Accumulation of Amino Acids in Muscle of Perfused Rat Heart

EFFECT OF INSULIN IN THE PRESENCE OF PUROMYCIN

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1. Rat heart was perfused with a mixture of amino acids and other ninhydrin-positive substances in the same concentrations as they are found in plasma, and with or without sufficient puromycin to suppress protein synthesis all but completely. 2. In the presence of puromycin, but not in its absence, insulin increased the concentrations of most of the individual amino acids in the intracellular water of heart. 3. The results accord with the conclusion that insulin increases the entry of most, if not all, amino acids into muscle.

In the preceding paper (Scharff & Wool, 1965) the results of a study of the effect of insulin on the actual concentrations of individual amino acids in heart muscle were reported. When heart was perfused with a mixture of amino acids and other ninhydrin-positive substances in the same concentrations as they occur in plasma, the hormone was without a clear or meaningful effect on the concentrations of amino acids in the medium or in the intracellular water of heart muscle.

The concentration, at any moment, in heart muscle of a particular amino acid must be the resultant of a number of vectors: of the net rate of entry, i.e., the rate of entry less the rate of exit; of the utilization of the amino acid, either for protein synthesis, in transamination reactions, or by deamination; of the synthesis of the amino acid, either by transamination, or by new synthesis. If the intention is to examine the regulation of amino acid entry into muscle then the other processes may seriously interfere with that purpose. Insulin is known to increase the utilization of amino acids for protein synthesis (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958; Wool & Krahl, 1959*a*); the hormone, has not, however, been shown to influence transamination, deamination, or the synthesis of amino acids in muscle. Castles & Wool (1964) have demonstrated that a failure to take into account the stimulation by insulin of protein synthesis may obscure the influence of the hormone on the transport of certain amino acids; to minimize that possibility they suggested the use of puromycin in the study of the mechanism and means of regulation of amino acid transport in muscle. Puromycin in the proper concentration will completely suppress protein synthesis (Yarmolinsky & de la Haba, 1959), and the antibiotic does so without interfering with the accumulation in muscle of aminoisobutyric acid (Fritz & Knobil, 1963).

We have retested the effect of insulin on the concentrations of individual amino acids in the medium and the intracellular water of perfused rat heart, following the methods and principles described in the preceding paper (Scharff & Wool, 1965), but now having suppressed protein synthesis by the addition of puromycin. In the presence of the antibiotic insulin increased the accumulation in heart muscle of most of the natural amino acids.

METHODS

Chemicals. The sources for most of the materials used are listed by Scharff & Wool (1965). The puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). The [U-^{14}C]histidine (240mc/m-mole) and the α-amino[1-^{14}C]isobutyric acid (9·96 mc/m-mole) were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.) and the [^{14}C]inulin (0·23μc/mg.) from Volk Radiochemical Co. (Skokie, Ill., U.S.A.).

Preparation and perfusion of hearts. The animals used and the technique of perfusion of isolated heart with a small volume of recirculating perfusion medium (medium *B*) were as described by Scharff & Wool (1965). Blood was washed from the hearts with 25–30ml. of medium *B*; insulin, puromycin and radioactive substrate, when present, were added at the beginning of the perfusion period. In every case perfusion was for 1hr.

Analytical procedures. The method of preparation of extracts of heart muscle and of medium and their analysis by column chromatography for amino acids and other ninhydrin-positive substances were as described by Scharff & Wool (1965), as was the means used to determine the inulin space and total water content of perfused rat heart. The accumulation in perfused heart of α-amino[^{14}C]isobutyric acid and the incorporation into protein of radio-

activity from [^{14}C]histidine were measured as described by Manchester & Wool (1963).

Calculations. The means for calculating the inulin space, the intracellular concentrations of ninhydrin-positive substances in muscle and the concentrations of citrulline and proline when they could not be separated by chromatography were all as described by Scharff & Wool (1965).

Table 1. *Effect of puromycin on the incorporation of* [^{14}C]*histidine into protein of perfused rat heart*

Rat heart was perfused for 1 hr. with medium *B* (Scharff & Wool, 1965) containing 0·1 μc of [^{14}C]histidine/ml. The values are the means ± S.E.M. of the numbers of observations in parentheses.

