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The Control by Insulin of Amino Acid Accumulation in Muscle

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1. The capacity of insulin to enhance the accumulation by muscle of several amino acids was studied. Reports that threonine uptake is enhanced by insulin were not confirmed, despite its enhanced incorporation into protein. Uptake of β-alanine and γ-aminobutyric acid also did not respond to the hormone. A stimulation of accumulation of alanine, histidine and ethionine was observed. 2. The capacity of inhibitors of protein synthesis to reveal a stimulation by insulin of accumulation of several amino acids, hitherto considered unresponsive to insulin in the absence of inhibitor, was confirmed. Cycloheximide was as effective as puromycin. However, two of these amino acids, alanine and histidine, here showed response to insulin in the absence of inhibitor. The presence of cycloheximide was found to enhance uptake of cycloleucine, ethionine and threonine; in its presence insulin enhanced uptake of β-alanine and α-methyltyrosine. 3. It is concluded that the influence of inhibitors of protein synthesis on amino acid accumulation and the response of amino acid accumulation to insulin are not adequately explained on the basis of inhibition of protein synthesis allowing amino acids to accumulate more readily. 4. The technical problems of whether linear rates of incorporation of amino acids into protein really indicate more than one cell pool are discussed and safeguards suggested. That initial rates of incorporation of label into protein are likely to be non-linear is shown.

Insulin added *in vitro* to muscle preparations stimulates their capacity to incorporate labelled amino acids into protein. The hormone also stimulates the accumulation by muscle of a number of amino acids, but whereas enhancement of incorporation is seen for all the amino acids (for review see Manchester, 1970) increase in accumulation is seen for only some amino acids; the present position is listed in Table 1. The reason why insulin can be seen to stimulate the accumulation of only some of the naturally occurring amino acids is not clear. It has been suggested that compartmentation of the cell pool obscures observation of an effect of the hormone on a fraction of the pool, or that the enhancement of incorporation in the presence of insulin drains amino acids away from the cell pool so rapidly as to prevent observable enhancement of accumulation occurring. The latter view is supported by observations of Castles & Wool (1964) and Wool, Castles & Moyer (1965) that in the presence of puromycin insulin enhances the accumulation of several amino acids hitherto considered unresponsive. Their experiments were carried out under conditions in which a high proportion of the total quantity of labelled amino acid in the tissue at the end of the incubation had become incorporated into protein. If the capacity of inhibitors of protein synthesis to reveal an influence of insulin on accumulation of amino acids does arises for this reason, we should find both that insulin stimulates accumulation of amino acids when they are presented to the tissue in higher concentrations such that the proportion of accumulated material going into protein is decreased, and also that, if the higher quantities of amino acid are such as to saturate the transport system, an effect of inhibitor to facilitate observation of the stimulation of amino acid uptake by insulin will not be seen. These possibilities have been explored together with measurements of the effect of insulin on the accumulation of a number of other amino acids.

The problems posed by compartmentation are more difficult to resolve. Its existence is postulated from findings of linear rates of incorporation during the early period of incubation before accumulation of the labelled amino acids reaches a steady state (Kipnis, Reiss & Helmreich, 1961), but the degree of linearity is somewhat variable for different tissues and amino acids (Manchester & Wool, 1963; Hider, Fern & London, 1969). If linear rates of incorporation are taken to indicate that incorporation occurs either as the amino acid enters the

cell or near the membrane (Hendler, 1962; Hider *et al.* 1969), we might expect that amino acids synthesized in the cell from non-amino acid precursors would exhibit non-linear incorporation rates because of their greater opportunity for mixing with the cell pool before incorporation. For example [^{14}C]acetate, which is incorporated into protein as [^{14}C]glutamate and [^{14}C]aspartate (Manchester, 1960), must enter the mitochondrion in the course of utilization and then move to the site of incorporation. Rosenberg, Berman & Segal (1963) found that the rate of oxidation of amino acids by kidney slices did indeed follow the extent of accumulation. The linearity or otherwise of incorporation into protein of ^{14}C from [^{14}C]acetate has therefore been measured.

