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Insulin and Incorporation of Amino Acids into Protein of Muscle

2. ACCUMULATION AND INCORPORATION STUDIES WITH THE PERFUSED RAT HEART*

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(*Received* 13 *March* 1963)

Kipnis & Noall (1958) found that insulin *in vitro* stimulated the accumulation of the non-utilizable amino acid, α-aminoisobutyric acid, by isolated rat diaphragm. Their discovery suggested that an action of insulin to promote the accumulation of amino acids might be the means by which the hormone enhances the incorporation of amino acids into protein in this tissue (Sinex, MacMullen & Hastings, 1952; Krahl, 1953). However, the extent to which insulin does in fact influence the intracellular accumulation of amino acids is still uncertain. Manchester & Young (1960) found insulin to be without effect on the uptake of alanine, leucine, phenylalanine, lysine, ornithine, aspartic acid and glutamic acid under the conditions in which it increased that of aminoisobutyric acid, although it did stimulate the uptake of glycine. Guroff & Udenfriend (1961) were unable to find a stimulation by insulin of the uptake of tyrosine or of another non-utilizable α-methyl amino acid, α-methyltyrosine. Akedo & Christensen (1962) have demonstrated an enhancement by insulin of the accumulation of sarcosine, L- and D-isovaline, cycloleucine, methionine and proline, though not of serine, valine, histidine or norleucine. Thus there is little support for the view that insulin promotes amino acid incorporation, an effect observable for every amino acid studied (Manchester & Young 1958; Wool & Krahl, 1959), through the stimulation of accumulation. However, the usefulness of experiments of this sort has been questioned by Kipnis, Reiss & Helmreich (1961). Because they were unable to find an increase in the rate of incorporation of [^{14}C]proline into diaphragm protein as its specific radioactivity in the cell amino acid pool rose, they suggested that the pool from which amino acids are taken for protein synthesis is more limited and not congruous with the total measurable amino acid pool. The lack of an effect of insulin on the entry of amino acids into the total measurable pool would not preclude an effect of the hormone on entry into a more limited fraction. However, the significance of the observation of Kipnis *et al.* (1961) could be simply that the specific radioactivity of ^{14}C-labelled amino acids penetrating into the cell rises to its maximum value in the region of the cell membrane and the ribosomes of the sarcoplasmic reticulum much more rapidly than in the total amino acid pool of the tissue which includes amino acids dissolved in water associated with the myofibrils.

In each instance cited the tissue studied has been the isolated rat diaphragm, either the cut or intact preparation (Kipnis & Cori, 1957). To both prepara-

* Part 1: Manchester (1961).

tions there are objections: the severed edges of the muscle fibres of the cut diaphragm provide a means of access to the cell interior not involving passage across the cell membrane; in the intact diaphragm the amino acid must penetrate the pleural and peritoneal membranes to enter the cells, and the uptake and release of amino acids by the intercostal muscle fibres adds a further complication. The perfused rat heart, which has been used extensively for studies of sugar penetration and carbohydrate metabolism (Bleehen & Fisher, 1954; Morgan, Henderson, Regen & Park, 1961), provides an alternative and advantageous muscle preparation in which to measure the rate and manner of accumulation and incorporation of ^{14}C-labelled amino acids. A study of the effect of insulin on the uptake of amino acids in this tissue and the time-course of their incorporation into protein has been our present purpose.

METHODS

Chemicals. The ^{14}C-labelled amino acids were obtained variously from Volk Radiochemical Co., Nuclear-Chicago Corp. and The Radiochemical Centre, Amersham. All were uniformly labelled except [1-^{14}C]glycine and amino[1-^{14}C]-isobutyric acid. The insulin used (Eli Lilly and Co.) was assayed to contain 25·2 units/mg. and contained less than 0·1% of glucagon.

Preparation and perfusion of hearts. Male rats of the Sprague-Dawley strain, weighing 250-300 g., were maintained under standard conditions and allowed free access to food and water at all times. Heparin was injected intraperitoneally about 1 hr. before the rats were killed.

