INCORPORATION IN VITRO OF LABELED AMINO ACIDS INTO BONE MARROW CELL PROTEINS*

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Nearly all experiments on the incorporation of labeled amino acids into tissue proteins *in vitro* have been done on tissues whose cell structure has been partially or completely disintegrated, *e.g.* tissue slices, segments, or homogenates. Since cell destruction reduces or abolishes the uptake of labeled amino acids (1), it seemed worth while to carry out studies on intact cells *in vitro*. Bone marrow cells were found to be suitable for this purpose. The labeled amino acids used were glycine-1-C^{14}, L-leucine-1-C^{14}, L-lysine-1-C^{14}, and L-lysine-6-C^{14}.

Preparation of Marrow Cells

In preliminary experiments it was found that the bone marrow cells of guinea pig, rabbit, and rat were all very active, but too many guinea pigs or rats were needed to obtain a convenient amount of bone marrow cells to work with. All the results reported here were obtained with rabbit marrow cells. Three or four rabbits were usually needed for an experimental run. The rabbits were obtained commercially; they were of both sexes and in normal nutrition. They were killed by a blow on the head and bled. The humerus, ulna, femur, and tibia were removed as quickly as possible; usually it took three workers 45 minutes to remove and clean these bones from four rabbits. Since it was too inconvenient to remove the bone aseptically, after the bones were removed (without any aseptic precautions), they were immersed in ice-cold 0.1 per cent phenol for 5 minutes, then in ice-cold 70 per cent ethanol for 5 minutes, and then transferred to ice-cold sterile 0.9 per cent NaCl. The ends of a bone were sawn off and the marrow was pushed out of the shaft and scraped out of the ends with a steel knitting needle. The marrow was collected in Krebs-Henseleit Ringer's solution (2), then passed through six layers of cheesecloth to break up the lumps, suspended in 10 volumes of the Ringer's solu-

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tion and centrifuged in a refrigerated centrifuge at 2° at about 2000 × *g* for 10 minutes. The sedimented cells were suspended in about 10 ml. of the Ringer solution and the pH was adjusted to 7.8.

The equipment used in the collection of the marrow cells was sterilized before use. There was a possibility that the incorporation of labeled amino acids was due to bacterial action. We checked the degree of bacterial infestation by plating out reaction mixtures which had been incubated for 6 hours at 38°. In no case did we find more than 50 colonies per ml. of reaction mixture; usually there were none. We compared the results in reaction mixtures with and without additions of 1000 units of penicillin G per ml. We had found earlier that, with this concentration of penicillin, reaction mixtures with an initial heavy bacterial infestation became nearly or completely bacteria-free at the end of a 6 hour experimental run. No difference was found in the uptake of labeled amino acids with and without added penicillin. As an additional precaution against bacterial action penicillin was used in most of the experiments.

Labeled Amino Acids

Glycine labeled with C^{14} in the carboxyl group was synthesized according to the method of Sakami *et al.* (3). The synthesis and resolution of L-leucine and of L-lysine labeled with C^{14} in their carboxyl groups (4) and of L-lysine in position 6 (5) have been described previously.

The activity of the labeled amino acids used in the experiments was as follows: glycine-1-C^{14}, 13,500 c.p.m. per mg.; L-leucine-1-C^{14}, 6000 c.p.m. per mg.; L-lysine-1-C^{14}, 16,500 c.p.m. per mg.; L-lysine-6-C^{14}, 15,000 c.p.m. per mg.

Procedure

The solvent and the suspending fluid used throughout was Krebs-Henseleit Ringer's solution. The reaction mixtures were made up in 20 ml. beakers and incubated in the Dubnoff apparatus (6) at 38° under 95 per cent O_2 and 5 per cent CO_2, except in a few cases in which the mixtures were anaerobic under 95 per cent N_2 and 5 per cent CO_2. The incubation time was 4 hours unless otherwise stated. The volume was either 1.0 or 1.01 ml. and contained, except as noted, in addition to other components, 0.1 ml. of a solution of penicillin G containing 10,000 units per ml., 0.01 ml. of 0.4 M $CaCl_2$ solution, pH adjusted to 6.0, and 0.1 ml. of a solution of each amino acid. At the end of the incubation 4 ml. of water were added, the pH was measured, more water was added to a volume of 80 ml., and, then, with stirring, 20 ml. of 35 per cent trichloroacetic acid. After standing overnight at room temperature, the precipitated protein was centrifuged, washed, and dried and its radioactivity measured as previously described (7).

The results given are the average of duplicates; they did not vary more than ± 10 per cent from the average.