METHODS

Diaphragm muscle was taken from albino rats (approx. 100g) who had had access to food and water at all times. Hemidiaphragms were incubated at 37°C in Krebs–Ringer bicarbonate (Krebs & Henseleit, 1932), gassed with O_2+CO_2 (95:5), to which no glucose or other oxidizable substrate was added other than the stated radioactive compounds. Stock insulin solutions were tested periodically to show that they had retained activity on recognized responsive parameters (i.e. aminoisobutyrate uptake).

Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., Department des Radioéléments, Gif-sur-Yvette, France, or from NEN Chemicals G.m.b.H., 6072 Dreieichenheim, West Germany, and were usually used within a few weeks of delivery. They were added to the medium either undiluted or diluted to a standardized concentration of 1mM as indicated. Puromycin came from Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A., and cycloheximide (actidione from *Streptomyces griseus*) from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. L-α-Methyl-*p*-tyrosine was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A.

At the end of the incubation period the tissue was removed from the incubation flasks, blotted and either plunged into boiling water to extract free amino acids or placed in 10% trichloroacetic acid for preparation of protein samples by the procedures described previously (Manchester & Young, 1960*a*; Manchester, 1966). The radioactivities of samples of ^{14}C-containing medium and of tissue extracts were measured by scintillation counting after addition to a mixture of 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (0.4%) in toluene–Triton X-100 (2:1, v/v); 1ml of aqueous ^{14}C-containing solution was added to 10ml of the toluene–Triton solution (Patterson & Greene, 1965). Efficiency of scintillation counting was about 80%. Protein samples, after washing with 10% trichloroacetic acid, NaOH and acetone were plated on stainless-steel planchets and radioactivity was measured in gas-flow equipment at about 25% efficiency.

Colorimetric estimations were carried out on deproteinized extracts. Histidine was estimated by the method of Macpherson (1946), tyrosine and α-methyltyrosine were estimated by the method of Udenfriend & Cooper (1952) and arginine was measured as described by Dubnoff (1957).

In the time-course experiments the tissue was incubated for 30min before addition of radioactive precursor in Warburg flasks sealed with skirted rubber caps after gassing. At the end of the period of preincubation the labelled acetate or amino acid was injected through the cap. Incubation was continued for the period stated in Fig. 1 and the tissue was then removed from the medium, blotted and placed in ice-cold 10% trichloroacetic acid. Protein samples were then prepared as usual. Cycloheximide (10μg/ml), when added, was added to the medium before the start of the preincubation to ensure that protein synthesis had ceased before introduction of the labelled material (Manchester, 1967).

The 'accumulation ratio' in the tables is defined as the amount of radioactivity or measured amino acid in the tissue water (i.e. tissue weight/0.75 to correct for content of solids) divided by that in an equal volume of medium. For the most part the ^{14}C-labelled amino acids were present in the medium at about 0.1μCi/ml, but occasionally higher. The results in the tables are presented as the mean ±S.E.M. for a given number of observations and the significance of the differences between means was assessed by Student's *t* test. Since the diaphragm yields two hemidiaphragms, one each of which went into separate groups, it is also possible to calculate the mean and S.E.M. of the paired differences and this has been done in some cases where inter-animal variations obscured an otherwise significant difference between groups.

RESULTS

Linearity of amino acid incorporation. Measurement of incorporation over short periods presents a technical problem in that an amount of non-specific contamination of protein samples due to incomplete efficiency in the washing procedures employed in their preparation which may be tolerable (e.g. 2%) in values obtained after incubation for 1h becomes a serious error (25%) with a sample incubated for 5min. The lower the proportion of label incorporated to that added to the medium, as when for example a non-amino acid precursor is employed, the greater is the potential error. To minimize therefore the error likely to result when short periods of incubation were used, controls preincubated with an amount of cycloheximide sufficient to produce virtually complete inhibition of protein synthesis (Manchester, 1967) were introduced. The counts in these protein samples after incubation and extraction exactly as for the uninhibited samples were deducted from experimental points.

The results of this procedure are shown in Fig. 1(*a*) in which incorporation from [^{14}C]acetate is followed. The points constituting the upper line are the experimental values before deduction of the correction of the cycloheximide-treated sample.

