pool in rapid equilibrium with the extracellular space (Hider, Fern & London, 1969, 1971). During an incubation *in vitro* the extracellular amino acid pool of rat extensor digitorum longus muscle has a specific radioactivity

lower than that of the medium (Hider *et al.* 1971). This difference probably arises because of an appreciable contribution of intracellular amino acids to the extracellular pool. As it has been reported that insulin decreases the rate of amino acid efflux from various tissues (Manchester, 1961; Guidotti *et al.* 1968; Pozefsky, Felig, Soeldner & Cahill, 1968; Hernandez & Coulson, 1969), the specific radioactivity of the extracellular pool of amino acid during an incubation *in vitro* might be increased. If this were so, any effect of the hormone on incorporation might merely reflect a change in the radioactivity of the precursor pool.

Thus, before any firm conclusions can be drawn as to whether insulin does stimulate protein synthesis, it is necessary to estimate the effect of insulin on the specific radioactivity of the extracellular amino acid pool. Such observations, together with evidence indicating that the total intracellular amino acid pool is not that utilized during the stimulation of incorporation by insulin, are the subject of the present paper.

MATERIALS

[U-¹⁴C]Leucine (10 mCi/mmol), [1-¹⁴C]leucine (50 mCi/mmol), [U-¹⁴C]glycine (5 mCi/mmol), D-[1-¹⁴C]mannitol (17 mCi/mmol), [U-¹⁴C]sorbitol (6 mCi/mmol), [carboxyl-¹⁴C]inulin (14 mCi/mmol) and [³⁵S]sulphate (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

* Present address: Department of Chemistry, University of Essex, Wivenhoe Park, Colchester, Essex, U.K.

METHODS

Male Sprague-Dawley-derived white rats (Animal Supplies Ltd., Knebworth, Herts., U.K.) weighing 50–60 g were starved for 18 h before decapitation. The extensor digitorum longus muscles (25–30 mg) were maintained at their natural length during incubation at 37°C as described by Hider *et al.* (1971). Each muscle was placed in a conical flask containing 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1949), and gassed with O₂ + CO₂ (95:5). During periods of transfer (1 min), the muscle and its frame were removed from one medium, washed twice in the Krebs–Ringer bicarbonate buffer and added to a second medium that had previously been preincubated for 1 min at 37°C. The muscle was then gassed with O₂ + CO₂ (95:5).

Recrystallized insulin (Novo Research Institute, Copenhagen, Denmark) was used at a concentration in the medium of 100 μ units/ml. Preliminary experiments with ¹²⁵I-labelled insulin had shown that bovine serum albumin (0.05%, w/v) prevented the binding of insulin to glassware, so this concentration of albumin was used subsequently in all the incubation media. Cycloheximide was used at a concentration of 100 μ g/ml. This concentration was found to decrease the incorporation of [¹⁴C]leucine into protein to 2% of that normally observed in extensor digitorum longus muscle. After incubation the extracellular amino acids were removed from the muscle preferentially by an incubation at 2°C (Hider *et al.* 1971). Insulin has no effect on amino acid flux at 2°C.

Intracellular solutes and protein were extracted and prepared for scintillation counting and automated amino acid analysis as described by Hider *et al.* (1971).

Determination of ¹⁴CO₂ was effected by the addition of 1 M-NaOH (0.6 ml) to the central well of an incubation flask, then 1 M-HCl (1.0 ml) was added to the incubation medium. The flask was incubated for a further 60 min at 37°C. A portion (0.5 ml) of the resulting alkaline extract was counted, as for radioactive protein.

After a 90 min incubation with [¹⁴C]glycine at least 95% of the water-soluble radioactivity in the tissue and medium was recovered as unmetabolized glycine, as determined by paper chromatography in butan-1-ol-acetic acid-water (12:5:3, by vol.); the corresponding

result with both [U-¹⁴C]- and [1-¹⁴C]-leucine was 80–85%.

The significance of differences was calculated by using Student's *t* test for paired variates.

RESULTS

To ascertain whether insulin affected the relative amount of intracellular amino acid incorporated into protein, muscles were preloaded with [¹⁴C]-glycine and then incubated in the presence of [¹²C]glycine with and without insulin. If insulin increased the contribution of intracellular glycine to protein, then a correspondingly increased incorporation of ¹⁴C label into protein would be observed, when compared with a paired control incubated in the absence of insulin. However, preincubation of muscles with insulin and glycine (500 μ M) for 45 min markedly decreased the sensitivity of the preparation towards insulin during the subsequent 45 min (Table 1), presumably because of carry-over of the insulin effect into the second incubation. To avoid this difficulty muscles were preincubated with [¹⁴C]glycine in the absence of insulin, before a subsequent incubation in [¹²C]glycine with and without insulin. As shown in Table 1, there was no appreciable difference between the incorporation of [¹⁴C]glycine in the presence and absence of insulin. Only when insulin and [¹⁴C]glycine were present together in the second incubation was there a significant increase in incorporation of label.

