The Effect of Insulin on Incorporation of Amino Acids into Protein of Normal Rat Diaphragm *in vitro*

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Sinex, MacMullen & Hastings (1952) found that, when insulin was added *in vitro* to diaphragm from normal non-fasting rats incubated in a medium containing DL-[1-^{14}C]alanine but lacking an easily oxidizable substrate such as glucose or pyruvate, the transfer of ^{14}C from the alanine into protein in the diaphragm was increased. Similar observations with [1-^{14}C]glycine in place of alanine were made by Krahl (1953). Sinex *et al.* (1952) found that transfer was less if glucose or pyruvate was present in the medium when no insulin had been added, and that in the presence of glucose or pyruvate addition of insulin raised to only a small extent incorporation into diaphragm protein of ^{14}C from the alanine. But Krahl (1953), on the other hand, observed that incorporation of ^{14}C from [1-^{14}C]glycine into protein of normal fasting rat diaphragm *in vitro* was raised by the addition of glucose alone to the medium, the presence of insulin in the medium in addition to glucose having no significant effect. Since these results were of importance with respect to the relative effects of insulin on carbohydrate and protein metabolism (see Ketterer, Randle & Young, 1957), we have repeated the experiments of these authors and extended their observations to include a variety of labelled amino acids.

MATERIALS AND METHODS

Radioactive materials. ^{14}C-Labelled materials were obtained from the Radiochemical Centre, Amersham. Glycine and DL-alanine, both 1-^{14}C-labelled, and uniformly ^{14}C-labelled ([U-^{14}C]) L-alanine were diluted to a specific activity of about 65 μc/m-mole. L-Leucine, L-isoleucine, L-phenylalanine, L-serine, L-lysine, L-arginine, L-glutamic acid and L-aspartic acid, all U-^{14}C-labelled, were diluted to a specific activity of 130 μc/m-mole. A radioactive protein hydrolysate, obtained by the acid hydrolysis of protein from *Chlorella* grown in the presence of $^{14}CO_2$ and containing a large number of [^{14}C]amino acids, was diluted with a mixture of amino acids (made by acid hydrolysis of *Chlorella*) to a specific activity of 130 μc/m-mole.

L-[^{35}S]Methionine, in the form of methionine sulphoxide, had a specific activity of 2·7 mc/mg. and was used at a concentration of 1 μc/flask.

Medium. Bicarbonate buffer (pH 7·4; Gey & Gey, 1936), gassed with $O_2 + CO_2$ (95:5, v/v), was used throughout. Amino acids had a final concentration of 2·7 μmoles/ml., except for methionine (0·2 μg./ml.). Glucose and sodium pyruvate were used at a concentration of 12·5 μmoles/ml., and NaCl was added to all media not containing sodium pyruvate in such amount as to keep the concentration of Na^+ ions of the medium constant.

Insulin (Boots crystalline insulin) was dissolved in 0·0017 N-HCl to give a concentration of 10 units/ml. Insulin solution (0·1 ml., or 0·1 ml. of 0·0017 N-HCl as control) was added to each flask to make a final insulin concentration of about 0·5 unit/ml.

Preparation of diaphragm. A female albino Wistar rat, which weighed 100–150 g. and had fasted 20–24 hr. before use, was decapitated and bled, and the diaphragm removed. The two halves of the diaphragm were washed in freshly gassed buffer, then gently blotted and transferred to small conical flasks containing 2 ml. of medium. One half of a diaphragm was used as control for the other half in experiments where the effect of insulin was studied. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr., the diaphragm was removed, blotted and homogenized in a Potter–Elvehjem homogenizer in 10 ml. of 10% trichloroacetic acid (TCA). The precipitate was spun down, resuspended in 10 ml. of 10% TCA and heated for 15 min. at 90°. The insoluble material was extracted twice with 1·5 ml. of 0·4 N-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% TCA. It was washed with 2 ml. of ethanol–ether (50:50, v/v), then with 2 ml. of ether, and was dried in a vacuum desiccator.

