INCORPORATION IN VITRO OF LABELED AMINO ACIDS INTO RAT DIAPHRAGM PROTEINS*

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We have reported the incorporation of labeled amino acids into the proteins of rabbit bone marrow cells *in vitro* (1), a study that was undertaken to compare the process in intact cells as compared with tissue slices and homogenates. Bone marrow cells are a mixture in different stages of maturity. The uptake of labeled amino acids by rat diaphragm was studied because it is an adult tissue with predominantly one type of cell and it can be removed from the animal with little damage. This preparation has been found useful in studies of carbohydrate metabolism of muscle *in vitro* (2).

The labeled amino acids used were C^{14}-labeled glycine, L-leucine, and L-lysine. The uptake of these amino acids was much slower in rat diaphragm than in bone marrow cells; otherwise the main features of the process were found to be the same in the two tissues.

Procedure

The synthesis of the labeled amino acids used has been described in a previous publication (3). Unless otherwise stated the activities of the amino acids were as follows: glycine-1-C^{14}, 13,400 c.p.m. per mg.; L-leucine-1-C^{14}, 6000 c.p.m. per mg.; L-lysine-1-C^{14}, 15,000 c.p.m. per mg.

The animals were adult white rats in normal nutrition. They were killed by a blow on the head and bled thoroughly. Usually ten to twelve animals were used for an experiment. The diaphragm was removed close to its origin and placed in a small beaker containing cold Krebs-Henseleit Ringer's solution (4). When all the diaphragms were collected, the thick margins, crura, and the central tendons of each were cut away and the remainder was cut along the central axis into two halves. All the halves were collected in a Petri dish containing 20 ml. of ice-cold Ringer's solution; two, taken at random, were then transferred to each reaction vessel.

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The reaction vessels, which were 20 ml. beakers, containing the reaction mixtures were incubated in the apparatus of Dubnoff (5) at 38° under 95 per cent O_2 and 5 per cent CO_2, except in the anaerobic experiments which were under 95 per cent N_2 and 5 per cent CO_2, for 4 hours unless otherwise stated.

At the end of the incubation water was added to each beaker, the pH, in the presence of the tissue, was adjusted to 5.0, the tissue and reaction mixture were homogenized in the apparatus of Potter and Elvehjem (6), and then trichloroacetic acid and water were added to give a volume of 100 ml. of 7 per cent trichloroacetic acid. The suspension stood overnight at room temperature; the precipitated protein was then washed, dried, and its radioactivity measured as previously described (7).

All of the equipment was sterilized before it was used. As a further protection against interference by bacterial action, all of the reaction mixtures contained 1000 units per ml. of penicillin G. We have found in these and other experiments that this concentration of penicillin G keeps the reaction mixtures practically bacteria-free and does not affect the incorporation of labeled amino acids.

The solvent for all the solutions used in making up the reaction mixtures was Krebs-Henseleit Ringer's solution. The volume of the reaction mixtures was 1.0 ml. One component was 0.1 ml. of a solution of penicillin G containing 10,000 units per ml., another the amino acid solution at pH 7.5.

Results

The rate of incorporation into the proteins of glycine, leucine, and lysine proceeded unslackened up to 5 hours (Fig. 1). Zero uptake at zero time indicated on the graph represents experimental values.

Fig. 2 shows that the rate of uptake of each of the three amino acids was a logarithmic function of the initial concentration up to an inflection point; beyond that point there was little or no increase in amino acid taken up with further increase in initial concentration. The inflection point with leucine and glycine was near 0.01 M, with lysine 0.003 M. The values at 0.001 M initial concentration are 0.52, 0.56, and 0.58 mM $\times 10^{-3}$ per gm. (dry weight) of protein for glycine, leucine, and lysine respectively.

Table I summarizes observations on the effects of anaerobiosis and of oxidation and phosphorylation inhibitors. Anaerobiosis, 0.001 M arsenite, or 0.001 M dinitrophenol inhibited the uptake of the three amino acids completely. 0.001 M arsenate and azide were somewhat less inhibitory and the degree of inhibition of the uptake of each of the three amino acids was nearly the same. These findings are very similar to those obtained with rabbit bone marrow cells (1).