Fig. 1 shows the effect of insulin on the rate of glycine incorporation. Although the two regression lines drawn between the 30 and 90 min time-points were significantly different, there was no difference between the slope of the two lines. These results could be interpreted as showing that insulin decreased the lag period before glycine incorporation but did not significantly affect the linear rate once it had become established.

Table 1. *Effect of insulin on incorporation into protein and intracellular accumulation of [¹⁴C]glycine*

The muscles were incubated, at 37°C, in Krebs–Ringer bicarbonate solution (2 ml) containing glycine (500 μ M). Insulin (100 μ units/ml) was present where indicated. See the Methods section for details of muscle transfer from one solution to another. The values are the means and standard errors of six determinations.

**** *P* = 0.001.

Incubation conditions		10 ⁻⁴ × Incorporation of [¹⁴ C]glycine into protein (Ci/mg of protein)	Distribution ratio of [¹⁴ C]glycine between total intracellular pool and incubation medium (extracellular fraction taken as 30%)
Preincubation (45 min)	Incubation (45 min)		
+ Insulin, [¹² C]glycine	+ Insulin, [¹⁴ C]glycine	4.3 ± 0.1	6.0 ± 0.2
	– Insulin, [¹⁴ C]glycine	4.2 ± 0.1	5.8 ± 0.1
– Insulin, [¹² C]glycine	+ Insulin, [¹⁴ C]glycine	4.4 ± 0.1****	5.5 ± 0.1
	– Insulin, [¹⁴ C]glycine	3.6 ± 0.1****	4.7 ± 0.1
– Insulin, [¹⁴ C]glycine	+ Insulin, [¹² C]glycine	4.1 ± 0.1	2.9 ± 0.1
	– Insulin, [¹² C]glycine	4.2 ± 0.1	2.7 ± 0.1

Fig. 1. Incorporation of [¹⁴C]glycine into protein as a function of time. The muscles were incubated in Krebs-Ringer bicarbonate solution, at 37°C, containing glycine (500 μM). Each point represents the mean of six observations; the bar represents ±1 s.e.m. ●, With insulin (100 μunits/ml); $y = 0.12x - 1.16$; ○, control, $y = 0.10x - 1.44$. Multiple regression analysis: significance of difference of intercepts, $P < 0.001$; two lines versus common line, $P < 0.001$; difference in slopes of lines, $P =$ not significant.

Table 2 illustrates the effect of insulin on the extracellular and intracellular pool sizes of [¹⁴C]glycine and [¹⁴C]leucine. Insulin increased the rate of incorporation of both [¹⁴C]glycine and leucine into protein, but had different effects on the intracellular concentrations of these amino acids. Whereas insulin increased both the concentration of glycine and the amount of [¹⁴C]glycine present in the intracellular pool, no change was observed in the leucine concentration; moreover, the hormone actually decreased the amount of free intracellular [¹⁴C]leucine. Insulin increased the amount of both glycine and leucine in the extracellular pool. No significant effect was observed on the amount of [¹⁴C]glycine or [¹⁴C]leucine in the extracellular pool. Thus clearly the specific radioactivity of both [¹⁴C]glycine and [¹⁴C]leucine was decreased by insulin in the extracellular pool.

Insulin has no significant effect on the water content of extensor digitorum longus muscle, or on the extracellular space as measured by small extracellular markers (Table 3). There was, however, a large difference in the insulin spaces.

In both untreated muscles and muscles treated with cycloheximide, insulin decreased the concentration of leucine in both the tissue and in the

Table 2. Effect of insulin on muscle pools of glycine and leucine

		10 ⁻⁴ × [¹⁴ C]Incorporation into protein (μCi/mg of protein)		10 ⁻⁴ × Intracellular amino acid pool size (μCi/mg of tissue wet wt.)		10 ⁻⁴ × Extracellular amino acid pool size (μCi/mg of tissue wet wt.)		10 ⁻⁴ × Extracellular amino acid pool size (μmol/mg of tissue wet wt.)	
Amino acid	Insulin	+	-	+	-	+	-	+	-
Glycine		4.3 ± 0.1 (12)****	3.4 ± 0.1	6.38 ± 0.15 (12)****	5.0 ± 0.4	67 ± 1 (3)****	56 ± 2	1.2 ± 0.06 (12) N.S.	64 ± 1 (3)**
Leucine		11.6 ± 0.66 (12)****	8.3 ± 0.27	1.13 ± 0.04 (12)****	1.34 ± 0.05	1.67 ± 0.037 (3) N.S.	1.88 ± 0.047	1.3 ± 0.05	55 ± 2
								1.65 ± 0.1* (12) N.S.	4.1 ± 0.2 (3)*
								1.8 ± 0.1	3.7 ± 0.1