The technique and apparatus used for the perfusion of hearts with a small volume of recirculating perfusate was that described by Morgan *et al.* (1961). Each rat was decapitated, and the heart quickly excised and placed in ice-cold 0·9% NaCl. After 1-2 min. in 0·9% NaCl the heart was fitted through its aorta on to the perfusion cannula and tied in position. In the perfusion vessel was placed 25 ml. of medium [Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932), gassed with O_2+CO_2 (95:5) or, when indicated, N_2+CO_2 (95:5), pH 7·4 at 37°]. With a head of pressure of about 5 cm. Hg, 15 ml. of the perfusion medium was run through the heart and discarded. Subsequently fluid emerging from the heart was collected at the base of the perfusion chamber and, after passage through a sintered-glass filter, was returned by a peristaltic pump to the reservoir from which the fluid again entered the heart. Regular cardiac contractions generally persisted throughout the periods studied, though the rate varied appreciably between different hearts. There was no apparent correlation between the rate of contraction and the rate of accumulation or total incorporation of amino acids.

Insulin, cyanide and azide, when present, were added to the system after the initial 15 ml. of buffer had passed through the heart. The ^{14}C-labelled amino acids were also added at this stage; in the experiments with higher concentrations of amino acids the unlabelled carrier was added to the medium from the beginning, the ^{14}C-labelled amino acid being added as indicated above. In the experiments in Fig. 2, by use of a three-way tap, hearts were switched at the commencement of the uptake period from plain buffer to medium in which the ^{14}C-labelled amino acid was already distributed.

Incubation of diaphragm. Hemidiaphragms from female Wistar rats were removed and incubated as described by Manchester & Young (1960). In the experiments of Fig. 3 the hemidiaphragms were incubated in Warburg flasks, the ^{14}C-labelled amino acids being contained in 0·1 ml. of 0·9% NaCl in the side arm and not added to the main contents until after 50 min of preincubation.

Accumulation of ^{14}C-labelled amino acids. At the end of the perfusion period the hearts were slipped from the cannula into ice-cold 0·9% NaCl, and a portion of the apex region of each was cut off, blotted, weighed and placed in 5 ml. of water in a boiling-water bath for about 5 min. A sample of perfusate was collected and diluted tenfold. Samples (1 ml.) of tissue extract and diluted medium were pipetted on to stainless-steel planchets each with a concentric ring and dried under an infrared lamp, and the radioactivities were assessed in a gas-flow Geiger counter (Nuclear-Chicago Corp. model D-47) having an efficiency of about 40%. Previous experiments (Manchester & Young, 1960) indicated that further isolation of ^{14}C-labelled amino acids was unnecessary. A similar procedure was adopted with diaphragm.

For the purpose of calculation, the total water content of heart muscle ('tissue water') was taken as 80% of the wet weight (Morgan *et al.* 1961). The intracellular water of the heart was assumed to be that not penetrated by sorbitol and given as 55% of the total water or 44% of the wet weight (Morgan *et al.* 1961). Under anoxic conditions total and intracellular water are 82 and 39% of the wet weight respectively, and these values were used for the calculation of the values in the presence of cyanide and azide and in the absence of oxygen. The total water content of diaphragm muscle was assumed to be 75% (Randle, 1956). Calculation of the intracellular concentration of an amino acid assumed that the extracellular fluid contained the same concentration of amino acid as the perfusate, and it was therefore the amino acid in the tissue less that in the extracellular fluid.

Free amino acids of heart and plasma. Three hearts from rats whose plasma was collected and pooled were perfused free of blood (1-2 min. of perfusion). The weighed hearts and a sample of plasma were quickly frozen and sent for analysis to the Analytic Corp., 118 East 28th Street, New York 16, U.S.A. The procedure for deproteinization and determination was as described by Moore, Spackman & Stein (1958).

Incorporation of ^{14}C-labelled amino acids into protein. The incorporation of radioactivity into protein was assessed on samples of heart or diaphragm prepared as described by Wool & Krahl (1959) or Manchester (1961).

Release of endogenous amino acids. Measurement was by the method described by Manchester (1961).

RESULTS

The results are best considered under two major heads: (1) the rate of uptake and the concentration of accumulated ^{14}C-labelled amino acids by heart, and the effect of insulin on this process; (2) the measurement of the rate of incorporation of amino

acids into protein of heart and diaphragm. An analysis of the extractable amino acids of rat heart muscle and plasma is contained in Table 1.

Accumulation of ^{14}C-labelled amino acids by heart

Rate and amount of accumulation. The entry of trace concentrations of ^{14}C-labelled amino acids into the extracellular space of perfused hearts occurred rapidly, and, in most cases, within 1 min. of the addition of the ^{14}C-labelled amino acid to the medium the space was nearly saturated (Table 2). Entry into the intracellular water took place at considerably different rates for different amino acids. With [^{14}C]glycine and amino[^{14}C]isobutyric acid the label was not detected in the intracellular water until the observation at 5 min., whereas with the other ^{14}C-labelled acids there was substantial intracellular accumulation within 1–2 min. [^{14}C]-Phenylalanine and [^{14}C]tyrosine in particular reached their maximum concentrations within 5 min., and [^{14}C]alanine by 20 min., whereas for most of the others there was still evidence of continuing accumulation after 80 min.