Concn. of puromycin (μg./ml.)	Radioactivity in protein (counts/min./mg.)
0	121 ± 9·7 (4)
100	2 ± 0·2 (6)
200	1 ± 0·3 (6)

Table 2. *Effect of insulin and puromycin on the inulin space and the total water content of perfused rat heart*

Rat heart was perfused for 1 hr. with medium *B* (Scharff & Wool, 1965) containing 0·01 μc of [^{14}C]inulin/ml. The concentration of insulin, when present, was 0·1 unit/ml. and that of puromycin was 100 μg./ml. The values are the means ± S.E.M. of four observations.

Addition to the medium	Water content (μl./g.)	Inulin space (μl./g.)
None	812 ± 1	324 ± 14
Insulin	807 ± 6	320 ± 9
Puromycin	810 ± 4	309 ± 7
Insulin and puromycin	811 ± 6	323 ± 10

RESULTS

To test the effect of puromycin in inhibiting protein synthesis, rat heart was perfused with medium *B* containing [^{14}C]histidine and puromycin. The incorporation of radioactivity from [^{14}C]histidine into myocardial protein was all but completely inhibited (98%) by 100 μg. of puromycin/ml. (Table 1); 200 μg. of puromycin/ml. produced only a slightly greater inhibition (99%). A concentration of puromycin less than 100 μg./ml. was not tested; however, in diaphragm muscle 50 μg. of puromycin/ml. produced 93% inhibition of [^{14}C]leucine incorporation into protein (Wool, Castles & Moyer, 1965).

Since the intention was to test the effect of insulin on amino acid accumulation in heart muscle in circumstances where protein synthesis had been suppressed with puromycin, it was important to establish the effect, if any, of puromycin and of insulin, and of the combination of the two, on the water content and the inulin space of perfused heart. When heart was perfused with medium *B* for 1 hr., the addition of insulin (0·1 unit/ml.), of puromycin (100 μg./ml.), or of the two, did not significantly alter the total water content or the inulin space (Table 2).

Rat hearts were perfused for 1 hr. with medium *B* (Scharff & Wool, 1965), a bicarbonate buffer containing amino acids and other ninhydrin-positive substances in the concentrations they are found in rat plasma, pyruvate (an energy substrate whose utilization is not influenced by insulin; Villee, White & Hastings, 1952) and albumin, and the effect of insulin on the concentrations of amino acids and like material in the medium and the intracellular water was determined in the presence of puromycin. To authenticate the occurrence of a physiological effect of insulin on the heart, amino-[^{14}C]isobutyric acid was added to the medium and

Table 3. *Effect of insulin and puromycin on the accumulation of α-amino*[^{14}C]*isobutyric acid by perfused rat heart*

Rat heart was perfused for 1 hr. with medium *B* (Scharff & Wool, 1965) containing 0·05 μc of α-amino[^{14}C]-isobutyric acid/ml. The concentration of insulin, when present, was 0·1 unit/ml. and that of puromycin was 100 μg./ml. The hearts are those analysed for the experiment recorded in Table 4; the effect of insulin on the accumulation of radioactivity from α-amino[^{14}C]isobutyric acid served to authenticate the occurrence of an insulin effect. The values are the means ± S.E.M. of five observations.

Additions to the medium		Accumulation $\left(\frac{\text{counts/min./ml. of intracellular water}}{\text{counts/min./ml. of medium}}\right)$
Insulin	Puromycin	
—	—	2·66 ± 0·42
+	—	4·07 + 0·65**
—	+	2·36 ± 0·42
+	+	3·35 ± 0·89*

* Difference due to insulin that is significant ($P < 0·05$); ** difference due to insulin that is very significant ($P < 0·001$).

the effect of the hormone on the concentration of radioactivity from the amino acid determined in samples from the same muscle and medium that were analysed for their amino acid concentrations. Insulin significantly increased the accumulation in heart muscle of radioactivity from α-amino-[^{14}C]isobutyric acid and the hormone did so whether puromycin was present or not (Table 3); the antibiotic alone had no significant effect on aminoisobutyric acid accumulation (Table 3). The findings with aminoisobutyric acid accord with those reported for diaphragm by Fritz & Knobil (1963).

Table 4. *Effect of insulin and puromycin on the concentration of ninhydrin-positive substances in the medium and muscle of perfused rat heart*

Rat heart was perfused for 1 hr. with medium *B* (Scharff & Wool, 1965); the concentration of insulin, when present, was 0·1 unit/ml. and that of puromycin was 100 μg./ml. The values are, with few exceptions, the means ± S.E.M. of five observations.