In experiments with methionine, the TCA precipitate was heated for 15 min. at 90° and then dissolved in 1 ml. of 98% formic acid, followed by 0·2 ml. of 100 vol. hydrogen peroxide. The solution was allowed to stand for 30 min. at room temperature, and was then precipitated by the addition of 10 vol. of 10% TCA. The precipitate was washed with ethanol and ether and dried as above.

Assessment of radioactivity of diaphragm protein. Two hemidiaphragms, weighing about 200 mg., yielded by the method described 25–30 mg. of protein, which had a nitrogen content of about 13% (consistent with a protein content of about 80%), and about 0·05% of phosphorus. The samples of protein were ground in a mortar, collected on 1 cm.^{2} plastic disks, and counted as infinitely thick samples (Popják, 1950), by using a Geiger–Müller tube with a thin mica end-window (thickness 1·5–2·5 mg./cm.^{2}). Sufficient counts were recorded to give a counting error of less than 5%.

Statistical treatment of results. Experiments were performed in groups of six, one each with and without addition of insulin in the absence of added substrate, with and

without addition of insulin in the presence of glucose, and with and without addition of insulin in the presence of pyruvate. Counts found were expressed as a percentage of the counts of the control. This procedure was repeated in most cases six times. The percentages so obtained were grouped and averaged, and the standard errors determined. Significance was assessed on the basis of Student's *t* test.

RESULTS

Alanine. Our results with DL-[1-^{14}C]alanine agree with the findings of Sinex *et al.* (1952), since incorporation of ^{14}C from this amino acid into the protein of diaphragm, incubated in a medium free from easily oxidizable substrate, rose in the presence of insulin (Table 2). Likewise, we agree with them that with DL-[1-^{14}C]alanine addition of glucose or pyruvate to the medium depresses the incorporation into protein of the ^{14}C from the alanine, and that the effect of insulin in promoting incorporation of the ^{14}C under these conditions is less than is found in the absence of added glucose or pyruvate (Tables 1–3). Generally similar results were obtained when L-[U-^{14}C]alanine was used instead of DL-alanine (Tables 1–3), although the incorporation with L-alanine was significantly greater than that with DL-alanine.

Other [^{14}C]*amino acids.* When experiments similar to those with [^{14}C]alanine were carried out either with [1-^{14}C]glycine or with various L-[U-^{14}C]-amino acids added singly, or with algal [^{14}C]protein hydrolysate, incorporation of ^{14}C into the protein of diaphragm was unaffected by addition of glucose or pyruvate either in the absence or in the presence of insulin (Tables 1 and 3). The addition of insulin to the medium resulted in a greater incorporation of ^{14}C either in the presence or absence of glucose or pyruvate (Table 2).

With L-[U-^{14}C]leucine, and to a smaller extent with L-[U-^{14}C]glutamic acid, incorporation of ^{14}C into protein was slightly greater in the presence of added glucose than in the absence of added substrate, insulin also being absent. Addition of insulin in the presence of glucose raised further the incorporation of ^{14}C, and the increase produced by the addition of insulin was roughly the same in the presence of glucose as in its absence. Addition of pyruvate diminished slightly incorporation of ^{14}C from L-[U-^{14}C]glutamic acid into protein (Tables 1–3).

Incorporation into protein of ^{14}C from L-[U-^{14}C]-aspartic acid was unaffected by addition of glucose but was significantly depressed when pyruvate was

Table 1. *Effect of the addition of glucose or of pyruvate to the medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

No insulin was added to the medium. The results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of *P* for a difference which is significant is given in brackets. The number of observations is given in parentheses.