The tissue was incubated in Krebs-Ringer bicarbonate buffer at 37°C, with either [¹⁴C]glycine (500 μM) or [¹⁴C]leucine (100 μM). Statistics, insulin versus control, for the number of experiments in parentheses: * $P = 0.05$; ** $P = 0.01$; *** $P = 0.005$; **** $P = 0.001$; N.S., not significant.

incubation medium (Table 4). Cycloheximide by itself increased the total amount of leucine in both the intracellular pool and in the medium. Leucine has a relatively low intracellular concentration and as there was initially no leucine in the incubation medium, small differences in concentrations could easily be observed. Analogous results were obtained with valine and isoleucine.

Insulin decreased the production of $^{14}\text{CO}_2$ from both $[1\text{-}^{14}\text{C}]$ - and $[\text{U-}^{14}\text{C}]$ -leucine when incubated with extensor digitorum longus muscles (Table 5).

DISCUSSION

As shown in Table 1, insulin does not increase the amount of ^{14}C label entering protein when the intracellular glycine pool is strongly labelled with $[^{14}\text{C}]$ glycine and when the extracellular pool is not. This indicates that insulin does not increase the contribution of intracellular amino acid to the immediate protein precursor pool. Similar results have been obtained with leucine and proline in rat caudofemoralis muscle, when the effect of extra-

Table 3. *Effect of insulin on tissue spaces and electrolyte content of extensor digitorum longus muscle*

The tissue was incubated in Krebs-Ringer bicarbonate solution (2 ml) at 37°C . Inulin and sulphate were present in the incubation medium in trace amounts, in contrast to mannitol (5 mM). Statistics, insulin versus control: * $P = 0.05$; ** $P = 0.01$; **** $P = 0.001$.

Insulin (100 μ units/ml)	Tissue space (% of wet tissue wt.)			Tissue water (% of wet tissue wt.)	Total K^+ (mM)	Total Na^+ (mM)
	Inulin	Mannitol (5 mM)	Sulphate			
+	$15.2 \pm 0.2^{**}$	31.4 ± 0.4	$29.8 \pm 0.3^*$	78.9 ± 0.1	$125 \pm 2^{****}$	$48 \pm 2^*$
-	18.3 ± 0.1	30.7 ± 0.8	28.4 ± 0.5	78.6 ± 0.1	108 ± 2	51 ± 1
No. of expts.	(8)	(6)	(6)	(6)	(6)	(6)
Incubation time (min)	120	120	120	120	60	60

Table 4. *Effects of cycloheximide and insulin on pools of leucine when the tissues have been incubated in medium from which amino acid was initially absent*

The muscles were incubated in Krebs-Ringer bicarbonate solution, at 37°C , for 60 min. The tissue leucine determination was made with the assumption that the tissue water is a uniform compartment. The values are the mean and standard errors of three determinations. Statistics: * $P = 0.05$; ** $P = 0.01$; *** $P = 0.005$; N.S., not significant.

Cycloheximide (100 $\mu\text{g/ml}$)	Insulin (100 μ Units/ml)	Leucine concentration (μM)		Total amount of leucine in system (mmol)
		Tissue	Medium	
+	-	$215 \pm 11^*$	$7.0 \pm 0.2^{***}$	18.5
-	-	187 ± 6.5	3.1 ± 0.15	10.0
-	+	161 ± 7.5 N.S.	$2.1 \pm 0.1^{**}$	7.0
-	-	155 ± 9.5	3.5 ± 0.15	10.5
+	+	$144 \pm 9.0^{**}$	$4.6 \pm 0.15^{**}$	12.5
+	-	192 ± 10	6.1 ± 0.25	16.5

Table 5. *Effect of insulin on $^{14}\text{CO}_2$ evolution from either $[1\text{-}^{14}\text{C}]$ leucine or $[\text{U-}^{14}\text{C}]$ leucine*

The muscles were incubated in Krebs-Ringer bicarbonate solution, at 37°C , for 60 min. The $^{14}\text{CO}_2$ was trapped in NaOH (see the Methods section). The values are the means of six determinations. Statistics, insulin versus control: in both experiments $P = 0.025$.