When the concentration of added [^{14}C]phenylalanine was raised to 1·0 mM a constant accumulation ratio was reached much more slowly, and it was a lower ratio than when traces were used. With the concentration of [^{14}C]glycine at 1·0 mM there was no evidence of substantial intracellular accumulation for 10–20 min., though a ratio higher than that for [^{14}C]phenylalanine was subsequently observed.

Table 1. *'Free' amino acids and like materials of rat plasma and heart muscle (Sprague–Dawley strain)*

Experimental details are given in the text.

	Concn. of 'free' amino acids and like materials	
	In plasma (μmoles/ 100 ml.)	In heart muscle (μmoles/ (100 g. wet wt.)
Taurine	24·1	754
Urea	621·0	—
Hydroxyproline	5·5	—
Aspartic acid	1·95	268
Threonine	24·3	91
Serine	29·2	154
Asparagine + glutamine	81·9	529
Proline	21·2	101
Glutamic acid	14·1	418
Citrulline	11·6	—
Glycine	40·1	153
Alanine	34·9	361
Valine	21·2	77
Methionine	6·1	53·4
Isoleucine	10·1	70
Leucine	15·9	123
Tyrosine	8·9	38·7
Phenylalanine	7·4	50
Ornithine	8·2	30·1
Ethanolamine	2·6	60
Ammonia	21·5	1087
Lysine	49·5	109
Histidine	6·0	55
Arginine	13·2	119

The rate of egress of ^{14}C-labelled amino acids from the heart was fairly rapid. If, after a period of accumulation, the medium passing through was changed to fresh buffer containing no added amino acid, about 75 % of the estimated initial uptake left the heart within 10 min., a similar value being observed for [^{14}C]lysine, [^{14}C]phenylalanine and amino[^{14}C]isobutyric acid.

Effects of metabolic inhibitors on accumulation. Although when trace concentrations of ^{14}C-labelled naturally-occurring amino acids are added to the perfusion fluid net uptake is not to be expected (in fact as Fig. 1 indicates there is a net outflow of amino acids from the heart during perfusion), it was decided to determine to what extent energy-requiring processes are involved in the penetration of ^{14}C-labelled amino acids into the cell. The accumulation of ^{14}C in the presence of various metabolic inhibitors was therefore determined. The addition of cyanide (1 mM) arrested contractions, but was without effect on the uptake of [^{14}C]-glycine or [^{14}C]phenylalanine over periods of 5 min. and gave unpredictable results when used for longer periods (Table 2). Presumably because of the constant passage of oxygen plus carbon dioxide through the perfusate, hydrocyanic acid was displaced, and after about 20 min. the hearts began to beat again and behave normally. The significance of the values in the presence of cyanide are therefore uncertain. As an alternative to cyanide, azide (10 mM) was used. Hearts perfused with medium containing azide did not recommence contractions; its presence brought about the release of K^+ ions into the perfusate (not seen in its absence or in the presence of cyanide), and also stimulated the release of endogenous amino acids (Fig. 1). Azide decreased substantially the accumulation of amino[^{14}C]isobutyric acid, [^{14}C]glycine, [^{14}C]alanine, [^{14}C]proline and [^{14}C]lysine (Table 2). Replacement of the gas phase by nitrogen plus carbon dioxide severely decreased the accumulation ratio of amino[^{14}C]isobutyric acid but to a less extent than that of several other ^{14}C-labelled amino acids studied (Table 3). However, even under adverse conditions concentration ratios greater than unity were attained, suggesting that exchange reactions not requiring metabolic energy may be responsible for much of the accumulation observed.

Effect of insulin. The addition of insulin to the perfusate stimulated the accumulation of amino-[^{14}C]isobutyric acid and [^{14}C]proline at trace external concentrations, and of [^{14}C]glycine at both trace and 1·0 mM concentrations (Table 2). These effects were most clearly seen after the longer

perfusion periods, and there was no evidence of a disappearance of the difference with time. An effect of insulin on the accumulation of [^{14}C]proline by the intact rat diaphragm has been shown by Akedo & Christensen (1962); a similar effect can also be seen with the cut diaphragm (the tissue water:medium ratio at 80 min. being $2{\cdot}07 \pm 0{\cdot}079$ and $1{\cdot}61 \pm 0{\cdot}045$ with and without insulin respectively). The addition of insulin was without observable effect on the accumulation by the heart of any of the other ^{14}C-labelled amino acids studied, in accordance with observations with diaphragm (Manchester & Young, 1960; Guroff & Udenfriend, 1961; Akedo & Christensen, 1962). However, an effect of insulin on the incorporation of [^{14}C]lysine or [^{14}C]glutamic acid into protein is as readily observable as with [^{14}C]glycine or [^{14}C]proline (Table 4).