Labelled amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Percentage of control value in presence of added: Glucose	Pyruvate
DL-[1-^{14}C]Alanine	57	62·6±3·0 (8) [P<0·001]	43·9±2·6 (8) [P<0·001]
L-[U-^{14}C]Alanine	103	65·5±3·2 (6) [P<0·001]	47·0±3·1 (6) [P<0·001]
[1-^{14}C]Glycine	84	108·5±5·3 (8)	88·0±9·6 (8)
L-[U-^{14}C]Leucine	524	115·0±6·6 (8) [P<0·1]	94·5±6·9 (8)
L-[U-^{14}C]Isoleucine	337	118·4±15·5 (6)	100·2±10·4 (6)
L-[U-^{14}C]Phenylalanine	104	102·7±5·4 (6)	100·7±5·6 (6)
L-[U-^{14}C]Serine	226	109·0±5·0 (6)	97·3±5·1 (6)
L-[U-^{14}C]Lysine	92	104·0±4·6 (6)	95·2±5·7 (6)
L-[U-^{14}C]Arginine	144	100·3±5·2 (6)	95·3±6·0 (6)
L-[U-^{14}C]Glutamic acid	34	110·0±3·4 (6) [P<0·05]	89·7±3·1 (6) [P=0·02]
L-[U-^{14}C]Aspartic acid	37	90·8±11·0 (6)	75·0±4·6 (6) [P<0·01]
L-[^{35}S]Methionine	382	130·8±9·4 (6) [P<0·05]	112·5±6·7 (6)
Algal ^{14}C-labelled protein hydrolysate	1800	101·0±4·8 (6)	93·7±3·4 (6)

Table 2. *Effect of the addition of insulin* (0·5 *unit/ml.*) *to the medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

The results are expressed as a percentage of the control value found in the absence of added insulin. The value of P for a difference which is significant is given in brackets. The number of observations is given in parentheses.

	Medium including					
	No added substrate Radioactivity in diaphragm protein		Glucose Radioactivity in diaphragm protein		Pyruvate Radioactivity in diaphragm protein	
Labelled amino acid added to medium	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin	Control experiments (counts/min./disk)	Percentage of control value in presence of added insulin
DL-[1-^{14}C]Alanine	57	135·7±4·8 (16) [P<0·001]	35	104·2±3·2 (8)	26	121·7±5·2 (8) [P<0·01]
L-[U-^{14}C]Alanine	106	129·0±6·0 (6) [P<0·01]	69	105·0±1·1 (6) [P=0·01]	49	135·7±3·7 (6) [P<0·001]
[1-^{14}C]Glycine	84	145·0±8·0 (8) [P=0·001]	91	141·0±7·3 (8) [P=0·001]	70	162·0±6·6 (8) [P<0·001]
L-[U-^{14}C]Leucine	524	117·9±1·8 (8) [P<0·001]	596	116·0±2·5 (8) [P<0·001]	491	121·7±2·9 (8) [P<0·001]
L-[U-^{14}C]Isoleucine	337	146·7±2·3 (6) [P<0·001]	422	156·5±5·9 (6) [P<0·001]	335	165·5±6·5 (6) [P<0·001]
L-[U-^{14}C]Phenylalanine	103	136·8±5·8 (6) [P<0·01]	106	138·7±3·2 (6) [P<0·001]	103	145·5±5·1 (6) [P<0·001]
L-[U-^{14}C]Serine	226	157·3±6·1 (6) [P<0·001]	244	140·2±4·1 (6) [P<0·001]	221	146·0±8·1 (6) [P<0·01]
L-[U-^{14}C]Lysine	92	161·8±9·2 (6) [P<0·01]	96	158·2±5·2 (6) [P<0·001]	89	172·7±5·2 (6) [P<0·001]
L-[U-^{14}C]Arginine	144	144·8±7·7 (6) [P<0·01]	144	139·0±7·6 (6) [P<0·01]	136	148·8±4·0 (6) [P<0·001]
L-[U-^{14}C]Glutamic acid	34	131·7±2·8 (6) [P<0·001]	38	125·7±6·0 (6) [P<0·01]	31	128·5±6·7 (6) [P<0·01]
L-[U-^{14}C]Aspartic acid	37	137·7±4·0 (6) [P<0·001]	33	124·0±3·4 (6) [P<0·001]	27	131·3±3·6 (6) [P<0·001]
L-[^{35}S]Methionine	382	217·8±7·0 (6) [P<0·001]	489	171·0±6·4 (6) [P<0·001]	426	216·0±10·2 (6) [P<0·001]
Algal ^{14}C-labelled protein hydrolysate	1800	154·8±4·3 (6) [P<0·001]	1810	152·5±6·2 (6) [P<0·001]	1690	154·7±6·2 (6) [P<0·001]