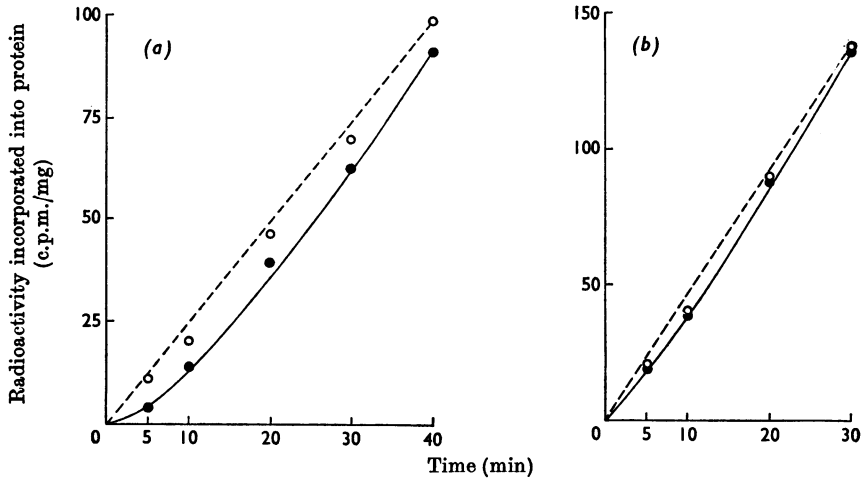

Fig. 1. Time-course of incorporation of ^{14}C from (*a*) acetate and (*b*) leucine into protein of isolated rat diaphragm. ○, Amount of incorporation actually observed; ●, incorporation remaining after subtracting the radioactivity found in samples similarly prepared but with the incubation carried out in medium containing cycloheximide. ----, A straight line connecting the last point and the origin to give some measure of the departure from linearity of the rate of incorporation. Radioactivity added: for acetate, 0.4 μCi/ml; for leucine, 0.08 μCi/ml.

This line is virtually linear, starting from the origin. After deduction of the counts apparently incorporated in the presence of cycloheximide, however, the lower curve is obtained in which a delay in commencement of incorporation can now be observed. Since the tissue was incubated in medium containing approx. 2.5×10^5 c.p.m./ml the contamination in this instance constitutes approx. 0.04%, although of course a large proportion of the counts would not have been taken up by the tissue in the first instance and may also include [^{14}C]-acetate incorporated into non-protein contaminants. This experiment illustrates the importance of including an adequate form of control in such experiments of short duration. Fig. 1(*b*) shows that for incorporation of [^{14}C]leucine, one of the most rapidly incorporated amino acids, there is again some correction in the shape of the graph to be made which then begins to show departure from linearity.

Promotion by insulin of amino acid accumulation. Table 1 lists the various amino acids on whose uptake by muscle (heart or diaphragm) the influence of insulin has been tested. For the majority of the amino acids, both those entering protein and a number of other species studied, insulin is without observable effect. A number of amino acids are included here for the first time and the placing of others has been reassessed. Thus (Table 2) insulin was found to be without effect on the accumulation of β-alanine or γ-aminobutyric acid, neither of which appeared to reach concentrations in the tissue water greater than in the medium. Insulin was also without effect on accumulation of *p*-fluorophenylalanine and canavanine, as might be expected from the lack of influence on uptake of phenylalanine and arginine (Manchester & Young, 1960*a*). By analogy with methionine Akedo & Christensen, 1962), uptake of ethionine was enhanced by the presence of insulin. There was negligible incorporation into protein of ^{14}C from ethionine, β-alanine, γ-aminobutyric acid or canavanine.

Of the naturally occurring amino acids accumulation of alanine has been regarded as unresponsive to insulin. However, on both past occasions on which its uptake was studied there was suggestion of a stimulation, though the difference was not statistically significant (Manchester & Young, 1960*a*; Akedo & Christensen, 1962). Reinvestigation of this point suggests that significant if not large enhancement of uptake of [^{14}C]alanine is observable (Table 2). With histidine there is a recent report of stimulation of its uptake by chick embryo heart (Guidotti, Lüneburg, & Borghetti, 1969). In the present series of experiments a small but significant effect of insulin to enhance uptake of histidine when added both at tracer concentration and at 1 mM was found. The response of both alanine and histidine is particularly relevant to the studies with inhibitors of protein formation (see below).