Table 2. *Accumulation of ^{14}C-labelled amino acids by the perfused rat heart*

Each value is the average for three hearts, or when indicated (*) two. The final concentrations of additions to the perfusate were: insulin, 0·1 unit/ml.; cyanide, 1 mM; azide, 10 mM; ^{14}C-labelled amino acids, 0·01 μc/ml., or when indicated (†), 0·1 μc/ml. Experimental details are given in the text.

				Radioactivity (counts/min./ml.) of tissue water / Radioactivity (counts/min./ml.) of medium							Radioactivity (counts/min./ml.) of intracellular water / Radioactivity (counts/min./ml.) of medium						
				After perfusion for (min.)							After perfusion for (min.)						
^{14}C-labelled amino acid	Expt. no.	Concn. (μM)	Additions to the perfusate	1	2	3	10	20	40	80	1	2	5	10	20	40	80
Aminoisobutyric acid	1	33†	—	0·30	0·34	0·63	0·74	1·47	1·86	3·65	0·00	0·00	0·33	0·53	1·85	1·56	5·82
			Cyanide	—	—	0·47	—	—	—	1·85	—	—	0·00	—	—	—	2·80
			Azide	—	—	—	—	—	—	1·66	—	—	—	—	—	—	2·49
	2	3·3	—	0·42*	0·45	0·58	0·67	1·22	2·33	3·58	0·00	0·00	0·24	0·40	1·40	3·42	5·70
			Insulin	0·39	0·48	0·54	0·82	1·62	3·51	5·29	0·00	0·05	0·16	0·67	2·13	5·55	8·60
Glycine	1	2·3	—	0·37	0·39	0·57	0·94	1·11	1·53	2·81	0·00	0·00	0·22	0·89	1·20	1·96	4·28
			Cyanide	—	—	—	—	1·08*	—	2·51	—	—	—	—	1·15	—	4·18
			Azide	—	—	—	—	0·99	—	1·36	—	—	—	—	0·98	—	1·76
	2	2·3	—	—	0·42	0·51	0·78	0·99	1·13	2·43	—	0·00	0·11	0·60	0·98	1·24	3·60
			Insulin	—	0·40	0·56	0·70	1·30	1·73	3·38	—	0·00	0·20	0·45	1·54	2·32	5·31
	3	1000	—	0·35	0·41	0·43	0·47*	0·74	1·11	1·67	0·00	0·00	0·00	0·04	0·53	1·20	2·22
			Insulin	—	—	—	—	—	1·64	—	—	—	—	—	—	2·16	—
L-Alanine	1	2·4	—	—	1·21	1·71	2·84	4·47	4·21	4·15*	—	1·38	2·29	4·34	7·32	6·83	6·72
			Insulin	—	—	1·67	3·56	4·60	3·68	3·96	—	—	2·21	5·65	7·57	5·88	6·40
			Azide	—	—	—	—	2·84	—	—	—	—	—	—	4·87	—	—
L-Valine	1	0·39	—	0·58	0·75	1·17	1·23	1·20	1·33	1·59	0·24	0·55	1·31	1·42	1·36	1·61	2·07
			Insulin	—	—	1·27	1·22	1·23	1·30	1·59	—	—	1·49	1·40	1·42	1·55	2·07
L-Proline	1	0·93	—	—	0·89	1·01	1·23	1·52	1·51	2·61	—	0·80	1·02	1·42	1·95	1·92	3·92
			Insulin	—	0·67	0·93	1·43	1·84	2·07	3·67	—	0·40	0·87	1·78	2·53	2·94	5·85
			Azide	—	—	—	—	—	—	1·45	—	—	—	—	—	—	1·94
	2	240	—	—	—	—	—	—	—	1·51	—	—	—	—	—	—	1·92
L-Lysine	1	1·7	—	0·41	0·68	1·24	2·30	4·20	5·73	6·63	0·00	0·42	1·44	3·46	6·80	9·40	11·2
			Insulin	—	0·72	1·21	2·04	4·63	5·75	7·03	—	0·48	1·38	2·89	7·60	9·60	11·9
			Azide	—	—	—	—	3·80*	—	—	—	—	—	—	6·05	—	—
L-Glutamic acid	1	0·37	—	0·34	0·53	0·59	0·84	1·13	1·80	1·96	0·00	0·15	0·25	0·71	1·24	2·45	2·75
			Insulin	—	—	—	—	—	1·80	—	—	—	—	—	—	2·45	—
L-Phenylalanine	1	2·2†	—	—	1·27	1·65	1·67	1·68	1·80	1·65*	—	1·49	2·18	2·22	2·24	2·45	2·18
	2	2·2†	—	0·86	1·55	1·85	1·80	—	—	—	0·74	2·00	2·54	2·45	—	—	—
			Insulin	0·79	1·45	1·88	1·78	—	—	—	0·62	1·82	2·60	2·42	—	—	—
			Cyanide	—	—	1·83	—	—	—	—	—	—	2·51	—	—	—	—
	3	1000	—	0·37	0·62	0·70	0·62	—	0·90*	1·03	0·00	0·31	0·44	0·31	—	0·82	1·06
			Insulin	0·40	0·45	0·53	0·58	—	0·92	0·85	0·00	0·00	0·15	0·24	—	0·86	0·73
L-Tyrosine	1	0·87	—	0·86	1·53	2·68	2·40	2·74	2·23	2·25	0·75	1·96	4·05	3·55	4·16	3·24	3·27
			Cyanide	—	—	1·88	—	—	—	—	—	—	2·60	—	—	—	—
	2	8·7		—	—	1·63	—	—	—	—	—	—	2·15	—	—	—	—