		Concn. in heart (μmoles/100 ml. of cell water)		Concn. in medium (μmoles/100 ml.)		Heart/medium concentration ratio	
	Puromycin	No insulin	Insulin	No insulin	Insulin	No insulin	Insulin
Amino acids							
Aspartic acid	−	144±12·8	176±30·6	5·40±0·36	5·09±0·86	26·9±1·20	34·5±8·20
	+	91·8±19·5	117±11·6	5·88±0·70	5·24±0·45	21·6±4·07	22·9±9·39
Threonine	−	129±10·9	132±5·70	24·2±0·55	25·5±1·26	5·42±0·51	5·32±0·39
	+	117±17·4	155±11·0	29·2±1·64†	25·5±1·53	4·14±0·78	6·19±0·62
Serine	−	308±22·7	411±22·5**	48·5±1·34	46·3±1·37	6·71±0·47	8·95±0·69*
	+	304±13·1	405±22·1**	51·1±1·02	44·8±2·18*	5·99±0·35	9·22±0·95*
Glutamic acid	−	895±35·3	952±15·8	13·4±0·15	13·4±1·25	67·0±3·21	63·9±6·56
	+	886±19·2	1000±51·4	16·2±1·79	13·0±0·42	57·3±6·05	77·5±4·93*
Proline	−	101±4·07	138±14·4*	29·0±1·03	27·9±1·14	3·78±0·41	4·73±0·38*
	+	95·1±8·11	143±6·34**	31·5±1·01	30·1±1·81	2·96±0·27	4·78±2·19***
Citrulline	−	19·8±2·69	30·3±3·46*	8·35±0·04	8·17±0·19	2·37±0·32	3·69±0·39*
	+	27·8±4·01	30·1±3·94	8·26±0·15	8·22±0·24	3·56±0·44	3·63±0·38
Glycine	−	178±16·9	182±14·1	29·8±0·89	28·7±0·67	6·02±0·66	6·37±0·48
	+	168±15·4	194±4·43	31·1±0·48	28·4±0·86*	5·39±0·39	6·90±0·28*
Alanine	−	422±24·9	411±23·2	46·9±1·40	40·6±1·26*	9·07±0·75	10·1±0·57
	+	374±34·2	491±20·1*†	47·4±0·85	42·7±1·20*	7·88±0·37	11·5±0·35***
Valine	−	41·5±2·81	36·1±3·55	17·3±0·29	17·0±0·44	2·41±0·63	2·13±0·25
	+	36·3±2·86	45·2±1·44*†	18·2±0·24†	17·5±0·58	2·00±0·18	2·50±0·14
Cystine (half)	−	6·54±0·90	5·49±0·88	1·48±0·18	1·45±0·18	4·86±1·13	4·22±1·10
	+	5·10±0·45	6·05±1·13	1·12±0·12	1·44±0·17	4·53±0·61	4·63±1·16
Methionine	−	16·3±1·26	15·9±0·98	4·33±0·04	4·10±0·09	3·76±0·31	3·89±0·29
	+	14·8±0·88	19·7±1·00**†	4·28±0·10	4·30±0·12	3·47±0·28	4·57±0·11**
Isoleucine	−	29·0±2·14	18·8±2·92*	7·71±0·20	7·40±0·18	3·80±0·37	2·28±0·14**
	+	22·6±1·47†	24·6±0·60	8·12±0·17	7·85±0·36	3·00±0·26	3·30±0·11††
Leucine	−	48·8±3·91	36·8±5·23	13·6±0·38	13·0±0·21	3·64±0·37	2·48±0·14*
	+	39·2±2·07	44·8±0·78*	14·2±0·39	13·7±0·50	2·77±0·20	3·39±0·08*†††
Tyrosine	−	19·5±2·16	17·2±1·59	7·40±0·17	6·65±0·21*	2·65±0·31	2·59±0·23
	+	22·2±4·05	19·5±0·92	7·72±0·39	7·37±0·31	2·91±0·54	2·66±0·15
Phenylalanine	−	18·7±2·07	17·9±2·44	7·81±0·11	7·82±0·42	2·41±0·29	2·31±0·94
	+	22·7±1·56	20·4±1·60	7·65±0·22	7·24±0·36	2·98±0·26	2·81±0·14
Ornithine	−	14·8±1·30	12·8±0·58	6·48±0·07	6·38±0·08	2·29±0·20	2·01±0·10
	+	11·9±0·85	15·4±0·89*†	6·51±0·15	6·47±0·27	1·84±0·17	2·38±0·09*
Lysine	−	122±5·86	110±6·58	35·2±0·46	32·8±1·20	3·49±0·21	3·37±0·34
	+	114±37·1	120±6·10	35·5±0·55	35·5±1·27	3·23±0·11	3·42±0·23
Histidine	−	25·9±3·01	28·9±1·53	7·07±0·22	6·95±0·10	3·70±0·51	4·19±0·22
	+	29·3±1·11	31·8±3·42	7·61±0·22	7·05±0·08	3·86±0·18	4·51±0·45
Tryptophan	−	9·36±1·48	8·74±1·38	6·13±0·24	6·54±0·71	1·54±0·88	2·40±0·76
	+	10·7±0·91	11·8±0·58	6·06±0·20	6·15±0·31	1·79±0·20	1·93±0·09
Arginine	−	138±4·74	123±5·25	36·3±2·58	37·4±1·26	3·82±0·21	3·30±0·22
	+	130±13·1	139±6·48	36·5±0·38	37·0±1·12	3·58±0·37	3·77±0·23
Non-amino acids							
Taurine	−	3900±111	3520±257	53·4±3·95	49·0±6·36	75·1±7·56	79·5±15·0
	+	4040±58·8	5010±362*†††	70·4±8·79	60·7±4·14	61·1±7·52	85·2±17·2*
Glutathione	−	865±121	1040±92·1	—	—	—	—
	+	1070±90·3	1070±165	—	—	—	—
Ammonia	−	1930±78·5	1650±83·0*	108±3·13	102±1·24	18·0±0·83	16·1±0·92
	+	1580±87·2†	1680±133	101±3·71	103±2·88	15·6±1·13	16·4±1·40
Total							
Amino acids	−	2990±216	2880±87·6	356±7·15	343±7·72	8·43±0·71	8·40±0·36
	+	2540±73·2	3010±118**	368±7·27	343±14·2	6·91±0·26	8·85±0·57*
Non-amino acids	−	6700±110	6210±351	161±39·6	152±7·04	42·0±2·22	41·2±9·30
	+	6690+104	7760±522†	172±10·2	164±5·20	39·5±2·30	47·7±4·07
Ninhydrin-positive substances	−	9690±254	9090±417	517±8·61	495±14·1	18·8±0·77	18·4±0·90
	+	9230±193	10770±587*†	540±16·9	507±14·6	17·1±0·66	21·3±1·01**