Uptake of serine has previously been found in one study to respond to insulin (Wool, 1964), but in another not to (Akedo & Christensen, 1962). A stimulation by insulin of serine uptake was noted

Table 1. *Amino acids whose accumulation by muscle is or is not enhanced by insulin*

References: [1] Manchester & Young (1960*a*), [2] Akedo & Christensen (1962), [3] Guroff & Udenfriend (1961), [4] Manchester & Wool (1963), [5] Wool (1964), [6] Elsas, Albrecht & Rosenberg (1968), [7] Christensen & Cullen (1969), [8] Guidotti *et al.* (1969), [9] Table 2. A more complete bibliography is given in Manchester (1970).

Uptake enhanced by insulin

Alanine[9]
Glycine[1,2,4]
Histidine[8,9]
Methionine[2]
Proline[2,4,5]
(Hydroxyproline[5])
Serine[5,9]
Threonine[5,8]
Aminoisobutyric acid[1,2,4]
Cycloleucine[2,6]
Ethionine[9]
Isovaline[2]
Sercosine[2]

Uptake not enhanced by insulin

Alanine[1,2,4]
Arginine[5]
Aspartic acid[1]
Cystine[5]
Glutamic acid[1,4]
Histidine[2,5]
Isoleucine[5]
Leucine[1,2,5]
Lysine[1,4]
Phenylalanine[1,4,5]
Threonine[9]
Tryptophan[5]
Tyrosine[3,4]
Valine[2,4]
β-Alanine[9]
γ-Aminobutyric acid[9]
Canavanine[9]
α,γ-Diaminobutyric acid[6]
p-Fluorophenylalanine[9]
α-Methyltyrosine[3]
Norleucine[2]
Ornithine[1]
α-Aminobicyclo[2,2,1]heptene-2-carboxylic acid[7]

in the present instance (Table 2). However, enhancement of threonine uptake observed by Wool (1964) and by Guidotti *et al.* (1969) was not confirmed when looked for in either the hemidiaphragm or intact preparation. Lack of response of threonine has also been noted by R. C. Hider, E. B. Fern & D. R. London (personal communication). The concentration of amino acid added could be of importance; the present work used slightly higher concentrations than did Wool (1964), but since diaphragm liberates amino acids into the medium during incubation (Manchester, 1961) a very low initial concentration of tracer soon undergoes substantial dilution and it is unlikely therefore that this constitutes a significant difference. In view of enhancement of uptake of histidine, uptake of another basic amino acid, namely arginine, was studied, but no effect of insulin seen. For both histidine and arginine colorimetric estimations were carried out in parallel with the radioactivity measurements, with similar results. Likewise, no effect of insulin on accumulation of tyrosine was seen by either form of measurement. Slightly higher concentration ratios were observed in the colorimetric as opposed to radioactive measurements. This may be indicative of the presence of amino acid in the tissue not exchanging with the labelled form, or merely that with a high external concentration there is negligible leakage of the amino acid out of the tissue and the endogenous pool plus the ^{14}C-labelled species concentrated (since the endogenous content is much lower than 1 mM) are additive.