added to the medium. Addition of insulin, however, stimulated incorporation both in the presence and absence of added substrate (Tables 1–3).

With L-[^{35}S]methionine, the incorporation of ^{35}S into diaphragm protein was promoted by addition of insulin to the medium whether or not glucose or pyruvate were also present (Tables 1–3).

In these experiments the percentage rise in incorporation of label differed from one amino acid to another, but the results were of the same general order.

The results are summarized diagrammatically in Fig. 1.

DISCUSSION

Methods. In their experiments with alanine Sinex *et al.* (1952) used non-fasting male rats, whereas we have used fasting females. Nevertheless, we agree with their findings with respect to the stimulating action of insulin on the incorporation of ^{14}C from DL-[1-^{14}C]alanine into diaphragm protein, and the depressing effect on this incorporation of the presence of glucose or pyruvate in the medium. This observation is of particular interest, because the results of Krahl (1953) suggested that diaphragm from fasting animals can behave differently from diaphragm from non-fasting rats. In a few experiments carried out on the possible influence of sex we have found no differences in behaviour between diaphragm from male or female albino rats.

Behaviour of alanine. Although addition to the medium of glucose or pyruvate diminishes markedly incorporation of radioactivity from [^{14}C]alanine into protein of diaphragm, incorporation of label from none of the other labelled amino acids we have studied is so strikingly depressed by addition to the medium of glucose or pyruvate. We therefore are forced to conclude that the behaviour of alanine is atypical, and must be due to factors peculiar to alanine. Sinex *et al.* (1952) considered the possibility that the diminution in incorporation of radioactivity from [^{14}C]alanine in the presence of glucose or pyruvate results from a dilution of the labelled alanine with unlabelled alanine produced by a process of transamination (Braunstein & Kritzmann, 1937). But if this explanation were true, one would expect to find that addition of glucose or pyruvate to the medium would diminish incorporation of radioactivity from [^{14}C]glutamic acid, aspartic acid and possibly other amino acids. We have observed no such phenomenon in our experiments, except possibly in the effect of the addition of pyruvate on incorporation of radioactivity from [^{14}C]aspartic acid. Alanine differs from all the amino acids we have examined for reasons which are at present under investigation (Manchester & Young, 1958). If we assume that

Table 3. *Effect of the addition of glucose or of pyruvate to an insulin-containing medium on the incorporation* in vitro *of radioactivity from amino acids into protein of normal rat diaphragm*

Insulin (0·5 unit/ml.) was present in each experiment. The results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. The number of observations is given in parentheses.

Labelled amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Percentage of control value in presence of added: Glucose	Pyruvate
DL-[1-^{14}C]Alanine	73	49·8±2·7 (8) [P<0·001]	38·3±3·6 (8) [P<0·001]
L-[U-^{14}C]Alanine	130	58·8±3·1 (6) [P<0·001]	51·7±2·7 (6) [P<0·001]
[1-^{14}C]Glycine	119	105·4±2·4 (8)	97·6±9·3 (8)
L-[U-^{14}C]Leucine	622	112·5±4·5 (8) [P<0·05]	96·0±3·3 (8)
L-[U-^{14}C]Isoleucine	495	124·5±13·8 (6)	113·0±11·8 (6)
L-[U-^{14}C]Phenylalanine	141	104·2±4·3 (6)	105·5±2·7 (6)
L-[U-^{14}C]Serine	351	97·2±2·2 (6)	90·2±5·2 (6)
L-[U-^{14}C]Lysine	152	102·0±4·1 (6)	102·5±6·1 (6)
L-[U-^{14}C]Arginine	207	96·5±6·4 (6)	98·5±6·9 (6)
L-[U-^{14}C]Glutamic acid	45	104·7±3·8 (6)	88·2±6·7 (6)
L-[U-^{14}C]Aspartic acid	51	81·5±7·7 (6)	72·3±4·5 (6) [P<0·01]
L-[^{35}S]Methionine	830	101·2±7·2 (6)	111·8±8·8 (6)
Algal ^{14}C-labelled protein hydrolysate	2720	99·3±4·6 (6)	93·5±4·1 (6)

