5. Magnesium can be totally replaced by manganese in the medium.

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The Effect of Insulin *in vitro* on the Accumulation of Amino Acids by Isolated Rat Diaphragm

BY K. L. MANCHESTER AND F. G. YOUNG
Department of Biochemistry, University of Cambridge

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It is now well established that insulin *in vitro* enhances the incorporation of labelled amino acids into the protein of isolated rat diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*). This effect of insulin, which is not reproduced *in vitro* by any other hormones examined in this respect (Manchester & Young, 1959; Manchester & Young, in preparation), is not dependent upon or affected by the presence of glucose in the medium, and addition of glucose alone under most conditions has no observable effect on amino acid incorporation.

Since insulin accelerates the rate of entry of glucose and of a variety of non-utilizable sugars into the muscle cell (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Helmreich & Cori, 1957; Park, Johnson, Wright & Batsel, 1957), it seemed to us possible that the stimulation by insulin of incorporation of labelled amino acids into the protein of diaphragm might be a consequence of an enhancement by this hormone of the rate of entry of amino acids into the tissue cells. Evidence in support of such a view was found by Kipnis & Noall (1958), when they showed that insulin *in vitro* enhanced the rate of accumulation, and the maximum accumulation effected, of a non-utilizable amino acid (α-amino*iso*butyric acid) by the isolated intact diaphragm preparation of Kipnis & Cori (1957). We have reinvestigated the effect of insulin on the accumulation of α-amino*iso*butyric acid by both intact and ordinary 'cut' diaphragm preparations, and have also studied the effect of insulin on the accumulation of a number of utilizable, naturally occurring amino acids.

METHODS

Radioactive materials. Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and α-amino*iso*butyric acid had ^{14}C in the carboxyl position; alanine, leucine, phenylalanine, arginine, lysine, aspartic acid and glutamic acid were all the L-isomers and uniformly (U) labelled with ^{14}C. The concentrations at which the amino acids were added to the medium are indicated in the tables and figures. In the experiments with intact diaphragm the amount of radioactivity added to the medium (irrespective of the quantity of amino acid) was 0·33 μc/ml.; in the experiments with cut diaphragm the amount of radioactivity added to the medium was 0·4 μc/ml.

[U-^{14}C]Ornithine was made from [U-^{14}C]arginine by a modification of the method of Mohamed & Greenberg (1945) with arginase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). It was purified by paper electrophoresis.

Medium. The balanced salt bicarbonate buffer of Gey & Gey (1936) was used throughout, gassed with O_2+CO_2 (95:5, v/v) or N_2+CO_2 (95:5, v/v) (pH 7·4 at 37°), with or without addition of glucose (2·5 mg./ml.). Ox insulin (Boots Pure Drug Co. Ltd.), six times recrystallized, was dissolved in 3·3 mN-HCl to a concentration of 20 units/ml. and diluted with Geys' buffer to 0·1 unit/ml. on the day of the experiment.

Rats. Diaphragms were removed from female albino Wistar-strain rats about 100–120 g. in weight. The rats had been bred in the Department and fed on a standard laboratory diet (MRC-41, Bruce & Parkes, 1949). They were deprived of food about 16 hr. before death, but had free access to water at all times.

Removal and incubation of diaphragm. Experiments with sugars (Kipnis & Cori, 1957; Randle & Smith, 1958) have suggested that the usual isolated hemidiaphragm, because of its cut edges, is not a satisfactory preparation for the measurement of a sugar 'space', and that the intact-diaphragm preparation, introduced by Kipnis & Cori (1957), in which the diaphragm remains uncut within the rib case, is more suitable in this respect. In the present investigation we have used both preparations, both being removed as soon as practicable after death and washed for about 10 min. at 10° in freshly gassed buffer containing no glucose, insulin or amino acid. Intact diaphragms were placed in 50 ml. conical flasks containing 5 ml. of medium. They were gassed, sealed and incubated at 37° with continuous shaking. In some experiments, involving the construction of a curve relating time and effect, hemidiaphragms were incubated individually in small conical flasks containing 1 ml. of medium and gassed and sealed before incubation. In other experiments involving only one period of time, four 50 ml. conical flasks were set up, each containing 6 ml. of medium. The medium in the first flask contained no insulin or glucose; the second, glucose but no insulin; the third, insulin but no glucose; and the fourth, both glucose and insulin. Hemidiaphragms were removed from 12 rats, systematically mixed so that hemidiaphragms from the same rat were never contained in the same flask, and six were added to each flask, the flasks being gassed and sealed as before. In the experiments in which the time allowed for the accumulation of labelled amino acids was 5–15 min. the labelled amino acid was added to the medium after incubation of the diaphragm had begun in order that the length of time for which the diaphragm was in contact with labelled amino acid at 37° might be precisely fixed.