Effects of puromycin and cycloheximide. It is possible that in the presence of puromycin enhanced accumulation results from incorporation of amino acids into peptide fragments (Smith, Traut, Blackburn & Monro, 1965), the small peptides being counted in the free amino acid rather than protein fractions. Cycloheximide inhibits protein synthesis by a mechanism not subject to this complication (Felicetti, Colombo & Baglioni, 1966; Heintz, Salas & Schweet, 1968). Cycloheximide enhanced the effect of insulin on amino acid accumulation (Table 3). The additional presence of actinomycin D at a concentration that inhibits RNA synthesis in diaphragm (Manchester, 1964; Wool & Moyer, 1964) was without influence. In the presence of cycloheximide a stimulating influence of insulin was again seen on the accumulation of alanine after a slightly shorter incubation period, and also for histidine (Table 4). Accumulation of these two amino acids showed the largest proportional response to insulin in the studies of Castles & Wool (1964) and Wool *et al.* (1965) but the significance of their observations in the presence of inhibitor now loses much of its force in the finding of increased accumulation of alanine and histidine with insulin in the absence of inhibitor (Table 2). With other amino acids a significant enhancement of tyrosine uptake was also noted, but no change was found in the accumulation of arginine. In these experiments the concentration of amino acid was initially 1 mM. Much lower concentrations were employed by Castles & Wool (1964) and Wool *et al.* (1965). The higher concentrations permitted use of colorimetric estimation, which showed similar if less clear-cut results. However, with arginine and threonine at lower concentrations no significant stimulation of uptake by insulin in the presence of cycloheximide was seen, though the value for threonine suggested a slight increase. An appreciable difference in the extent of accumulation of threonine in the absence of insulin as a result of the addition of cycloheximide appeared possible from comparison of the values in Tables 2 and 4. Direct comparison (Table 5) showed some difference though no more marked than for several other amino acids. Uptake of β-alanine in the presence of cycloheximide also showed a small stimulation in response to insulin.

Table 2. *Effect of insulin on the accumulation of amino acids by isolated rat diaphragm*

Each value is the mean ± S.E.M. of six observations. The values of P for differences that are significant are indicated. Incubation was for 1 h.

Amino acid	Concn. (μM)	Accumulation ratio measured: For ^{14}C-labelled amino acid − Insulin	For ^{14}C-labelled amino acid + Insulin	By chemical determination − Insulin	By chemical determination + Insulin
Alanine	16	1.85 ± 0.03	2.31 ± 0.04		
		$P < 0.001$			
Alanine	1000	1.84 ± 0.04	2.52 ± 0.09		
		$P < 0.001$			
Arginine	1000	2.01 ± 0.05	2.02 ± 0.063	2.67 ± 0.21	2.46 ± 0.10
Histidine	3.2	3.56 ± 0.13	4.01 ± 0.27		
		$\Delta = 0.451 \pm 0.16$			
		$P < 0.05$			
Histidine	1000	2.97 ± 0.15	3.36 ± 0.09	3.04 ± 0.16	3.52 ± 0.21
		$P = 0.05$			
		$\Delta = 0.379 \pm 0.089$			
		$P < 0.01$			
Serine	1.1	3.94 ± 0.15	4.55 ± 0.27		
		$P < 0.02$			
		$\Delta = 0.618 \pm 0.19$			
		$P < 0.05$			
Threonine	40	2.85 ± 0.12	3.00 ± 0.05		
Threonine*	8	2.30 ± 0.07	2.24 ± 0.04		
Tyrosine	1000	2.02 ± 0.053	2.03 ± 0.025	3.11 ± 0.06	3.14 ± 0.04
β-Alanine	15	1.07 ± 0.03	1.08 ± 0.02		
γ-Aminobutyric acid	32	0.82 ± 0.01	0.82 ± 0.02		
Ethionine	120	1.66 ± 0.052	2.24 ± 0.12		
		$P < 0.01$			
		$\Delta = 0.585 \pm 0.078$			
		$P < 0.001$			
p-Fluorophenylalanine†	1000	1.54 ± 0.08	1.46 ± 0.06		
Canavanine†	5	1.44 ± 0.02	1.40 ± 0.02		

* Intact diaphragm preparation.
† DL-Form.

Table 3. *Effect of inhibitors of protein and nucleic acid synthesis on the uptake of* [^{14}C]*alanine by isolated rat diaphragm*

Incubation was for 1.5 h; [^{14}C]alanine was originally at a concentration of 1 mM. Concentrations of inhibitors were: puromycin, 200 μg/ml; cycloheximide, 100 μg/ml; actinomycin, 10 μg/ml. Each value is the mean ± S.E.M. of six observations.