the process of transamination is one that occurs outside of the mitochondrion, while the enzymes of the tricarboxylic acid cycle which are necessary for the formation of, for example, α-oxoglutarate from glucose or pyruvate, are situated within the mitochondrion, the difference between the behaviour of alanine and of glutamic acid might be explicable.

It is also possible that in the presence of pyruvate there is appreciable fixation of pyruvate to form oxaloacetate (Ochoa, 1945). The oxaloacetate might then be transaminated to aspartic acid, and this unlabelled aspartic acid would dilute the [^{14}C]-aspartic acid entering the tissue. Incorporation of radioactivity would therefore be less. We have investigated this possibility in more detail (Manchester & Young, 1958).

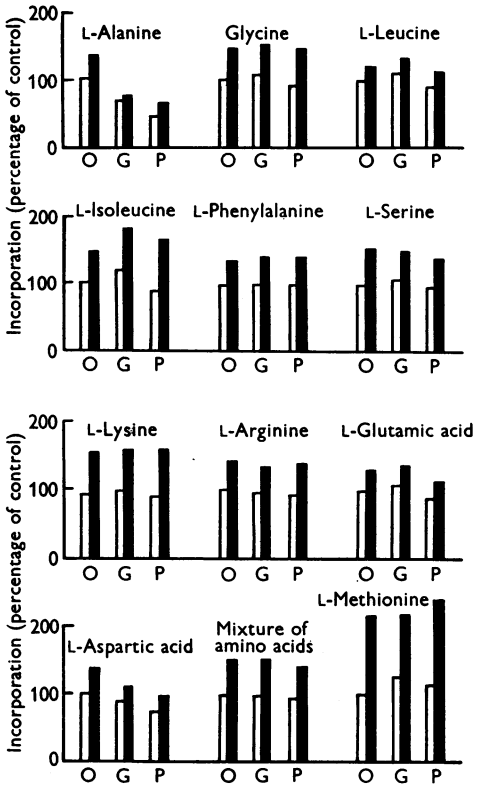

Fig. 1. Effect of insulin, glucose and pyruvate on incorporation *in vitro* of radioactive material from labelled amino acids into protein of normal rat diaphragm. The figures on the ordinate express incorporation as a percentage of that found in the absence of insulin, glucose and pyruvate. Open blocks represent incorporation in the absence of insulin, solid blocks incorporation in the presence of insulin. The letter O signifies that no oxidizable substrate had been added to the medium, G that glucose was present and P that pyruvate was present.

Effect of insulin on protein metabolism. Administration of insulin to a normal animal lowers the plasma level of amino acids (Luck, Morrison & Wilbur, 1928; Kiech & Luck, 1928; Kerr & Krikorian, 1929), and injection of insulin suppresses the increase of amino acid level in plasma that occurs after evisceration (Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947). These observations suggest that insulin influences the nitrogen metabolism of extra-hepatic tissue. Frame & Russell (1946) and Ingle *et al.* (1947) found that this action of insulin was not related to the induced changes in blood-glucose level, though Flock, Block, Mann, Grindlay & Bollman (1952) found that the infusion of glucose could suppress the rise of the plasma amino acid level after hepatectomy.