Time-course of incorporation of ¹⁴C-labelled amino acids into protein of heart and diaphragm

Heart. Wool & Manchester (1962) have recorded that insulin can increase the rate of incorporation of [¹⁴C]glycine into protein of the rat heart, and Table 4 shows that insulin also increases the rate of incorporation of ¹⁴C-labelled amino acids whose total accumulation it does not observably enhance. For both [¹⁴C]glycine and [¹⁴C]leucine at moderate concentrations the rate of incorporation seems to remain fairly constant as perfusion proceeds (Fig. 2). However, with low concentrations more irregular curves were obtained, partly because there was a tendency as time proceeded for the hearts to have removed from the perfusate most of the ¹⁴C-labelled amino acid. Particularly was this so with [¹⁴C]leucine, and a similar phenomenon was encountered with [¹⁴C]arginine and [¹⁴C]proline (not shown). At first glance the curves confirm the observation of Kipnis *et al.* (1961), with the incorporation of [¹⁴C]proline into protein of diaphragm, that the rate of incorporation of ¹⁴C-labelled amino acid into protein is constant even when the amount of radioactivity accumulated in the tissue is rising fast. However, the curve for [¹⁴C]glycine at a low concentration (Fig. 2) does show a distinct concavity and departure from linearity. Such a delay in the onset of the full rate of incorporation is to be expected if the label takes an appreciable time to rise to its maximum specific radioactivity in the pool from which amino acids are taken for protein synthesis. On the other hand, if the specific radioactivity rises at differing rates in differing parts of the cell owing to different accessibilities, and if amino acids used in protein synthesis are in an area of the cell where the specific radioactivity rises rapidly, a constant rate of incorporation might speedily be attained while accumulation of ¹⁴C is still taking place elsewhere. That an initial delay in incorporation should be observable with [¹⁴C]-glycine rather than with the other ¹⁴C-labelled amino acids might be predicted from the obser-

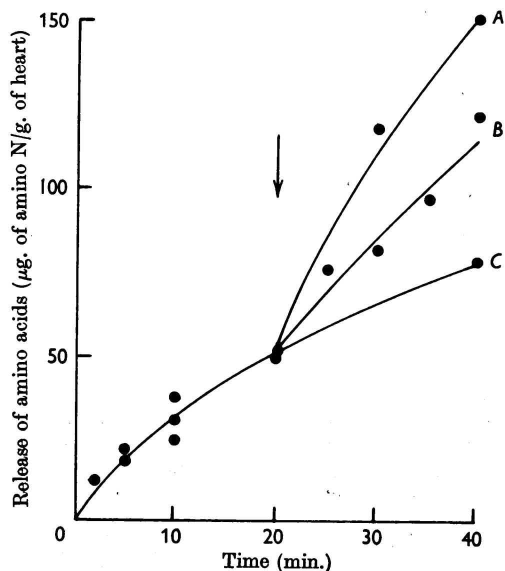

Fig. 1. Effect of cyanide and azide on the release of endogenous amino acids by the perfused rat heart. The inhibitors were added after perfusion of hearts for 20 min. (at the point marked by the arrow). *A*, Azide added; *B*, cyanide added; *C*, control. Experimental details are given in the text.