Results

Fig. 1 shows that the rates of incorporation of the three amino acids into the marrow cell protein did not slacken off greatly until after 3 hours.

Fig. 2 shows how the amount of labeled amino acid incorporated varied with the initial concentration of the amino acid. In all three instances the relationship was logarithmic until the initial amino acid concentration that gave maximum incorporation was reached. With higher concentrations the uptake gradually became less, especially with leucine. The marrow cells incorporated the labeled amino acids from concentrations as low as those in the blood, and, in this range, the rate of incorporation varied most with the initial concentration of the amino acid.

The maximum amounts of glycine, leucine, and lysine incorporated on the basis of the observed radioactivity of the proteins were 5.5, 8.9, and 5.4 mM $\times 10^{-3}$ per gm. (dry weight) of protein respectively in 3 hours. Over this time interval the average rates were only a little less than the initial rates (Fig. 1).

Only about one-quarter of the counts in the protein after incubation with labeled glycine was due to glycine incorporated as such into the proteins; with leucine or lysine practically all the counts in the protein were due to the amino acids incorporated as such. The evidence for these conclusions is as follows.

A bone marrow cell suspension was incubated for 4 hours at 38° under 95 per cent O_2 and 5 per cent CO_2 in a reaction mixture containing 0.2 mg. per ml. of labeled glycine whose specific activity was 58,000 c.p.m. per mg. The proteins were precipitated, washed, and dried and gave 19.3 c.p.m. per mg. On treatment with ninhydrin (with non-radioactive leucine added as a CO_2 carrier) the CO_2 collected as $BaCO_3$ gave no counts. 237.8 mg. of protein were completely hydrolyzed and the hydrolysate was chromatographed on starch with a mixture of 0.1 N HCl, *n*-propanol, and *n*-butanol, in the proportions of 1:2:1, respectively, as eluting solvent according to the method of Moore and Stein (8). 100 mg. of non-radioactive glycine were added to the hydrolysate before it was chromatographed. The glycine fraction of the eluate was collected and evaporated to dryness, 600 mg. of non-radioactive glycine were added, and the mixture was acetylated by the method of Hebrst and Shemin (9). The crystalline acetylglycine obtained was recrystallized four times. Its specific activity was the same after the fourth recrystallization as after the first crystallization.

A summary of the results and calculations is as follows: protein hydrolyzed, weight 237.8 mg.; c.p.m. per mg. 19.3; total counts 4589 per

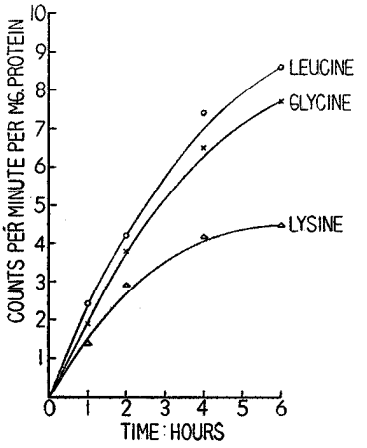

FIG. 1. Rates of incorporation of labeled amino acids by rabbit bone marrow cells. The reaction mixtures contained 0.8 ml. of marrow cell suspension, penicillin G, and $CaCl_2$, and the following amounts of amino acids: glycine 0.75 mg., leucine 1.31 mg., and lysine-1-C^{14} 0.047 mg.

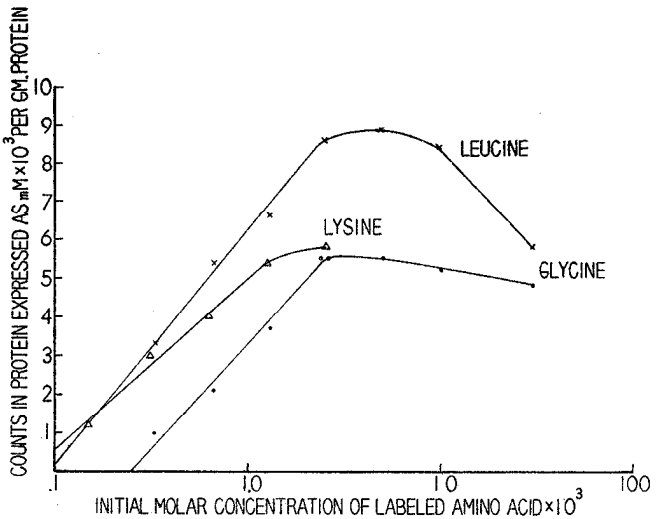

FIG. 2. Effect of initial concentration of labeled amino acid on its rate of incorporation by rabbit bone marrow cells. The results shown are the averages of experiments with and without penicillin G; there were no consistent differences between them, and the differences were within the usual experimental variations. The incubation time was 3 hours, 0.8 ml. of marrow cell suspension was used, and lysine-1-C^{14} was employed. The scale of the abscissa is logarithmic.