Additions to medium	Accumulation ratio: − Insulin	+ Insulin
Puromycin	2.21 ± 0.06	3.41 ± 0.15
	$P < 0.001$	
Cycloheximide	1.90 ± 0.03	2.84 ± 0.16
	$P < 0.001$	
Cycloheximide + actinomycin	1.98 ± 0.04	2.93 ± 0.16
	$P < 0.001$	

As pointed out by Wool *et al.* (1965), if the presence of an inhibitor of protein synthesis facilitates observation of the effect of insulin to enhance amino acid accumulation because it stops the drain of amino acids away from the cell pool into protein, we might likewise expect to see enhanced accumulation also resulting from addition of the inhibitor alone. Wool *et al.* (1965) did not find very marked evidence for this. Table 5 shows that puromycin and cycloheximide can themselves stimulate amino acid accumulation. This is shown for cycloleucine and ethionine, which do not incorporate into protein, and for threonine and α-methyltyrosine. Whether the last is incorporated into protein is not known. Neither the response of β-alanine to insulin in the presence of cycloheximide nor the capacity of the inhibitor to enhance accumulation of amino acids not contained in protein is consistent with its effects resulting specifically from decreased protein synthesis.

Lastly it is important to ask whether the proportion of the uptake of amino acid that enters

Table 4. *Effect of insulin on the accumulation of amino acids by isolated rat diaphragm in the presence of cycloheximide*

Each value is the mean ± S.E.M. of six observations. The values of P for differences which are significant are indicated. Incubation was for 1 h.

Amino acid	Concn. (μM)	Accumulation ratio measured: For ^{14}C-labelled amino acid, −Insulin	For ^{14}C-labelled amino acid, +Insulin	By chemical determination, −Insulin	By chemical determination, +Insulin
Alanine	1000	1.91 ± 0.034	2.46 ± 0.065		
		$P < 0.001$			
Arginine	0.5	3.14 ± 0.11	3.23 ± 0.12		
		$\Delta = 0.092 \pm 0.15$			
Arginine	1000	2.16 ± 0.064	2.17 ± 0.052	2.76 ± 0.14	2.74 ± 0.09
Histidine	1000	3.00 ± 0.055	3.35 ± 0.048	3.90 ± 0.21	4.23 ± 0.10
		$P < 0.001$			
Threonine	40	4.21 ± 0.13	4.67 ± 0.21		
		$\Delta = 0.459 \pm 0.20$			
Tyrosine	1000	1.84 ± 0.032	1.98 ± 0.015	2.04 ± 0.15	2.16 ± 0.47
		$P < 0.01$			
β-Alanine	15	1.06 ± 0.021	1.15 ± 0.019		
		$P = 0.01$			

Table 5. *Effect of puromycin and cycloheximide on the accumulation of various amino acids by isolated rat diaphragm*

Each value is the mean ± S.E.M. of the number of observations in parentheses. The values of P for differences which are significant are indicated. α-Methyltyrosine was estimated colorimetrically and puromycin (200 μg/ml) was the inhibitor used. Cycloleucine and ethionine were measured as ^{14}C and cycloheximide (100 μg/ml) was the inhibitor employed. Incubation was for 1 h.

Amino acid	Concn. (μM)		Accumulation ratio: −Inhibitor	+Inhibitor
α-Methyltyrosine	1000	(12)	2.39 ± 0.045	2.59 ± 0.078
			$P < 0.05$	
Cycloleucine	70	(6)	1.60 ± 0.076	1.82 ± 0.048
			$P < 0.05$	
Ethionine	120	(6)	1.72 ± 0.038	2.07 ± 0.049
			$P < 0.001$	
Threonine	40	(6)	3.51 ± 0.15	3.97 ± 0.20
			$\Delta = 0.464 \pm 0.102$	$P < 0.01$

protein does affect the nature of the response of the amino acid to insulin. Table 6 compares for some of the same experiments reported in Table 2 the amount of radioactivity that is extractable from the tissue at the end of the incubation with that calculated to have entered protein. Since loss of protein occurs during washing of the samples it is necessary to calculate the amount of incorporation into protein from the specific activity of the samples multiplied by the protein content (approx. 200 mg) of each 1 g of tissue. As is to be expected the proportion of radioactivity entering the tissue that is incorporated into protein is less when the amino acid is presented at 1 mM than at lower concentrations, the values in the absence of insulin being 16, 7.1, 3.7 and 5.5% respectively for alanine, arginine, histidine and tyrosine added at 1 mM, and 29, 29, 32 and 43% for alanine, histidine, serine and threonine added at tracer concentrations. Despite this variation in the proportion of accumulated amino acid incorporated into protein an enhancement of accumulation is observed (Table 2) for histidine and serine but not for arginine, threonine and tyrosine. There is thus no obvious correlation between the proportion of amino acid going into protein and whether there is response to insulin. Similar values for glycine and proline on the one hand as opposed to leucine and phenylalanine have been presented elsewhere (Manchester, 1969). It must also be remembered that the incorporation is given as the