Table 3. *Effect of anoxia on the accumulation of ¹⁴C-labelled amino acids by the perfused rat heart*

Each value is the average for three hearts. The concentrations and radioactivities of the ¹⁴C-labelled amino acid were as in Table 2. Experimental details are given in the text.

¹⁴C-labelled amino acid	Concn. (μM)	Gas phase	Radioactivity (counts/min./ml.) of tissue water / Radioactivity (counts/min./ml.) of medium — After perfusion for (min.)			Radioactivity (counts/min./ml.) of intracellular water / Radioactivity (counts/min./ml.) of medium — After perfusion for (min.)		
			10	20	80	10	20	80
Aminoisobutyric acid	3·3	$O_2 + CO_2$	—	—	5·97	—	—	10·1
		$N_2 + CO_2$	—	—	2·20	—	—	3·53
Glycine	2·3	$O_2 + CO_2$	—	—	3·04	—	—	4·71
		$N_2 + CO_2$	—	—	2·06	—	—	3·23
L-Alanine	2·4	$O_2 + CO_2$	—	4·83	—	—	7·98	—
		$N_2 + CO_2$	—	4·05	—	—	7·42	—
L-Lysine	1·7	$O_2 + CO_2$	—	5·28	—	—	8·80	—
		$N_2 + CO_2$	—	2·85	—	—	4·90	—
L-Tyrosine	0·87	$O_2 + CO_2$	2·74	—	—	4·17	—	—
		$N_2 + CO_2$	2·11	—	—	3·35	—	—

Table 4. *Effect of insulin on the incorporation of* ^{14}C-*labelled amino acids by the perfused rat heart*

The concentration of label was 0·1 μc/ml. for glycine and glutamic acid and 0·01 μc/ml. for proline and lysine. The numbers of observations are shown in parentheses. Perfusion was for 1 hr. Experimental details are given in the text. The values for glycine are taken from Wool & Manchester (1962).

^{14}C-labelled amino acid	Concn. (μM)	Insulin added (0·1 unit/ml.)	Radioactivity in protein (counts/min./mg.)	
Glycine*	5·0	−	38 ± 5·1 (6)	$P < 0.02$
		+	73 ± 10·6 (6)	
L-Proline	0·93	−	21 ± 1·7 (4)	$P < 0.01$
		+	29 ± 1·0 (4)	
L-Lysine	1·7	−	14 ± 1·5 (4)	$P < 0.002$
		+	27 ± 2·6 (4)	
L-Glutamic acid	3·7	−	18 ± 2·3 (6)	$P < 0.001$
		+	38 ± 3·6 (6)	

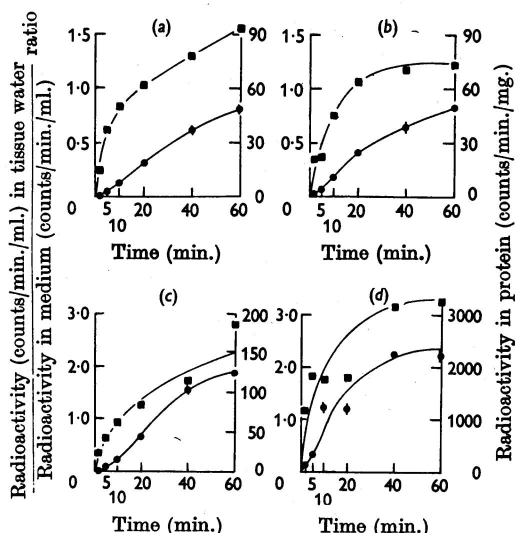

Fig. 2. Accumulation and incorporation into protein of ^{14}C-labelled amino acids by the perfused rat heart: (*a*) glycine (1 mM); (*b*) leucine (5 mM); (*c*) glycine (5μM); (*d*) leucine (1·4μM). ●, Incorporation into protein; ■, accumulation in tissue water (mean of three observations). Vertical lines through circles are S.E.M. (where vertical lines are not shown the S.E.M. was smaller than the circle size). Experimental details are given in the text.