Substrate	Incubation conditions	$[^{14}\text{C}]\text{CO}_2$ (c.p.m./mg of muscle)
$[\text{U-}^{14}\text{C}]\text{Leucine}$	+ Insulin	1924 ± 58
	- Insulin	2217 ± 107
	Control (no muscle)	440
$[1\text{-}^{14}\text{C}]\text{Leucine}$	+ Insulin	2327 ± 137
	- Insulin	2621 ± 153
	Control (no muscle)	150

cellular specific radioactivity on insulin stimulation of ^{14}C -incorporation was studied (Goldstein & Reddy, 1970). Thus, probably in both the presence and the absence of insulin, extracellular amino acids are utilized in preference to those of the intracellular pool. This conclusion is not invalidated by the observations of Wool & Krah1 (1959) and Manchester & Krah1 (1959) with 'cut' diaphragm; for, as we have shown (Hider *et al.* 1969), even with the relatively 'unleaky' extensor digitorum longus preparation, there is, at 37°C , a significant efflux of amino acids into the medium. These amino acids could be utilized for incorporation into protein. Although this point was considered by Wool & Krah1 (1964), experiments with 'cut' diaphragm and extensor digitorum longus muscle (R. C. Hider, S. Barnes & L. Meade, unpublished observations) have demonstrated that, despite the precautions taken by Wool & Krah1 (1964), there is still a large release of ^{14}C -labelled amino acid generated from [^{14}C]pyruvate into the extracellular pool.

Unexpectedly, insulin decreased the specific radioactivity of both glycine and leucine in the extracellular pool. As the specific radioactivities of amino acids in this pool have been shown to be closely related to those of the amino acids incorporated into protein (Hider *et al.* 1971), it is unlikely that the insulin effect on amino acid incorporation results from any increase in the specific radioactivity of amino acid entering protein; but rather, there is a true increase in the rate of protein synthesis. In this context it is noteworthy that insulin increases the specific radioactivity of aminoacyl-tRNA isolated from heart muscle after perfusion with ^3H -labelled amino acids (Davey & Manchester, 1969). In these experiments total tRNA was isolated and it is conceivable that insulin increased the ratio of aminoacyl-charged to uncharged tRNA, rather than simply increasing the specific radioactivity of amino acids esterified by tRNA (Manchester, 1970). In fact Germanyuk & Mironenko (1969) have suggested that insulin insufficiency impairs the formation of aminoacyl-tRNA molecules in liver.

Insulin increased the amount of both glycine and leucine in the extracellular pool (Table 2). Munro (1968) has shown that the amino acid supply has a profound effect on the ribosomal profile in liver and Jefferson & Korner (1969), who obtained similar results, have suggested that the availability of amino acids regulates the binding of ribosomes to mRNA. Rat liver polyribosomes disaggregate on starvation but are readily re-formed by feeding with a complete diet (Sox & Hoagland, 1966). Wittman, Lee & Miller (1969) have shown that in alloxan-diabetic rats, the liver polyribosome profile is restored to normal by insulin treatment. Manchester (1970) has suggested that the

availability of amino acids may exert a controlling influence on the protein-synthetic rate by reducing the amount of uncharged tRNA. Earl, Broadus & Morgan (1967) showed that an increase in the concentration of amino acids supplied to perfused hearts increased the rate of incorporation but diminished the response to insulin. The insulin-induced increase of amino acid concentration in the extracellular space of extensor digitorum longus muscle could also affect the ribosomal profile and hence the protein-synthetic rate.

Estimation of the size of the extensor digitorum longus extracellular space with various markers has revealed evidence of extracellular compartmentation (Hider *et al.* 1971). That insulin changes the inulin space but not that of mannitol and sulphate (Table 3) suggests that there is some modification of extracellular compartmentation in the presence of insulin. This effect of the hormone on the inulin space of skeletal muscle has been noted by Creese & Northover (1961) and Fritz & Knobil (1963). This observation could be closely linked to the finding that insulin increases the amount of amino acids present in the extracellular spaces of extensor digitorum longus muscles.

The experiments utilizing muscles treated with cycloheximide showed that insulin decreased the amount of free leucine present in the system. This decrease could have arisen from either a corresponding decrease in the rate of protein catabolism, or from an increase in the rate of amino acid catabolism. Insulin has been reported to inhibit protein catabolism in liver (Christophe & Wodon, 1963; Mortimore & Mondon, 1970) and in adipose tissue (Minemura, Lacy & Crofford, 1970), and also to increase the rate of amino acid catabolism in chick embryo heart (Guidotti *et al.* 1968b) and in adipose tissue (Goodman, 1964; Jeanrenaud, Touabi, Cushman & Heindel, 1971). However, in the present work, with extensor digitorum longus muscle it was found that insulin significantly decreased the rate of catabolism of both [^{14}C]leucine and [$1\text{-}^{14}\text{C}$]leucine, as measured by $^{14}\text{CO}_2$ evolution. Thus it would appear that the insulin-induced decrease of leucine concentration results from an inhibition of proteolysis.