Measurement of the accumulation of soluble radioactive material. After incubation the diaphragm tissue from intact diaphragms was dissected away from the rib cage. Diaphragm tissue so obtained, and also hemidiaphragm (cut tissue), was carefully blotted, weighed and placed in 2 ml. of water at 100° for 5 min. After centrifuging, a 1 ml. sample of the soluble extract was transferred to a 2 cm. diam. aluminium planchet that had been painted with pale gold lacquer to bind the residue, and the extract was evaporated to dryness under an infrared lamp and counted beneath a thin end-window counter. A sample (0·2 ml.) of the medium after incubation was diluted to 5 ml. with water and 1 ml. of the diluted material was plated, dried and counted in a like manner. Counts/min. ranged between 500 and 3000. The number of counts/unit wt. of diaphragm water was calculated on the assumption that the diaphragm tissue contains 75% of water, and this figure was divided by the number of counts remaining in the incubation medium to give a concentration ratio for tissue/medium.

Although extracts of the tissue and medium prepared in this fashion contain a little protein into which amino acids may have been incorporated, calculation shows that the contribution of this protein to the amount of radioactivity measured in these extracts is negligible. In preliminary experiments with glycine, phenylalanine and lysine it was found that more than 95% of the radioactivity present in the extract of the tissue was recoverable by paper chromatography in the fraction corresponding to the amino acid added to the medium. Kipnis & Noall (1958) reported a similar finding for α-amino*iso*butyric acid.

Measurement of incorporation. Samples of protein for measurement of their content of radioactivity were prepared as described by Manchester & Young (1958*a*). Incubation at 12° reduced incorporation to a few per cent of the amount at 37°. Negligible incorporation of α-amino*iso*-[^{14}C]butyric acid into protein was observed. Small but measurable incorporation from [^{14}C]ornithine into protein was found, but whether this was a property of the ornithine or an artifact due to contamination of the ornithine by arginine was not certain.

Design of experiments. Experiments on the effect of insulin on the accumulation of free radioactive amino acids in diaphragm have been carried out in two ways. First, the accumulation at a number of different time intervals has been measured; this, of practical necessity, has been based on single observations at multiple times. Secondly, observations of the accumulation during a single period of incubation have been made. The latter procedure has the advantage that statistical analysis can be applied to the figures obtained; the former procedure ensures that one does not start by doing a large number of experiments, designed to provide statistically significant information, at a time period when the insulin effect is less apparent than at others.

Christensen & Streicher (1949) found that for there to be net uptake of amino acid from the medium by isolated rat diaphragm the amino acid must be added to the medium in a concentration greater than that in which it is normally present in blood plasma; for a concentration of amino acid less than that in plasma there was a net outflow of amino acid from the tissue into the medium. Within a physiological range of amino acid concentrations the ratio of the amount of amino acid in the tissue to that in the medium remained constant and did not vary with changes in the external concentration. In our investigation it appeared probable that addition of labelled amino acids to the medium at the lowest practicable concentration, when the net inflow of amino acid in the absence of insulin is at a minimum, would be the conditions under which an effect of insulin on accumulation of amino acid by diaphragm would be most easily demonstrable. We have done experiments of this sort, mostly involving the construction of a curve relating time and effect (e.g. Figs. 1–3), and we have also studied the effect of insulin on the accumulation of amino acids by diaphragm when the amount of amino acid added to the medium is considerably in excess of that found in

plasma (see Tables 1 and 2). Insulin is already known to stimulate incorporation of amino acid into protein of isolated rat diaphragm at all the concentrations of amino acids used in these experiments (compare figures in Manchester & Young, 1958*a*, 1959; Manchester, Randle & Smith, 1958).