minute; glycine added to hydrolysate 700 mg.; glycine in the hydrolyzed protein determined by microbiological analysis 15 mg.; total glycine as

acetylglycine 1115 mg.; c.p.m. per mg. of acetylglycine 1.12; total counts in acetylglycine 1249; per cent of total counts in the protein recovered as acetylglycine 1249/4589 × 100 = 27. Therefore 27 per cent of the counts in the protein were due to glycine incorporated as such. Some of the remaining counts were undoubtedly in the heme formed from the labeled glycine (10, 11).

The acetylglycine gave the following elementary analysis.

$C_4H_7NO_3$.	Calculated.	C 41.02, H 6.02, N 11.96
117.10	Found.	" 41.27, " 6.20, " 11.87

The same general method of hydrolysis and chromatography, followed by isolation with non-radioactive carrier, was applied to the proteins obtained after incubating marrow cells with labeled leucine and with labeled lysine. The leucine was isolated as such and accounted for 75 per cent of the counts originally in the protein.

The leucine isolated gave the following analysis.

$C_6H_{13}O_2N$.	Calculated.	C 54.93, H 9.6, N 10.4
131.17	Found.	" 55.00, " 9.76," 10.5

The lysine was isolated as the monopicrate and accounted for 87 per cent of the counts originally in the protein. The lysine monopicrate isolated gave the following analysis.

$C_{12}H_{17}O_9N_5$.	Calculated.	C 38.36, H 4.57
375.30	Found.	" 39.13, " 4.74

Anaerobiosis and inhibitors of oxidation and phosphorylation inhibited the incorporation of glycine, leucine, and lysine (Table I). The inhibitors used were arsenate, arsenite, azide, and dinitrophenol at 0.001 M concentration in the reaction mixtures. For the anaerobic experiments the reaction vessels were 50 ml. Erlenmeyer flasks fitted with inlet and outlet tubes in rubber stoppers. The flasks were evacuated and then filled with a mixture of 95 per cent N_2 and 5 per cent CO_2 until the pressure was a little above atmospheric; this procedure was repeated six times; the reaction mixtures were then incubated under the N_2-CO_2 gas mixture.

The results in Table I show that anaerobiosis inhibited completely the incorporation of the labeled amino acids into the proteins. The inhibition was a consequence of the anaerobiosis and not of the evacuation and gassing procedure; this was proved by the finding that the counts given by the proteins were the same when the incubation was carried out under O_2-CO_2, whether in beakers or in Erlenmeyer flasks with the repeated evacuation and regassing.

0.001 M arsenite or dinitrophenol was completely inhibitory; arsenate

and azide were nearly so. The relative effectiveness of the four inhibitors was practically the same with the three amino acids.

These observations are in accord with those of other workers. The uptake of methionine into their proteins by non-proliferating *Escherichia coli* is inhibited by azide, fluoride, and cyanide (12). Oxygen consumption and glycine uptake into the proteins of rat liver homogenate go together and the process is inhibited by anaerobiosis (13); anaerobiosis (14) and dinitrophenol (15) inhibit the uptake of alanine into the proteins of rat liver slices.

TABLE I

Effects of Anaerobiosis and of Oxidation and Phosphorylation Inhibitors on Incorporation of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rabbit Bone Marrow Cells

Inhibitor	Labeled amino acid					
	Glycine		L-Leucine		L-Lysine	
	Counts per min. per mg. protein	Inhibition	Counts per min. per mg. protein	Inhibition	Counts per min. per mg. protein	Inhibition
		per cent		*per cent*		*per cent*
None	3.4		4.2		4.3	
Anaerobiosis	0	100	0	100	0	100
Arsenite	0	100	0	100	0	100
Dinitrophenol	0	100	0	100	0	100
Arsenate	0.15	96	1.0	77	0.9	80
Azide	0.57	84	1.0	77	1.4	68

The reaction mixtures contained 0.7 ml. of marrow cell suspension. In the experiments with inhibitors 0.1 ml. of Ringer's solution was replaced by 0.1 ml. of one of the following: 0.01 M sodium arsenate (Na_2HAsO_4), arsenious oxide (calculated as As_2O_3), sodium azide, or 2,4-dinitrophenol; the pH in every case was adjusted to 7.0. The amino acids added were glycine 0.2 mg., leucine 0.35 mg., and L-lysine-6-C^{14} 0.047 mg.