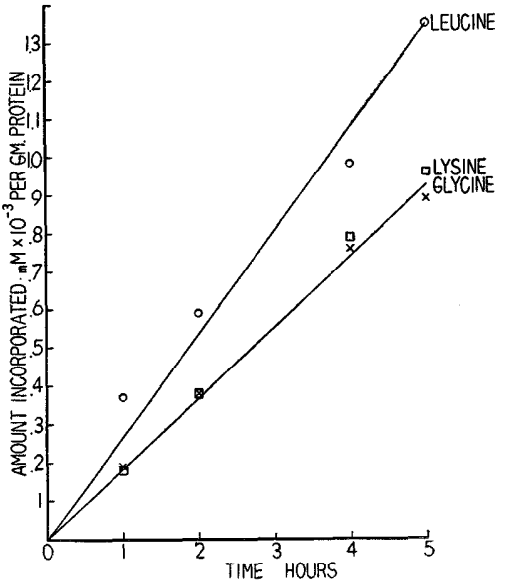

FIG. 1. Rates of incorporation of labeled amino acids by rat diaphragm. The reaction mixtures contained 0.4 mg. of glycine, 0.65 mg. of leucine, and 0.35 mg. of lysine. The zero values at zero time were experimental points.

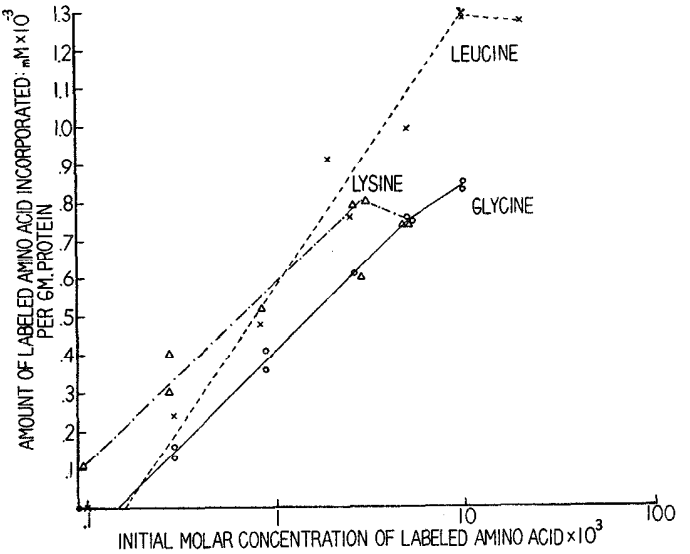

FIG. 2. Effect of initial concentration of labeled amino acid on its rate of incorporation by rat diaphragm. The points on the graphs were from three experiments in which the range of initial concentrations of the three amino acids overlapped. The abscissa scale is logarithmic.

When the tissue was incubated with labeled glycine, leucine, and lysine together, the count subsequently given by the protein was the sum of those when the tissue was incubated with each of these amino acids separately (Table II). This was the same result as that which was found with rabbit bone marrow cells (1) and with a sedimented fraction of guinea pig liver homogenate (3). It indicates that here also the amino acids are taken up independently of each other.

Unlike rabbit bone marrow cells the ability of the diaphragm to incorporate glycine, leucine, and lysine was not completely lost after its cell structure was disrupted by homogenization. The rate of uptake of each of the three amino acids in the homogenized tissue was one-quarter

TABLE I

Effect of Anaerobiosis and of Inhibitors of Oxidation and Phosphorylation on Incorporation of Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rat Diaphragm

Inhibitor	Glycine		Leucine		Lysine	
	Counts per min. per mg. protein	Per cent inhibition	Counts per min. per mg. protein	Per cent inhibition	Counts per min. per mg. protein	Per cent inhibition
None	0.76		0.65		1.63	
Anaerobiosis	0	100	0	100	0	100
Arsenite	0	100	0	100	0.05	97
Dinitrophenol	0	100	0	100	0	100
Azide	0.11	85	0.14	78	0.24	85
Arsenate	0.25	67	0.27	58	0.60	63

The reaction mixtures contained 0.40 mg. of glycine, 0.74 mg. of leucine, and 0.65 mg. of lysine, and 0.01 ml. of 0.01 M inhibitor, its pH adjusted to 7.5. The inhibitors were sodium arsenate (Na_2HAsO_4), arsenious oxide (calculated as As_2O_3), sodium azide, and 2,4-dinitrophenol.

of that in the intact tissue (Table III). When the diaphragm was boiled prior to its addition to the reaction mixture, there were no counts in the protein at the end of the incubation with either glycine, leucine, or lysine.