* Differences due to insulin that are significant: *$P<0·05$; **$P<0·01$; ***$P<0·001$.
† Differences due to puromycin that are significant: †$P<0·05$; ††$P<0·01$; †††$P<0·001$.

Puromycin tended to decrease the concentrations of individual amino acids in the intracellular water of heart muscle. The changes were small and the difference was significant only for isoleucine (Table 4). In the presence of the antibiotic the concentrations of amino acids in the medium were greater at the completion of perfusion, although the differences were small and significant only for threonine and valine (Table 4). Because of the direction of the change in the concentrations of amino acids in the intracellular water of heart and in the medium, the concentration ratio (heart/medium) when puromycin was included in the medium was in most cases decreased. Apparently, puromycin in some way interferes with the ability of the heart to maintain the normal concentration gradient for amino acids.

Insulin alone significantly increased the concentrations in intracellular water of heart of only three amino acids (serine, proline and citrulline). (No effect of insulin on the concentrations in heart of citrulline was observed by Scharff & Wool, 1965.) The concentrations in heart water of several amino acids were actually decreased by the hormone, but in most instances there was no change; the net result was to leave the total intracellular concentration of amino acids unaltered (Table 4).

In the presence of sufficient puromycin to suppress protein synthesis all but completely, insulin increased the total intracellular concentration of amino acids as well as the concentration gradient between the intracellular water and the medium (Table 4). The concentrations in heart muscle of many of the individual amino acids were increased by insulin when puromycin was present (the only certain exceptions were tyrosine and phenylalanine), and the increases were of sufficient magnitude to be statistically significant in a number of cases (serine, proline, alanine, valine, methionine, leucine and ornithine) despite the small number of observations. The non-amino acid taurine was also significantly increased in concentration by insulin when puromycin was present. In the presence of puromycin the changes in the concentrations of amino acids in the medium due to insulin were not striking, but what changes did occur, were, in general, in accord with an effect of the hormone to increase the net transfer of amino acid from the medium to the cell water of the heart. In experiments with perfused heart, the volume of fluid in equilibrium with the extracellular compartment is more than 20 times larger than is the intracellular compartment (i.e., 12ml. of perfusion medium and only approx. 0·5ml. of cell water); for that reason, transfer of amino acids from one compartment to the other will produce a far greater change in their concentrations in cell water than in the perfusion medium. A further consequence is that changes in the amounts of amino acids in the intracellular compartment are just as was found, reflected in changes in the concentration ratio, insulin increasing the concentration ratio for most amino acids when puromycin was present.