It is conceivable that the extracellular amino acid supply could control both protein synthesis and degradation, a plentiful amino acid supply promoting the synthesis and inhibiting the degradation and a sparse supply having the opposite effect.

A model for the mechanism of action of insulin on protein synthesis in muscle is shown in Fig. 2. Insulin increases the bidirectional flux of free amino acid across the cell membrane and between extra- and intra-cellular fluid compartments. It also stimulates the incorporation of extracellular amino acid into protein and reduces protein catabolism.

Fig. 2. Summary of the observed effects of insulin on the movement of amino acids in muscle incubated *in vitro*. The intensity of the line is an indication of the flux rates ($\pm$ insulin) between compartments. (a) Normal conditions; (b) with insulin. Abbreviations: I.C.F., intracellular fluid; E.C.F., extracellular fluid.

The net result is to lower the concentration of the external source of supply, which is either the incubation medium in studies *in vitro* or the plasma *in vivo*.

This study was initiated with a grant from the Wellcome Trust and completed with financial aid from the Medical Research Council. We are most grateful to both these bodies for their support.

REFERENCES

- Christophe, J. & Wodon, C. (1963). *Archs int. Physiol. Biochim.* **71**, 126.
- Creese, R. & Northover, J. (1961). *J. Physiol., Lond.*, **153**, 343.
- Davey, P. & Manchester, K. L. (1969). *Biochim. biophys. Acta*, **182**, 85.
- Earl, D. C. N., Broadus, A. E. & Morgan, H. E. (1967). *Abstr. FEBS 4th Meet., Oslo*, no. 5.
- Fritz, G. R. & Knobil, E. (1963). *Biochim. biophys. Acta*, **78**, 773.
- Germanyuk, Y. L. & Mironenko, V. I. (1969). *Nature, Lond.*, **222**, 486.
- Goldstein, S. & Reddy, W. J. (1970). *Archs Biochem. Biophys.* **140**, 181.
- Goodman, H. M. (1964). *Am. J. Physiol.* **206**, 129.
- Guidotti, G. G., Borghetti, G., Gaja, G., Loreti, L., Ragnotti, G. & Foa, P. P. (1968). *Biochem. J.* **107**, 565.
- Hernandez, T. & Coulson, R. A. (1969). *Am. J. Physiol.* **217**, 1846.
- Hider, R. C., Fern, E. B. & London, D. R. (1969). *Biochem. J.* **114**, 171.
- Hider, R. C., Fern, E. B. & London, D. R. (1971). *Biochem. J.* **121**, 817.
- Jeanrenaud, B., Touabi, M., Cushman, S. W. & Heindel, J. J. (1971). *Biochem. J.* **122**, 1 p.
- Jefferson, L. S. & Korner, A. (1969). *Biochem. J.* **111**, 803.
- Manchester, K. L. (1961). *Biochem. J.* **81**, 135.
- Manchester, K. L. (1970). *Biochem. J.* **117**, 457.
- Manchester, K. L. & Krah, M. E. (1959). *J. biol. Chem.* **234**, 2938.
- Minemura, T., Lacy, W. W. & Crofford, O. B. (1970). *J. biol. Chem.* **245**, 3872.
- Mondon, C. E. & Mortimore, G. E. (1967). *Am. J. Physiol.* **212**, 173.
- Mortimore, G. E. & Mondon, C. E. (1970). *J. biol. Chem.* **245**, 2375.
- Munro, H. N. (1968). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **27**, 1231.
- Pozefsky, T., Felig, P., Soeldner, J. S. & Cahill, G. F. (1968). *Trans. Ass. Am. Physns*, **81**, 258.
- Sox, H. C. & Hoagland, M. B. (1966). *J. molec. Biol.* **20**, 113.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 119. Minneapolis: Burgess Publishing Co.
- Wittman, J. S., Lee, K. L. & Miller, O. N. (1969). *Biochim. biophys. Acta*, **174**, 536.
- Wool, I. G. & Krah, M. E. (1959). *Nature, Lond.*, **183**, 1399.
- Wool, I. G. & Krah, M. E. (1964). *Biochim. biophys. Acta*, **82**, 606.