RESULTS

Accumulation in vitro *of amino acids by isolated intact and cut rat diaphragm*

A comparison of the figures in Tables 1 and 2 reveals that in 90 min. a greater accumulation of most amino acids is effected by cut diaphragm than by the intact preparation. The reason for this is uncertain. If cut diaphragm lost amino acid through its cut edges lower figures for accumulation by cut diaphragm than for intact would be expected. It is possible that accumulation is initially more rapid with cut diaphragm than with the intact, although similar values would ultimately be attained.

In the majority of our experiments the ratio of the concentration of free labelled amino acid in tissue water to that in the medium was greater than unity, which suggests that a concentration gradient is maintained between the tissue and the medium. The abnormally low ratios for glutamic acid and aspartic acid (Table 1) are probably a consequence of the rapid breakdown and metabolism of these acids in diaphragm tissue *in vitro* (Manchester & Young, 1958*b*). Christensen & Streicher (1949) found a ratio of 3–5 for glycine for isolated diaphragm. Noall & Kipnis (1959) obtained a figure of 7 for the concentration ratio of α-amino*iso*butyric acid between diaphragm and plasma *in vitro*.

Cooling to 12°, which interferes with energy production both by oxidation and by glycolysis, reduced the ratio for phenylalanine and glycine to unity or below, both in the presence and the absence of added glucose (Tables 1 and 2). Anoxia, which interferes with energy production by oxidation but not by glycolysis, reduced the ratio for glycine and arginine nearly to unity in the absence of added glucose, but in the presence of glucose the ratio was significantly higher than in its absence (Table 2).

Effect of insulin in vitro *on accumulation of amino acids by diaphragm*

Addition of insulin to the medium accelerated the rate at which α-amino*iso*[^{14}C]butyric acid entered the intact-diaphragm preparation (Fig. 1). The time curve for the accumulation of this amino acid rose steeply over the first hour of incubation, but was still rising, though slowly, after 3 hr. At the end of 90 min. of incubation insulin had significantly increased the concentration ratio both with the intact-diaphragm preparation and the cut preparation (Tables 1 and 2). Although the accumulation was greater for the cut than for the intact preparation, the effect of the added insulin, on a percentage basis, was about the same (240 and 220% respectively). Addition of glucose in no case significantly affected the accumulation of α-amino*iso*butyric acid (Tables 1 and 2).

As was found with α-amino*iso*butyric acid, insulin raised the accumulation of [^{14}C]glycine by diaphragm, intact (Table 1) or cut (Table 2), both in the presence and the absence of added glucose in the medium. The insulin effect, however, was not as striking as with α-amino*iso*butyric acid. A possible effect of insulin was seen in the time curves for glycine (Figs. 1 and 2) at a majority of the time intervals between 30 min. and 3 hr., but the significance of the differences is doubtful. Addition of glucose had no consistent effect on glycine accumulation.

Addition of insulin had no stimulating effect on the accumulation, after 90 min., of [^{14}C]alanine, [^{14}C]phenylalanine, [^{14}C]lysine, [^{14}C]glutamic acid and [^{14}C]aspartic acid by the intact diaphragm (Table 1) or of [^{14}C]leucine, [^{14}C]phenylalanine and [^{14}C]lysine by the cut preparation (Table 2). Similar results are apparent from the time curves for alanine and phenylalanine (Fig. 1) and for phenylalanine, lysine, glutamic acid and ornithine (Fig. 2).

One of the difficulties of showing an effect of insulin on glucose transport is the rapid rate at which glucose is utilized once it has entered the tissue. Park *et al.* (1955) found that by incubating isolated rat diaphragm at 12° utilization of glucose was sufficiently retarded to allow free glucose to accumulate within the diaphragm. Under these conditions they found that insulin accelerated the entry of glucose. The possibility that insulin, by direct or indirect action, so raised the rate of transfer of labelled amino acids from the cell pool into protein that the observation of an effect of insulin on the accumulation of amino acids was masked, seems most unlikely, since the amount of free radioactivity present in the tissue as free amino acid at any time is vastly in excess of the amount being incorporated into protein. Nevertheless, we have studied the effect of insulin on the accumulation in diaphragm of amino acids under conditions where the incorporation of these amino acids into protein is reduced to negligible proportions. Incorporation has been inhibited (*a*) by cooling to 12°, which greatly slows down enzymic reactions, (*b*) by anoxia, which interferes with the production of energy-rich phosphate needed for incorporation (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959). Under neither of these conditions did insulin have an observable effect on the accumulation of glycine, phenylalanine or arginine (Tables 1 and 2).