Evidence that the labeled amino acids had been incorporated into the proteins was obtained by hydrolyzing them completely with HCl and chromatographing the hydrolysates on starch columns according to the method of Moore and Stein (8). In each case the non-radioactive form of the labeled amino acid with which the tissue had become labeled was added to the hydrolysate before the latter was chromatographed. In the eluates the fractions corresponding to the three labeled amino acids were thus identified by the order in which they emerged and by the increased

color (from the amino acids added to the hydrolysates) they gave with the ninhydrin reagent. And radioactivity was found in the fraction corresponding to the amino acid with which the protein had become labeled.

TABLE II

Incorporation of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rat Diaphragm When Incubated Separately and Together

Labeled amino acid	Unlabeled amino acids	Counts per min. per mg. protein observed
Glycine		0.78
"	Leucine + lysine	0.72
Leucine		0.70
"	Glycine + lysine	0.95
Lysine		2.20
"	Glycine + leucine	2.32
Glycine + leucine + lysine		3.46
Calculated from sum of glycine, leucine, and lysine separately.....		3.68

The reaction mixtures contained 0.4 mg. of glycine, 0.65 mg. of leucine, and 0.74 mg. of lysine.

TABLE III

Incorporation of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine into the Proteins of Intact, Homogenized, and Boiled Rat Diaphragm

Labeled amino acid	Intact	Homogenized	Boiled
	mM × 10^{-8} per gm. protein		
Glycine.....	0.83	0.20	0
Leucine.....	1.10	0.28	0
Lysine.....	0.74	0.18	0

The reaction mixtures contained 0.75 mg. of glycine which gave 26,800 c.p.m. per mg., 1.31 mg. of leucine, and 0.37 mg. of lysine. When homogenized diaphragm was used, it was homogenized in the Ringer solution, its pH was adjusted to 8.1, and 0.8 ml. was added to each of the reaction mixtures indicated.

The radioactive amino acids were not isolated and positively identified from the hydrolysates because the uptake of the labeled amino acid by the diaphragm protein was so low. A very large number of animals and of the labeled amino acids would have been required for this purpose, and the cost seemed unwarranted.

DISCUSSION

The incorporation *in vitro* of labeled amino acids into proteins is much slower in diaphragm (rat) than in bone marrow cells (rabbit). For example, at 0.001 M initial concentration, diaphragm took up glycine, leucine, or lysine at a rate of approximately 0.1 mM $\times 10^{-3}$ per gm. of protein per hour, whereas the rates in rabbit bone marrow cells from the same initial concentration of the amino acids were 0.5, 2.9, and 1.8 $\times 10^{-3}$ mM per gm. of protein per hour respectively.

This difference is in accord with the findings *in vivo*. Thus the data of Greenberg and Winnick (9) give, after intravenous injection of C^{14}-glycine into the rat, after $\frac{1}{4}$ hour rates of 2.6 and 0 mM $\times 10^{-3}$ per gm. of protein per hour, in bone marrow and muscle respectively, and after 6 hours, hourly rates of 1.2 and 0.88 mM $\times 10^{-3}$ respectively per gm. of protein.

SUMMARY

1. Rat diaphragm incorporated C^{14}-labeled glycine, L-leucine, and L-lysine into its proteins *in vitro*. The rates were of the order of one-tenth of that in rabbit bone marrow cells *in vitro*. This difference is in accord with the findings *in vivo*.

2. In the case of each of the labeled amino acids the rate of incorporation was a logarithmic function of the initial concentration of the amino acid up to a certain concentration. Beyond that concentration there was little or no further increase in the amount of the amino acid incorporated into the proteins.

3. Anaerobiosis and inhibitors of oxidation and phosphorylation inhibited the uptake of labeled amino acids into the proteins.

4. The rate of uptake of one of the labeled amino acids was unaffected by the presence of the others.

5. The rate of uptake of the amino acids by homogenized diaphragm was one-quarter of that by the intact tissue.

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