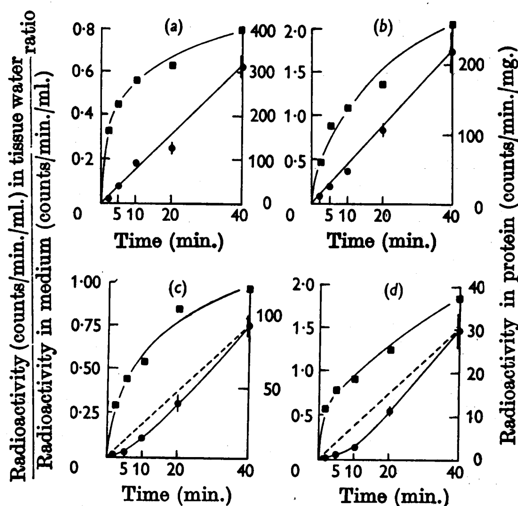

Fig. 3. Accumulation and incorporation into protein of ^{14}C-labelled amino acids by isolated rat diaphragm: (*a*) leucine (10μM); (*b*) arginine (10μM); (*c*) proline (20μM); (*d*) glycine (134μM). ●, Incorporation into protein; ■, accumulation in tissue water (mean of four observations for leucine and arginine, and of eight observations for proline and glycine). Vertical lines through circles are S.E.M. (where vertical lines are not shown, for points at 2, 5 and 10 min., the S.E.M. was smaller than the circle size). Each broken line is a straight line connecting the 40 min. point and origin.

vation (Table 2) of its relatively slow rate of initial penetration. It would have been desirable to record more observations of this sort with heart muscle, but in view of various practical difficulties in the use of this preparation it was thought easier to follow up the question of the linearity of the initial rates of incorporation in diaphragm.

Diaphragm. It is just possible that the absolute rate of protein synthesis in diaphragm declines considerably during the first 30 min. of incubation *in vitro*, and that in the experiments of Kipnis *et al.* (1961) a constant rate of incorporation of ^{14}C-labelled amino acids into protein arose as the result of a balance between a declining rate of protein synthesis and a rise in the specific radioactivity of amino acid incorporated. In the present work, therefore, the diaphragms were incubated for 50 min. at 37° before the addition of the ^{14}C-labelled amino acid. A further advantage of this procedure is that some degree of equilibration will have been reached by this time between the rate of release and uptake of unlabelled amino acids before the ^{14}C-labelled

amino acids are added. Under these conditions the incorporation of [^{14}C]leucine or [^{14}C]arginine into protein of the diaphragm does follow a linear course (Fig. 3), despite variations in the amount of ^{14}C in the total pool, but with [^{14}C]proline and even more so with [^{14}C]glycine a distinct departure from linearity is observed, the rate of incorporation increasing as the incubation proceeds.

DISCUSSION

The concentration ratios observed in the present study (Table 2) for the perfused heart are similar to those found previously with isolated diaphragm (Manchester & Young, 1960; Guroff & Udenfriend, 1960; Kipnis *et al.* 1961; Akedo & Christensen, 1962). They do not show any obvious correlation with the initial amino acid content of the heart (Table 1).

We have been impressed by the variation in uptake characteristics between different amino acids: at the two extremes the slow initial penetration and long-continued accumulation of glycine by comparison with the rapid initial uptake of the aromatic amino acids that quickly reach a constant concentration. It could be that the relatively smaller molecular size of glycine and, say, alanine enables these ^{14}C-labelled amino acids to exchange much more readily with their unlabelled counterparts within the body of the myofibrils than can the more bulky ^{14}C-labelled amino acids. Guroff & Udenfriend (1960) found that in the diaphragm added [^{14}C]tyrosine equilibrated with only a portion of the total 'free' tyrosine in the tissue. A rapid exchange of aromatic amino acids near the cell membrane and possibly throughout the sarcoplasmic reticulum, but a very much slower interchange with molecules dissolved in fluid associated with the myofibrillar elements, would explain both our observations and those of Guroff & Udenfriend (1960). It is noteworthy that non-utilizable sugars taken up by muscle never appear to penetrate the whole of the cell water (Park, 1955; Park, Reinwein, Henderson, Cadenas & Morgan, 1959). Moreover, Peckham & Knobil (1962) have found that damaging muscle fibres either by crushing or by treatment with iodoacetate increases the total accumulation of amino acids.