Table 1. *Effect of insulin and glucose* in vitro *on the accumulation of labelled amino acids by the isolated intact rat-diaphragm preparation*

Insulin was used at 0·1 unit/ml. and glucose at 2·5 mg./ml. Each figure is the average of six observations. In each instance the mean result is given ±S.E.M. Length of incubation was 90 min. in every case except for the experiment marked with an asterisk (*), where it was 120 min. N.S., not significant.

Amino acid added to the medium	Concn. of amino acid (μmole/ml.)	Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium				Significance of differences
		No insulin; no glucose (*a*)	No insulin; glucose (*b*)	Insulin; no glucose (*c*)	Insulin and glucose (*d*)	
Concn. of added amino acid greater than that in rat plasma						
α-Amino*iso*[1-^{14}C]butyric acid	0·15	0·77±0·026	0·74±0·042	1·63±0·09	1·67±0·13	(*c*) – (*a*) $P < 0·001$, (*d*) – (*b*) $P < 0·001$
[1-^{14}C]Glycine	1·0	1·19±0·03	1·36±0·07	2·09±0·04	1·70±0·07	(*c*) – (*a*) $P < 0·001$, (*d*) – (*b*) $P < 0·01$, (*b*) – (*a*) P 0·05, (*c*) – (*d*) P 0·001
L-[U-^{14}C]Alanine	1·0	1·24±0·049	1·33±0·024	1·38±0·086	1·36±0·072	N.S.
L-[U-^{14}C]Phenylalanine	1·0	1·33±0·054	1·32±0·040	1·26±0·029	1·07±0·085	(*b*) – (*d*) $P < 0·05$
L-[U-^{14}C]Lysine	1·0	1·14±0·048	1·14±0·086	0·90±0·055	1·01±0·051	(*a*) – (*c*) $P < 0·01$
L-[U-^{14}C]Glutamic acid	1·0	0·71±0·02	0·75±0·04	0·74±0·04	0·70±0·02	N.S.
L-[U-^{14}C]Aspartic acid	1·0	0·61±0·026	0·59±0·028	0·64±0·028	0·58±0·020	N.S.
Concn. of added amino acid less than that in rat plasma						
[1-^{14}C]Glycine*	0·1	—	1·54±0·09	—	2·47±0·25	(*d*) – (*b*) $P < 0·01$
L-[U-^{14}C]Lysine	0·05	—	1·04±0·036	—	1·08±0·05	N.S.
Incubation carried out at 12°						
L-[U-^{14}C]Phenylalanine	1·0	1·04±0·037	0·95±0·037	1·03±0·033	1·08±0·14	N.S.

Table 2. *Effect of insulin and glucose* in vitro *on the accumulation of labelled amino acids by the isolated cut rat-diaphragm preparation*

Insulin was used at 0·1 unit/ml. and glucose at 2·5 mg./ml. Each figure is the average of six observations. In each instance the mean result is given ±S.E.M. The length of incubation was in every case 90 min.