DISCUSSION

In the presence of sufficient puromycin to suppress protein synthesis all but completely, insulin increased the concentrations in muscle of perfused heart of almost all the individual amino acids; in the absence of the antibiotic the hormone did not do so. The results support the conclusion (Castles & Wool, 1964) that insulin acts on muscle to accelerate the transfer of most, if not all, amino acids from the extracellular space into the cell interior. The effect of the hormone on amino acid accumulation is not, as was suggested by Manchester & Young (1961), dependent on a concomitant stimulation of protein synthesis, a point made by Fritz & Knobil (1963) on the basis of their finding that insulin increased the accumulation in diaphragm of amino[^{14}C]isobutyric acid when protein synthesis had been completely inhibited by puromycin.

The accumulation by rat heart of ornithine and taurine, two substances not found in protein, is also increased by insulin only in the presence of puromycin (Table 4). That cannot be explained and, moreover, is not consistent with the general interpretation we have given the results. However, the movement of each amino acid is probably dependent on the movement of many others, through competition for the limited number of transport sites and through exchange processes using the same sites (Heinz & Walsh, 1958; Christensen, 1962). Therefore, in the presence of a mixture of amino acids, a variation of the concentration in intracellular water of one amino acid may affect the distribution of others.

That the increased concentrations of natural amino acids in rat heart muscle in the presence of insulin were the result of increased net transport of amino acids from the extracellular compartment into the cell water is supported by several observations. First, the changes in the concentrations of amino acids in the perfusion medium brought about by the hormone are consonant with that conclusion; there were small, but consistent, decreases in the concentrations of the amino acids whose intracellular concentrations were increased by insulin. The magnitude of the changes in the concentrations in the medium was more than sufficient to account for the increases in the intracellular amino acid concentrations; 3·0μmoles of amino acid were lost from the medium [(3·68 − 3·43) × 12; cf. Table 4] while 2·35μmoles were gained by the heart [(30·1 − 25·4) × 0·5].

Secondly, alternative explanations of the increase in the cellular content of amino acids are inconsistent either with the evidence of the present experiment or with established effects of the hormone. If insulin acted to raise the cellular content of amino acids by decreasing amino acid catabolism or by increasing amino acid formation (by synthesis of amino acid or from proteolysis) a net increase in the amount of amino acids present is to be expected. In point of fact, just the contrary, namely a net decrease, occurred, the amount lost from the medium being greater than the amount gained by the heart. Moreover, the general anabolic effect of insulin (Manchester & Young, 1961) makes increased proteolysis most unlikely. Since several of the amino acids (threonine, methionine, valine and leucine) whose concentrations in heart were increased by insulin are essential for the rat, the increase cannot have been the result of an increase in their synthesis.

Finally, it might be argued that insulin had increased the rate of exchange of one amino acid for another ('heteroexchange'; Heinz & Walsh, 1958) and thereby increased the concentration of one amino acid at the expense of another. However, exchange cannot produce a net increase in the total of the intracellular concentration of amino acids (or, for that matter, a net decrease in extracellular concentration) as was actually found.

The findings therefore suggest that insulin stimulates the intracellular accumulation of natural amino acids in perfused rat heart in a manner entirely analogous to its action on α-aminoisobutyric acid, i.e. by stimulating the transfer of amino acids from the extracellular to the intracellular compartment. This stimulation is not dependent on protein synthesis and for certain amino acids can be seen only in its absence.

The results are not to be interpreted as proving that insulin increases protein synthesis in muscle solely by an influence on amino acid accumulation. The evidence that the effect of the hormone on the two processes is separate has been reviewed by Wool & Scharff (1965). Briefly, insulin can enhance protein synthesis in muscle by an action at a site distal to amino acid transport, presumably by an effect on some intracellular process (Wool & Krahl, 1959*b*, 1964; Manchester & Krahl, 1959). The converse, as we have seen here, is also true, that insulin can accelerate amino acid transport in the absence of a stimulation by the hormone of protein synthesis. That one is not of necessity dependent on the other is of extreme import in analysing the mechanism of insulin action. In physiological circumstances, the response to insulin is presumed to be integrated; the hormone accelerates protein synthesis and provides, at one and the same time, the material (amino acids) and a supply of energy (glucose) to keep the synthetic process primed.

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