Table 6. *Comparison of the amount of labelled amino acid found in protein of diaphragm after incubation with that extractable from the tissue*

Each value is the mean and for incorporation ± S.E.M. of six observations. The stimulation of incorporation was in each case significant either on the stated values or the paired differences. Incubation was for 1 h. Radioactivity was present at about 0.08 μCi/ml, except for threonine where it was for the first experiment 0.80 μCi/ml and for the second about 0.16 μCi/ml.

		10^{-3} × Radioactivity			
		In tissue (d.p.m./g wet wt.)		Incorporated into protein (d.p.m./200 mg of protein)	
Amino acid	Concn. (μM)	−Insulin	+Insulin	−Insulin	+Insulin
Alanine	16	218	295	62 ± 4.7	94 ± 5.2
Alanine	1000	229	292	37 ± 2.1	52 ± 2.5
Arginine	1000	243	241	17.2 ± 0.60	22.9 ± 0.89
Histidine	3.2	368	398	108 ± 11	157 ± 12
Histidine	1000	355	374	13.3 ± 0.80	18.9 ± 0.62
Serine	1.1	393	411	125 ± 13	164 ± 18
Threonine	40	2960	3150	1265 ± 113	1681 ± 108
Threonine*	8	207	202	130 ± 14	189 ± 19
Tyrosine	1000	239	242	13.2 ± 0.65	18.8 ± 0.88

* Intact diaphragm preparation.

amount of radioactivity that has been incorporated steadily during the hour's incubation, whereas for many of the amino acids accumulation will by this time have reached a steady state.

DISCUSSION

Effects of insulin

The present work extends the list of amino acids whose uptake is not enhanced by insulin, namely by the addition of β-alanine and γ-aminobutyric acid. Since neither showed any sign of concentrative uptake lack of response to insulin is not surprising. Christensen (1964) noted that β-alanine was strongly concentrated by ascites cells. Because of its similarity to methionine, enhancement by insulin of ethionine uptake is to be expected. The finding of enhancement of alanine uptake removes an anomaly in that in Christensen's nomenclature insulin appears to stimulate amino acids using the alanine-preferring site (glycine, aminoisobutyrate and now alanine) rather than those taken up by the 'L site' (Oxender & Christensen, 1963). Why threonine should in these studies be unresponsive to insulin is not clear, but the author's interest was first aroused in noticing that threonine both forms a very stable aminoacyl-tRNA species, and that it can also interchange with leucine in variant protein sequences (Smith, 1967). These are properties more typical of the hydrophobic unresponsive amino acids than of the hydrophilic amino acids. The rationale of the position of histidine in the list (Table 1) is not apparent. Probably we would be wiser to consider a continuous spectrum of behaviour of amino acid uptake towards insulin rather than regarding them as simply responsive or unresponsive, though it must be stressed that enhancement of their incorporation into protein is uniformly responsive.

The original intention of this work was to seek an alternative explanation of why uptake of several amino acids appeared to respond to insulin in the presence of puromycin though not in its absence, since the reasoning of Wool *et al.* (1965) seemed inadequate. In the hope of avoiding possible complications due to formation of small peptides cycloheximide has been used here rather than puromycin. From the present results it now seems questionable whether the phenomenon remains to be explained. The two amino acids found by Castles & Wool (1964) to show the largest proportional change in response with inhibitor, alanine and histidine, appear in fact to show a response in the absence of inhibitor, and no very convincing influence of cycloheximide to reveal a response of arginine or threonine to insulin was found. Nor did the response to insulin in proportional terms change very much with the concentration of amino acid employed. Since the proportion of labelled uptake entering protein varies with the concentration presented to the tissue (Table 6) it might be expected either that the response to insulin in the absence of inhibitor would be more easily seen as the concentration of added amino acid is raised or that the influence of the inhibitor would be less marked at higher concentrations, but no evidence for either