Amino acid added to the medium	Concn. of amino acid (μmole/ml.)	Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium				Significance of differences
		No insulin; no glucose (*a*)	No insulin; glucose (*b*)	Insulin; no glucose (*c*)	Insulin and glucose (*d*)	
		Incubation at 37°; O_2+CO_2				
α-Amino*iso*[1-^{14}C]butyric acid	0·22	2·03±0·10	2·40±0·17	5·35±0·28	5·30±0·33	(*c*) − (*a*) $P < 0.001$, (*d*) − (*b*) $P < 0.001$
[1-^{14}C]Glycine	1·0	3·57±0·16	3·63±0·12	4·36±0·35	4·44±0·39	(*c*) − (*a*) $P < 0.1$, (*d*) − (*b*) $P < 0.1$
L-[U-^{14}C]Leucine	1·0	3·69±0·23	3·64±0·18	4·02±0·18	3·32±0·12	(*c*) − (*d*) $P < 0.01$
L-[U-^{14}C]Phenylalanine	1·0	1·67±0·31	1·61±0·046	1·54±0·024	1·53±0·027	N.S.
L-[U-^{14}C]Lysine	1·0	2·68±0·064	2·47±0·073	2·49±0·098	2·31±0·099	(*a*) − (*b*) $P < 0.05$
		Incubation at 12°; O_2+CO_2				
[1-^{14}C]Glycine	1·0	0·85±0·036	0·89±0·024	0·86±0·033	0·87±0·028	N.S.
		Incubation at 37°; N_2+CO_2				
[1-^{14}C]Glycine	1·0	1·18±0·011	1·49±0·034	1·24±0·019	1·56±0·035	(*b*) − (*a*) $P < 0.001$, (*d*) − (*c*) $P < 0.001$
L-[U-^{14}C]Arginine	1·0	1·18±0·021	1·33±0·036	1·23±0·013	1·51±0·034	(*b*) − (*a*) $P < 0.01$, (*d*) − (*c*) $P < 0.001$, (*d*) − (*b*) $P < 0.01$

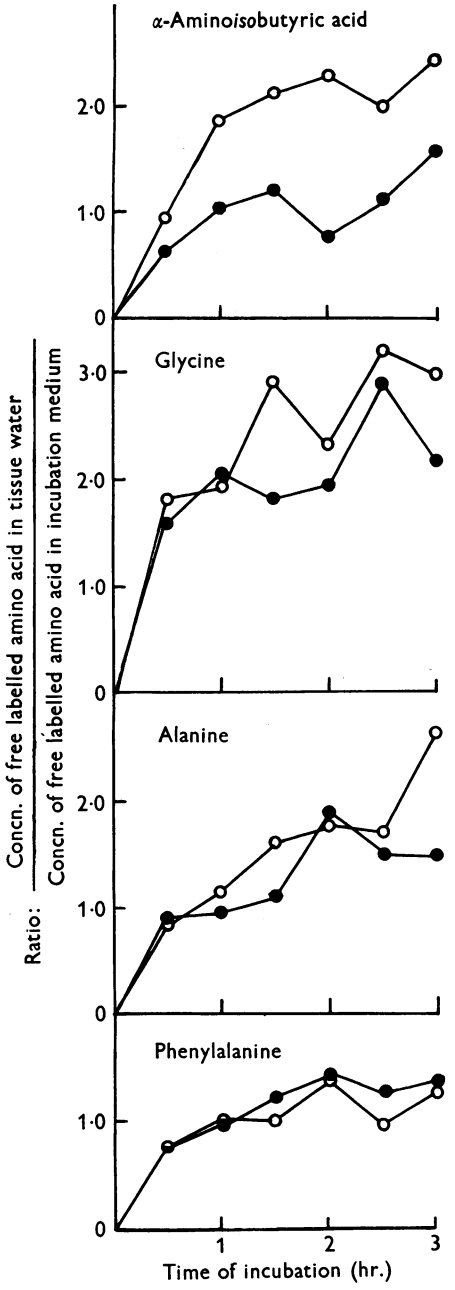

Fig. 1. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated intact rat-diaphragm preparation. ●, No insulin added; ○, insulin added. Each point represents a single observation. No glucose was added to the medium. The concentrations (μmole/ml.) of amino acids added to the medium were: α-amino*iso*butyric acid, 0·1; glycine, 0·1; L-alanine, 0·06; L-phenylalanine, 0·035.

