THE INCORPORATION OF LABELED LYSINE INTO THE PROTEINS OF GUINEA PIG LIVER HOMOGENATE*

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(Received for publication, December 29, 1948)

When C^{14}-labeled lysine is incubated with guinea pig liver homogenate, α-aminoadipic, α-ketoadipic, and glutaric acids are formed from the lysine (1). These transformations were established by finding the radioactivity of the C^{14} tracer in the metabolic products. The homogenate proteins coagulated by boiling at pH 5 also contained radioactivity. The counts given by the proteins corresponded to about 0.02 to 0.03 per cent of that added as lysine; the extent of lysine incorporation into the proteins was of the same order of magnitude as Melchior and Tarver (2) had found after incubating S^{35}-labeled methionine and Winnick *et al.* (3, 4) C^{14}-labeled glycine with rat tissue homogenates. Yet we could not satisfy ourselves that the radioactivity remaining in the proteins in our experiments, although it persisted through exhaustive extraction, did not come from traces of adsorbed radioactive lysine. Some counts were found in the protein when the homogenate was boiled prior to incubation with isotopic lysine.

The practical solution to the problem, it seemed, was to find experimental conditions in which very much more of the radioactivity added as lysine would remain in the protein after thorough washing, and little or no radioactivity in the protein of the controls; then we could conclude that the lysine was incorporated into the protein molecule and not adsorbed. The question of the mode of linkage would remain open.

Eventually two sets of conditions were found in which relatively large amounts of labeled lysine are incorporated into the proteins. In the one case, with the whole homogenate as the enzyme system, the optimum pH is in the neighborhood of 6.1, and calcium is required, the optimum concentration being above 0.003 M; the reaction proceeds hardly at all without the addition of calcium. In the other case the enzyme system was the centrifugate obtained by centrifuging the diluted homogenate at 2500*g;* we

* This work is a part of that done under contract with and joint sponsorship of the Office of Naval Research, United States Navy Department, and the United States Atomic Energy Commission. The C^{14} used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

shall refer to it as the sedimented fraction. The favorable condition when the sedimented fraction is used is at about pH 7.3; it is nearly inoperative at the optimum pH region (6.1) of the whole homogenate; it is not dependent on added calcium, though it is accelerated a little by calcium added to a final concentration of 0.004 M. The evidence points to either two different reactions or to two enzyme systems or substrates which incorporate labeled lysine.

In 2 hours at 38° in the whole homogenate reaction at pH 6.1 with a calcium concentration above 0.003 M, 3 per cent of the added radioactivity was incorporated into the mixed proteins, and 0.22 per cent of the lysine in the proteins was labeled. When the sedimented fraction was used at pH 7.3, in 2 hours at 38° 3 to 5 times as much labeled lysine were incorporated into the proteins as with the whole homogenate. These figures correspond to rates respectively 60 and more than 200 times faster than any reported hitherto on the incorporation of a labeled amino acid into the proteins of tissue homogenate (2–4). It is at the lower limit of the values found when tissue slices (2, 5–7), tissue segments (8), or resting bacteria (9) were incubated with labeled amino acids. Weissman and Schoenheimer (10) found after feeding labeled lysine to the rat that in 4 days 13.4 per cent of the lysine in the proteins was derived from the isotope. This rate of 0.14 per cent per hour is about the same as in the guinea pig liver whole homogenate under our best conditions.

Preparations

L- and D-lysine labeled with C^{14} in the ϵ position were prepared by methods described in a previous communication (1). These were diluted before use with the normal (*i.e.* not radioactive) isomers.

Procedure

The livers used in these experiments were taken from commercially procured adult guinea pigs, kept without food for 20 hours before use, killed by stunning, and bled thoroughly. The liver was washed in ice-cold saline and, without dilution with saline, minced in a Waring blendor for 1½ minutes, then homogenized in the apparatus of Potter and Elvehjem (11), and finally strained through two layers of cheese-cloth. By using undiluted homogenate a relatively large amount of tissue could be contained in a small volume of reaction mixture (0.5 ml. was quite convenient) and we could thus economize on labeled lysine.

The sedimented fraction was prepared as follows: the homogenate was suspended in 15 times its volume of ice-cold Krebs-Henseleit Ringer's solution (12) modified to contain twice the amount of bicarbonate. It was centrifuged in a refrigerated centrifuge at 500*g* for 3 minutes, and

the supernatant solution decanted off and centrifuged at 2500*g* for 15 minutes. The resulting supernatant solution was discarded, and the sediment was resuspended in a volume of the modified Ringer's solution equal to that of the diluted homogenate originally used and again centrifuged at 2500*g* for 15 minutes.