of these propositions was seen now or in any earlier studies (e.g. Manchester & Young, 1960*a*, Manchester & Wool, 1963). Scharff & Wool (1965) measured by chemical means the concentrations of amino acids in hearts perfused with and without insulin and puromycin. They found higher concentrations of taurine and ornithine in the presence of insulin when puromycin was also present and a significant stimulation by insulin in the presence of inhibitor for valine and leucine as well as for serine, proline, alanine and methionine (though not threonine). However, the significance of several of the differences with insulin depend as much on the apparent decline in the puromycin-without-insulin values as on an increase in the insulin values and the pattern of changes is complex. Moreover, it is surprising that puromycin did not consistently increase the amounts of free amino acids, assuming that protein breakdown was continuing at its normal rate. For a rate of incorporation into protein of 5μmol of amino acid/h per g of tissue (Manchester & Young, 1961; Hechter & Halkerston, 1964; Buse & Buse, 1967) we require roughly 0.25μmol of each amino acid/h per g of muscle, which is considerably in excess of the concentration of at least half of the amino acids. As pointed out by Gan & Jeffay (1967) reutilization of amino acids resulting from proteolysis is an important source of material for new protein synthesis. In short, the author would question whether there is now any conclusive evidence that inhibition of protein synthesis does facilitate observation of an influence of insulin on amino acid accumulation, and we must accept for the present that insulin can enhance to an appreciable extent the uptake of only a limited number of species.

Two further factors may be relevant to possible stimulation of amino acid uptake by puromycin. First, its glycogenolytic action (Appleman & Kemp, 1966) may increase the availability of energy for amino acid transport. It may be noted that concentration ratios greater than 1 were found even when the amino acids were presented at 1mM. This implies active accumulation even for species such as tyrosine since the initial endogenous concentrations are very much lower. Secondly, the dislocation of polyribosomes in the cell resulting from the presence of the inhibitors may increase the rate or extent of mixing of labelled amino acids with the endogenous cell pool. The extent of this possibility is difficult to assess, not least in part because, as the data of Fig. 1 are intended to demonstrate, and has been found elsewhere (Manchester & Wool, 1963; Hider *et al.* 1969), the extent and nature of compartmentation of the amino acid pool in muscle is uncertain. The rapid incorporation of ^{14}C from acetate and the ready oxidation of several labelled amino acids by diaphragm (Manchester, 1965) suggests that the mitochondria, which are usually seen between the bundles of fibrils, are not a space into which newly arrived amino acids have difficulty in penetrating. Finally there are several anomalous findings concerned with amino acid transport in muscle to which the actions of the two antibiotics may be related. These are: first, the fact that aminoisobutyrate uptake by the intact diaphragm is very much slower than that carried out by the hemidiaphragm (Manchester, 1966) and that the capacity for active uptake can be enhanced by, for example, crushing the tissue with a glass rod (Peckham & Knobil, 1962). Secondly, diaphragm from acutely diabetic animals appears to accumulate aminoisobutyrate and proline at an enhanced rate and exhibits a greater than normal response to insulin (Castles, Wool & Moyer, 1965) as well as showing an apparently enhanced rate of incorporation (Manchester & Young, 1960*b*).

Thirdly, trypsin treatment of muscle reportedly enhances proline uptake (Rieser & Rieser, 1964) and phlorrhizin enhances aminoisobutyrate uptake by kidney slices (Segal, Blair & Rosenberg, 1961). The enhanced entry of sugars into muscle induced by anoxia has suggested the possibility that the cell normally limits the rate of glucose entry (Smith, Randle & Battaglia, 1961). It is possible that with amino acids there is again a mechanism imposing a limitation on their rate of entry, for example, to minimize expenditure of energy, but that inhibitors of protein synthesis and other agents overcome this mechanism and allow accumulation to occur more rapidly and to respond to insulin. Alternatively, if despite inhibition of protein synthesis breakdown proceeds at the usual rate, one might expect formation of an enhanced intracellular pool of amino acid with which more exchange could occur, so leading to enhanced uptake of the labelled form.

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