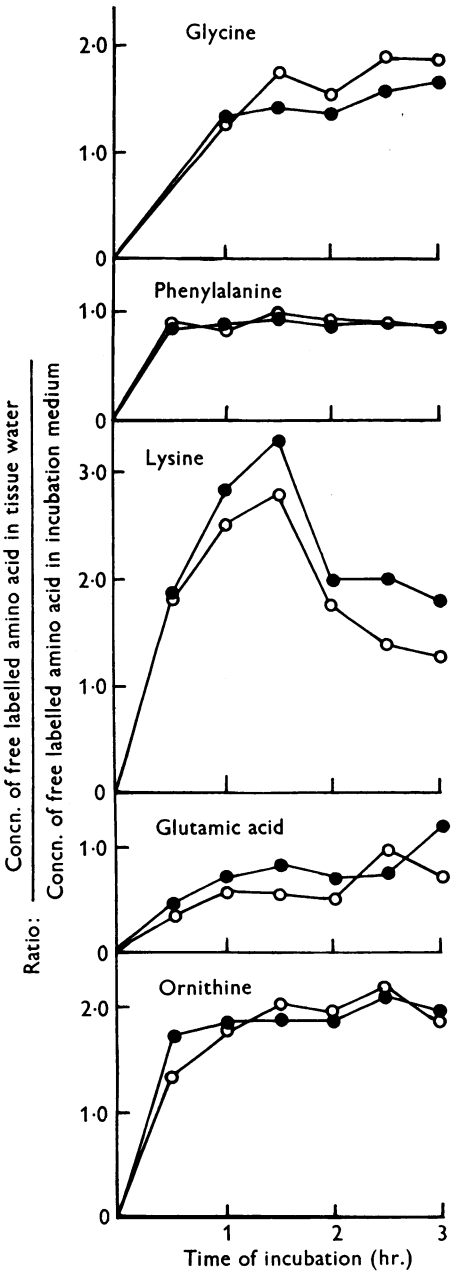

Fig. 2. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated cut rat-diaphragm preparation. ●, No insulin added; ○, insulin added. Each point represents a single observation. Glucose (2·5 mg./ml.) was added to the medium. The concentrations (μmole/ml.) of amino acids added to the medium were: glycine, 0·1; L-phenylalanine, 0·035; L-lysine, 0·055; L-glutamic acid, 0·08; L-ornithine, 0·1.

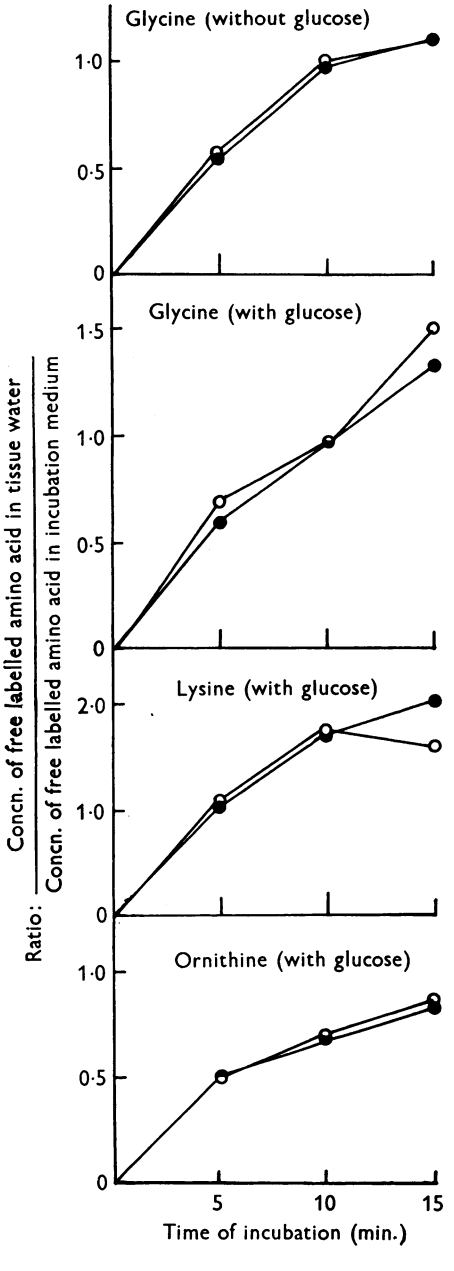

Fig. 3. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated cut rat-diaphragm preparation. ●, No insulin added; ○, insulin added. With glycine and lysine each point represents a single observation. Each point for ornithine is the average of two observations. The concentrations (μmole/ml.) of amino acids added to the medium were: glycine, 0·1; L-lysine, 0·055; L-ornithine, 0·1.