We have examined the effects of the chlorides of calcium, cobalt, copper, magnesium, and manganese and of potassium phosphate on the incorporation of glycine, leucine, and lysine into the proteins of bone marrow cells. Calcium and magnesium chloride added to a final concentration of 0.004 M were neither stimulatory nor inhibitory.

In the presence of 0.004 M potassium phosphate the uptake of glycine, leucine, and lysine was 77, 67 and 75 per cent of those without added phosphate.

Cobalt, copper, and manganese chloride were inhibitory. Table II gives the averages of results of a number of trials. 0.005 M $MnCl_2$ appeared to be slightly less inhibitory than 0.001 M.

Evidence has been given above that only about one-quarter of the counts of the protein after incubation with labeled glycine could be accounted for as incorporated glycine. It is noteworthy, in view of this finding, that the different inhibitory agents reduced the total counts in the protein after incubation with glycine to approximately the same

TABLE II

Effects of $CoCl_2$, $CuCl_2$, and of $MnCl_2$ on Uptake of Glycine, L-Leucine, and L-Lysine by Proteins of Rabbit Bone Marrow Cells

Molar concentration in reaction mixture of added salt	Uptake as per cent uptake without added metal salt		
$CoCl_2$	Glycine	Leucine	Lysine
	100	100	100
0.0001	97	97	100
0.0005	96	65	80
0.001	79	59	60
0.005	58	57	37
$CuCl_2$			
	100	100	100
0.0001	71	89	97
0.0005	55	73	65
0.001	46	41	48
0.005	4	7	9
$MnCl_3$			
	100	100	100
0.0001	100	95	97
0.0005	85	62	68
0.001	29	13	23
0.005	31	30	35

The reaction mixtures contained 0.7 ml. of marrow cell suspension and 0.1 ml. of the metal salt, 10 times the concentration indicated in the table; when no metal salt was added it was replaced by 0.1 ml. of the Ringer solution; 0.2 mg. of glycine 0.325 mg. of leucine, and 0.047 mg. of lysine-6-C^{14}.

degree as in the cases of leucine and lysine, in which practically all the counts in the protein could be ascribed to the incorporation of the amino acid as such.

We have found, in the case of guinea pig liver homogenate, evidence that amino acids are incorporated independently of each other (4). The same occurs in marrow cells. Table III gives the result of a typical experiment. The count given by the protein when marrow cells were incubated with three labeled amino acids was the sum of those when each was in the reaction mixture alone. When a reaction mixture contained

one of the three amino acids in the radioactive form and the two others in their non-radioactive forms, the counts were the same as those when only the radioactive amino acid was added to the reaction mixture.

There was no incorporation of labeled amino acids into the proteins when the bone marrow cell suspension was so treated that all the red cells were disrupted. Hemolysis with water or ether, lyophilization, or freezing and thawing six times gave the same result with all three amino acids. With partial hemolysis the amounts of the three amino acids taken up were in proportion to the degree of hemolysis.

TABLE III

Incorporation of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine into Proteins When Incubated with Rabbit Bone Marrow Cells Separately and Together

Labeled amino acid	Unlabeled amino acid	Counts per min. per mg. of protein
Glycine		1.7
"	Leucine + lysine	1.7
Leucine		2.2
"	Glycine + lysine	2.1
Lysine		4.6
"	Glycine + leucine	3.9
Glycine + leucine + lysine		8.6
Calculated from sum of glycine, leucine, and lysine separately		8.5

The reaction mixtures contained 0.5 ml. of marrow cell suspension. Both the radioactive and non-radioactive amino acids were at the same concentration, expressed as mg. per ml., glycine 2.0, leucine 0.44, and lysine-6-C^{14} 0.47.

DISCUSSION

The rates of incorporation of labeled glycine, leucine, and lysine into the proteins of rabbit bone marrow cells from optimum initial concentrations of the amino acids were from the data of Fig. 2, respectively, 0.5, 2.9, and 1.8 mM $\times 10^{-3}$ per gm. of protein per hour; the value for glycine is on the basis that only 27 per cent of the counts given by the protein came from incorporated glycine. These rates are higher than those that have been observed in tissue slices or homogenates. Rat liver slices formed aspartic acid and glutamic acid from C^{14}-labeled $NaHCO_3$, and the sum of the rates of uptake of the two amino acids was 1.4 $\times 10^{-3}$ mM per gm. of protein per hour (16). Expressed on the same basis (mM $\times 10^{-3}$ per gm. of protein per hour), the rate of incorporation by rat liver slices of alanine was 0.4 (17), of methionine as methionine and cystine 0.003 (18), and of glycine 0.2 to 0.6 (19); the latter values are too high

by 30 (20) to 80 per cent[1] because of adsorbed phosphatidylserine. The rates in the most active fractions of guinea pig liver homogenate (4) were for glycine 0.13, leucine 0.15, and lysine 2.1 mM $\times 10^{-3}$ per gm. of protein per hour; the value for lysine was that found after 2 hours, at which time the process stops, whereas in bone marrow cells it continues, slowing down only a little during 6 hours.