Variation in the rates of penetration may also explain why for only a minority of ^{14}C-labelled amino acids is it possible to observe an increase in the rate of their incorporation into protein as the intracellular radioactivity rises (Figs. 2 and 3). In the majority of cases, if the bulk of the incorporation takes place by means of ribosomes (Winnick & Winnick, 1960; Florini, 1962), which are believed to be associated with the sarcoplasmic reticulum (Moore & Ruska, 1957; Porter & Palade, 1957; Muscatello, Andersson-Cedergren, Azzone & Decken, 1961) and are therefore spatially separate from the myofibrils, or by the cell membrane itself (Hendler, 1962), the rate of rise of specific radioactivity at these loci may be too rapid for a gradual rise in the rate of incorporation to be apparent. But this does not necessarily mean that the total amino acid pool of the muscle cell is divided into fundamentally separate fractions, and it is not unreasonable to suppose that over longer periods of time there will be complete equilibration of label throughout the pool of the whole tissue.

The effects of insulin on the uptake of amino acids by heart are substantially similar to those observed with diaphragm; the hormone clearly stimulates the total accumulation of glycine and proline as well as of aminoisobutyric acid. The response to insulin of these three amino acids, as well as of isovaline, cycloleucine and methionine (Akedo & Christensen, 1962), therefore stands out as different from the others studied. No satisfactory explanation can be put forward to account for the different responses to the presence of insulin. However, the results reinforce previous evidence (Manchester & Young, 1960; Guroff & Udenfriend, 1961; Akedo & Christensen, 1962) that the mechanism by which insulin promotes the incorporation of amino acids into protein is not by an influence on their entry into the cell.

It might be argued that since uptake of radioactivity is not a measure of the specific radioactivity of the cell pool, it is an inadequate parameter to use as a basis for comparisons if the size of the cell pool changes under the influence of insulin. However, we can consider the alternatives. If insulin were to increase the size of the amino acid pool in the tissue, a given amount of radioactivity in the pool would have a lower specific radioactivity in the presence of the hormone than in its absence and this could not be the explanation of the enhanced rate of incorporation of label into protein. If, on the other hand, insulin were to decrease the size of the amino acid pool, for which some evidence has been put forward (Manchester, 1961), a constant amount of radioactivity would now possess a higher specific radioactivity than in the absence of insulin. The increased entry of label into protein might result from such an occurrence, but if so would indicate that the primary action of insulin is not to stimulate uptake of amino acid but to bring about a decrease in pool size. This could result from: (*a*) an increased release of amino acids from the tissue (this would be contrary to the available evidence; Manchester & Young, 1960; Manchester, 1961; Guroff & Udenfriend, 1961; Akedo & Christensen, 1962); (*b*) an increased rate of catabolism of amino acids (which is improbable on general consideration, and is unlikely in view of

the fact that insulin stimulates the incorporation into protein of ^{14}C derived from materials converted into amino acids in the tissue; Manchester & Krahl, 1959); and (*c*) an increased rate of protein synthesis or a decreased rate of its breakdown. Thus, either way, the arguments suggest that, in influencing the incorporation of amino acids into protein, insulin is affecting primarily the net rate of protein synthesis rather than any aspect of amino acid uptake.

SUMMARY

1. The accumulation and incorporation into protein of ^{14}C-labelled amino acids by the perfused rat heart and the effect of insulin thereon has been studied.

2. The entry of label into intracellular water was usually observed within a few minutes, and might continue to rise for as long as 80 min. or reach a constant concentration in as short a period as 10 min. Accumulation was decreased in various degrees by cyanide, azide and anoxia.

3. The accumulation of glycine, proline and α-aminoisobutyric acid was stimulated by insulin; that of alanine, valine, lysine, glutamic acid, phenylalanine and tyrosine was not. Insulin stimulated the incorporation into protein of each of four amino acids studied (glycine, proline, lysine and glutamic acid).

4. The rate of incorporation of amino acids into protein was fairly constant throughout the perfusion, despite a continual rise in the amount of radioactivity accumulated. With diaphragm some evidence of a lag in the onset of incorporation during the initial accumulation of glycine and proline was observed.

5. The results do not support the concept that insulin stimulates the incorporation of amino acids into protein of muscle solely through an enhancement of their accumulation, nor are they entirely consistent with the view that the amino acids entering are derived from a pool functionally distinct from the total free amino acids of the tissue.

K. L. M. is grateful to the Council of the Royal Society for a Jaffé Donation Studentship and financial support. Part of the expenses of this work was met by grants to I. G. W. from the National Institutes of Health (A-4842), the John A. Hartford Foundation and the Life Insurance Medical Fund.

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