Since the time curves described so far suggest that the attainment of equilibrium between the free amino acid in the tissue and that in the medium takes place rapidly, it seemed possible that the effect of insulin on this process, if in fact there is one, might be more apparent in the early stages of incubation than at the later time periods studied; insulin, although not affecting the final equilibrium, might accelerate the rate at which this equilibrium was reached. We have therefore looked for an effect of insulin on the accumulation of labelled amino acids by diaphragm during the first few minutes of incubation. No effect of insulin on the rate of accumulation of glycine, lysine or ornithine could be detected in periods of 5–15 min. (Fig. 3).

DISCUSSION

The fact that ratios approaching unity were obtained at 12° and under anoxic conditions in the absence of glucose suggests that amino acids are able to penetrate approximately the whole of the tissue water of isolated rat diaphragm in the absence of an active process of concentration (cf. measurements for xylose by Kipnis & Cori, 1957, and by Randle & Smith, 1958). Nevertheless the concentration of amino acid may not be uniform throughout the compartments of the tissue, and in particular may be different in the extracellular and intracellular fluids. If the extracellular fluid always contains the same concentration as the medium, a mean concentration ratio for the whole tissue which is above unity may be seriously less than the true ratio for the intracellular fluid and medium.

The finding of Kipnis & Noall (1958) that insulin stimulates the accumulation of α-amino*iso*butyric acid by the intact-diaphragm preparation has been confirmed; we further find that the insulin effect is equally apparent when cut diaphragm is used. We have also found that insulin stimulates accumulation of glycine by diaphragm, both cut and intact, but with none of the other eight naturally occurring amino acids investigated have we found any evidence to suggest that insulin stimulates their accumulation by either cut or intact diaphragm. Although our results do not preclude the possibility that insulin stimulates the transfer of amino acids into some small fraction of the cell volume, e.g. microsomes or mitochondria, an effect which would be too small to be observable in this type of experiment, we are forced to conclude that with the possible exception of glycine we have failed to obtain evidence that the stimulation of the incorporation of labelled amino acids into protein follows an enhancement by insulin of the accumulation of these amino acids in the intracellular fluid of the tissue. Such a conclusion is in accord with two recent observations. Wool & Krahl (1959*b*)

find that insulin *in vitro* stimulates the incorporation into diaphragm protein of labelled amino acids accumulated *in vivo* before removal of the diaphragm; that is, under conditions where the bulk of the free amino acid is probably intracellular and where the extracellular concentration of labelled amino acid has been reduced during the presoaking of the diaphragm. Furthermore, Manchester & Krahl (1959) find that insulin stimulates the incorporation into diaphragm protein of ^{14}C from a number of ^{14}C-labelled carboxylic acids and from $^{14}CO_2$, results which suggest that insulin enhances the incorporation of amino acids, formed presumably intracellularly, from various precursors, under conditions where transport of amino acids from extracellular to intracellular fluid is not involved. An effect of insulin on the accumulation of carboxylic acids by diaphragm was looked for but not found. In contrast with the concentration ratios for most amino acids studied those for many carboxylic acids are less than unity (Manchester & Krahl, 1959).

If indeed insulin has no effect on the accumulation of most amino acids by diaphragm, why should α-amino*iso*butyric acid and glycine behave exceptionally in this respect? Structurally, α-amino-*iso*butyric acid differs from all the naturally occurring amino acids since its α-carbon atom carries no hydrogen atom. Such a structure makes the α-carbon a potential tertiary radical, the α-carbon of the other amino acids in proteins, with the exception of glycine, being potential secondary radicals. The figures in the time curves suggest that the rate of attainment of the maximum level of accumulation of α-amino*iso*butyric acid may be slower than for naturally occurring amino acids. It is conceivable that the presence of a second group on the α-carbon in some way, perhaps by steric hindrance, retards the rate of entry of α-amino*iso*-butyric acid into the muscle cell and at the same time makes it more susceptible to an effect of insulin in easing its entry. Incidentally, both α-amino*iso*butyric acid and glycine, the two amino acids whose accumulation by diaphragm is stimulated by insulin, differ from other naturally occurring amino acids in that they are not optically active. The negative results with ornithine are of particular interest in that ornithine is an amino acid which is believed not to be incorporated into protein (see Methods section), i.e. another non-utilizable amino acid as is α-amino*iso*butyric acid. Whereas the effect of insulin on accumulation of α-amino*iso*butyric acid was very marked there was no observable effect with ornithine.