It has been known for many years that administration of insulin stems the loss of nitrogen from the diabetic animal and induces a positive nitrogen balance. This could be due to either a decreased breakdown or an increased formation of protein in the presence of insulin. In so far as experiments with isolated diaphragm are a measure of what takes place *in vivo*, our results clearly indicate that insulin stimulates the transfer of the carbon of amino acid from extracellular fluid into protein.

Krahl (1953) found that incorporation of ^{14}C from [^{14}C]glycine into glutathione and protein in liver and into diaphragm protein, in tissue removed from the fasting rat, is stimulated by addition of insulin or of glucose, and that in the presence of glucose insulin has no stimulating action. He has suggested therefore (Krahl, 1956) that insulin stimulates peptide synthesis by favouring carbohydrate utilization, a process which would make available more energy and possibly more pentose for the synthesis of ribonucleic acid and protein. But we regard as more significant the observations of Sinex *et al.* (1952), Krahl (1953) and ourselves, that incorporation *in vitro* of radioactivity from [^{14}C]amino acid into protein of normal rat diaphragm is stimulated by insulin in the absence of added carbohydrate, i.e. in circumstances where the action of insulin on carbohydrate metabolism is minimal. These observations suggest that insulin stimulates incorporation of radioactivity by a process which is largely or wholly independent of a stimulation of carbohydrate metabolism. The fact that incorporation of radioactivity of amino acids into protein, and its enhancement in the presence of insulin, is unaffected by addition of glucose or pyruvate adds support to this view.

Ketterer *et al.* (1957) suggested the possibility of a competition between carbohydrate metabolism and protein synthesis with respect to the action of insulin. Circumstances in which insulin promotes carbohydrate utilization could, according to this

view, lead to a diminution of its action on protein synthesis. Our results provide no support for this idea.

Mode of action of insulin on protein synthesis. The fact that addition of oxidizable substrates does not accelerate incorporation into protein of radioactivity from [^{14}C]amino acids indicates that in diaphragm from a rat which has been fasted 24 hr. protein formation is unlikely to be limited by availability of energy. Formation of protein might be limited either by the rate at which the protein-synthesizing mechanism can work or by the rate at which amino acids can cross the cell membrane from the extracellular fluid into the muscle cell. The action of insulin could therefore be to stimulate the protein-synthesizing mechanism, or to increase the transfer of amino acids across the cell membrane, or possibly to exert an effect on both processes. Our results do not enable us to differentiate between these two main possibilities, but in view of the evidence obtained by Park, Bornstein & Post (1955), Park & Johnson (1955) and Levine & Golstein (1955) that insulin promotes the utilization of glucose by accelerating its rate of entry into the muscle cell, insulin might be thought to stimulate the entry of amino acids into the cell in a similar fashion.

SUMMARY

1. The effect of addition of insulin, glucose and pyruvate on incorporation *in vitro* into protein of normal rat diaphragm of ^{14}C from [^{14}C]amino acids and of ^{35}S from a [^{35}S]amino acid has been studied.

2. Incorporation of radioactivity from the [^{14}C]amino acids glycine, leucine, isoleucine, phenylalanine, serine, lysine, arginine, glutamic acid and aspartic acid, each studied separately, and from a mixture of [^{14}C]amino acids, was increased by addition of insulin both in the presence and absence of glucose or pyruvate. Neither glucose nor pyruvate greatly affected incorporation in the presence or absence of insulin.

3. Incorporation of ^{35}S from [^{35}S]methionine sulphoxide was accelerated by addition of insulin both in the presence and absence of glucose or pyruvate. Addition of glucose slightly increased incorporation in the absence of insulin, but neither glucose nor pyruvate affected incorporation in the presence of insulin.

4. Incorporation of ^{14}C from alanine was increased in the presence of insulin in the absence of glucose or pyruvate, but was markedly decreased, in the absence of insulin, by addition of pyruvate or, to a less extent, of glucose; in the presence of these substrates insulin did not stimulate incorporation. The possibility is discussed that this behaviour of added alanine is attributable to the formation in muscle of alanine from pyruvate or glucose by a process of transamination.

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