The rabbit bone marrow cells took up glycine, leucine, and lysine into their proteins as fast as the maximum rates of protein turnover observed *in vivo*, after injecting labeled amino acids (21–23) or after feeding N^{15}-labeled glycine (24).[2]

In the calculation of the above rates in marrow cells it was assumed that there was no reduction in specific activity of the labeled amino acid in the reaction mixture as a result of dilution by the same but unlabeled amino acid present initially in the cells or formed during the incubation period. The following considerations indicate that it is unlikely that such dilution was large. The volume of the cells was not more than 5 to 8 per cent of that of the whole reaction mixture. The total amino nitrogen concentration in liver is of the order of 0.03 M. As a rough approximation we may assume that the concentration in the marrow cells was the same and that the concentration of any one amino acid is 1/20 of this value, *i.e.* 0.0015 M. In our experiments the optimum initial amino acid concentration was 0.001 M. The reduction of the specific activity of the labeled amino acid by the same amino acid (unlabeled) in the cells was, therefore, of the order of 5 per cent, and the actual turnover rates were, therefore, greater than those given above by about 5 to 8 per cent.

The uptake of labeled amino acids *in vitro* is much faster in embryonic and malignant than in normal adult tissues (17, 25). The data of Zamecnik *et al.* (17) on the uptake of labeled alanine by hepatoma slices give a rate of 1.0×10^{-3} mM per gm. of protein per hour. This rate is slower than that of leucine and lysine and only twice that of glycine in rabbit bone marrow cells. These cells belong, of course, with embryonic tissues.

[1] Personal communication from Dr. D. M. Greenberg.

[2] Sprinson and Rittenberg's figures give for protein turnover in the whole animal and in the viscera, respectively, 19 and 57 $\times 10^{-3}$ mM of nitrogen per gm. of protein per hour and in man 4.1 and 33 $\times 10^{-3}$. These figures were obtained after feeding N^{15}-labeled glycine on the assumption that its N^{15} was distributed through all the compounds in the body's labile nitrogen pool and the N^{15} in the proteins was derived predominantly from the nitrogen pool rather than only from the labeled glycine. The validity of this assumption is attested to by the finding that the rate of protein turnover so obtained was nearly the same as that found after feeding deuterium-labeled leucine (24). Sprinson and Rittenberg's figures refer to all the amino acids in the protein. A rough approximation, giving the order of magnitude of rate of average turnover of an individual amino acid, is obtained by dividing the rate of turnover of total protein nitrogen by 20.

Circulating rabbit erythrocytes do not incorporate labeled amino acids into their proteins *in vitro*. The rate in marrow cells appears, therefore, to be associated with their immaturity.

The incorporation of the labeled amino acids into the proteins of bone marrow cells was inhibited by anaerobiosis and inhibitors of oxidation and phosphorylation. This was to be expected if one assumes that amino acid turnover in a protein involves first the rupture and then the reconstitution of peptide bonds (1). But the incorporation of lysine into the proteins of guinea pig liver homogenate is inhibited neither by anaerobiosis, nor, to any marked extent, by the inhibitors of oxidation and phosphorylation, which were effective in bone marrow cells (7). It is premature, therefore, to generalize that the process immediately involved in amino acid turnover in proteins, whether *in vivo* or *in vitro*, is directly coupled with respiration and phosphorylation.

SUMMARY

1. Bone marrow cells incorporate rapidly C^{14}-labeled glycine, L-leucine, and L-lysine into their proteins *in vitro*.

2. After incubating the cells with labeled glycine, leucine, or lysine, 27, 75, and 87 per cent respectively of the counts given by the proteins were accounted for by these amino acids isolated from them.

3. The rate of uptake was, in the case of each of the three labeled amino acids, a logarithmic function of the initial concentration of the amino acid.

4. Anaerobiosis, arsenate, arsenite, azide, dinitrophenol, and disruption of the cells inhibited the uptake of all three labeled amino acids.

5. The chlorides of cobalt, copper, and manganese and potassium phosphate inhibited the uptake of the three labeled amino acids. Calcium chloride and magnesium chloride were neither stimulatory nor inhibitory.

6. Evidence is presented that glycine, leucine, and lysine are incorporated into the proteins independently of each other.

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