Kipnis & Noall (1958) observed that addition of glucose in either the presence or the absence of insulin slightly depressed the accumulation of α-amino*iso*butyric acid. This observation we have not confirmed. In our experiments glucose did not enhance accumulation of amino acids, except in the experiments under anoxic conditions with cut diaphragm (Table 2), in which it may have provided energy by glycolytic breakdown. Nor did it appear consistently to compete with and therefore retard the entry of amino acids (Tables 1 and 2). Competition between different sugars (Battaglia & Randle, 1959) and different amino acids (Christensen, Cushing & Streicher, 1949) for entry into the diaphragm-muscle cell has been previously observed. No evidence has been found for the competition of glucose with amino acids for entry into isolated rat diaphragm in our experiments, which suggests that sugars and amino acids are carried into the cell by different transport mechanisms. Since sugars do not appear to be actively concentrated, whereas amino acids are, this non-identity of transport mechanisms is not surprising. It is also in accord with the observation with isolated diaphragm that addition of phlorrhizin at a concentration in which it strongly depresses uptake of glucose and penetration by xylose has no effect on amino acid incorporation into protein (Battaglia, Manchester & Randle, in preparation).

In all our experiments it is the movement and accumulation of label that has been measured, not that of the amino acid itself. It is not possible to deduce from these figures how much unlabelled amino acid is lost by the tissue to the medium during the course of the uptake of labelled amino acid. Nor is it possible to assess from our measurements to what extent entry of labelled amino acid represents net uptake of amino acid by the tissue, what proportion of the label entering the tissue does so through the entry of labelled amino acid in exchange for an equal quantity of unlabelled amino acid of the same species leaving the tissue, or to what extent labelled amino acid enters the tissue as amino acid of another variety leaves (Christensen, Hess & Riggs, 1954). The existence of so many circumstances in which labelled amino acids can accumulate in diaphragm complicates attempts to explain the enhancement by insulin of incorporation of labelled amino acids into protein as a consequence of an effect of insulin on the transport of amino acids into the cell. Since the rate of entry of labelled amino acids from the medium into diaphragm is so rapid, the rate-limiting process, which is presumably accelerated by insulin, may well be elsewhere in the sequence of reactions by which labelled amino acids move from the medium into the protein of diaphragm.

SUMMARY

1. The rate of penetration into isolated rat diaphragm, both ordinary 'cut' preparation and 'intact' preparation, of ^{14}C present in the medium

in certain [^{14}C]amino acids has been investigated, together with the effect of insulin thereon. No important differences between the behaviour of the cut and intact preparations was observed.

2. With alanine, α-amino*iso*butyric acid, glycine, leucine, lysine, ornithine and phenylalanine the ratio of concentration of radioactivity in tissue water to that in the medium exceeded unity. With glutamic acid and aspartic acid the ratio was below unity, probably because of the rapid metabolism of these amino acids in isolated rat diaphragm.

3. The addition of insulin to the medium significantly raised the ratio for glycine and α-amino*iso*butyric acid, but not for the other amino acids under study. The effect of insulin was similar whether or not glucose was added to the medium. It was abolished by anoxia and by cooling the system to 12°.

4. Under anoxic conditions, both in the presence or absence of insulin, the ratio was significantly raised by the addition of glucose to the medium. A similar effect of glucose was not seen under any other conditions studied.

5. No substantial evidence has been obtained to suggest that insulin promotes the incorporation of amino acids into protein in isolated diaphragm by enhancing the rate of entry of amino acids into the cytoplasm of the cells from the medium.

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Phosphoinositides

1. CONFIGURATION OF THE INOSITOL PHOSPHATE IN LIVER PHOSPHATIDYLINOSITOL

By J. N. HAWTHORNE
Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15

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Work from several different Laboratories suggests that the inositol-containing lipid which occurs in liver and heart has structure (I) (Faure & Morelec-Coulon, 1954; Hawthorne, 1955*a*; McKibbin, 1956; Hanahan & Olley, 1958). The L-α-glycerophosphate structure, which might be expected from comparison with phosphatidylcholine, has not yet been rigidly proved. There is also doubt about the point of attachment of the phosphoric acid to the *myo*inositol ring, which is the