The viscous sediment was loosened and mixed and used as such or after mixing with a saline-bicarbonate solution. This fraction contained nuclei, mitochondria, submicroscopic granules, red cells, and probably some débris (13). We intend later to study the incorporation of lysine (labeled) into the separate nuclear, mitochondrial, submicroscopic granular, and cytoplasmic fractions.

The reaction mixtures were made up in Krebs-Henseleit Ringer's solution modified as indicated in the protocols.

The reaction mixtures were made up in 20 ml. Pyrex beakers and incubated at 38° in the apparatus of Dubnoff (14). At the end of an experimental run the contents of each beaker were transferred to a 250 ml. beaker with 80 ml. of water, the small clumps broken with a stirring rod, and then 20 ml. of 35 per cent trichloroacetic acid added. After standing overnight at room temperature the precipitated protein was washed either by filtration or by repeated centrifugation. Both methods gave the same results. In the filtration procedure the protein was filtered with suction onto a weighed circle of filter paper on a coarse sintered glass filter. After all the solution had passed through, the protein remaining on the filter was washed with six 50 ml. portions of 7 per cent trichloracetic acid, sucked dry after each addition, and then washed with three 50 ml. portions of water; the water was then removed by washing three times with 95 per cent ethanol followed by ether. The proteins were dried in a vacuum desiccator over solid NaOH. The centrifugation procedure was as follows. The next day, after the protein had been precipitated with 7 per cent trichloroacetic acid, most of the clear supernatant solution was removed by gentle suction. The remaining suspension was transferred to a weighed 15 ml. thick walled test-tube and centrifuged. The supernatant solution was poured off, and the sediment resuspended and broken up in about 12 ml. of 7 per cent trichloroacetic acid and centrifuged again. This was repeated nine times with trichloroacetic acid and twice with acetone. The protein remaining in the test-tube was then dried in an air-bath at 55°. When there are many protein samples to be washed, the latter procedure is less time-consuming and requires less trichloroacetic acid. The efficacy of either washing procedure is attested to by the very low or negative radioactivity of the proteins in the controls and when certain inhibitors were used.

The protein, after it was dry, was weighed and ground to a fine powder.

For the measurement of its radioactivity 20 mg. were spread evenly on an aluminum plate over a circle 19 mm. in diameter marked on it. Toward the end of the work reported here the circle was cut into the plate as a shallow depression; the spreading of the protein was easier and more even. The thickness of the counting samples was thus 7.05 mg. per sq. cm. The method of measuring the radioactivity was the same as in previous experi-

TABLE I

Influence of Hydrogen Ion Concentration on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	Buffer	Buffer concentration in reaction mixture	pH of buffer	Gas mixture	pH at end of run	Counts per min. per mg. protein
	molal		*molal*				
1	0.0006	$NaHCO_3$	0.009		O_2	7.7	0.08
	0.0006	"	0.009		" + CO_2	7.1	0.2
	0.0006	"	0.005		"	6.8	0.4
	0.0006	"	0.005		" + CO_2	6.6	0.55
	0.0006	None			" + "	6.6	0.60
	0.0006	Phosphate	0.011	6.6	" + "	6.6	0.6
	0.0006	"	0.011	6.0	" + "	6.4	0.8
	0.0006	"	0.011	4.5	" + "	6.1	1.0
2	0.003	$NaHCO_3$	0.009		" + "	7.2	1.0
	0.003	Phosphate	0.011	6.6	" + "	6.5	1.35
	0.003	"	0.011	6.0	" + "	6.4	1.6
	0.003	"	0.011	5.7	" + "	6.15	2.3
	0.003	"	0.011	5.6	" + "	6.05	2.4
	0.003	Succinate	0.002	5.8	" + "	6.05	2.4
	0.003	Phosphate	0.011	4.5	" + "	5.7	1.45

Reaction mixtures incubated at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.03 M $CaCl_2$ in Experiment 1 and 0.15 M in Experiment 2; 0.1 ml. of buffer solution; 0.1 ml. of Ringer's solution without bicarbonate adjusted to pH 6.5 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); and 0.3 ml. of undiluted homogenate. The pH values at the end of the run were after the addition of 4 ml. of water.

ments (1). All the values given are corrected for self-absorption and are the averages of duplicates or triplicates; the individual values did not vary from the average by more than ±10 per cent.

All the glassware and the saline solutions were sterilized before use by autoclaving for 20 minutes under 15 pounds steam pressure.

Results

Influence of Hydrogen Ion Concentration—The use of undiluted liver homogenate permitted only approximate pH control. The pH of the

homogenate immediately after its preparation varied between 6.9 and 7.1. It became acid during incubation whether under 100 per cent oxygen or 95 per cent oxygen and 5 per cent carbon dioxide. The desired final pH was attained approximately by addition of buffers and incubation under either pure oxygen or 95 per cent oxygen plus 5 per cent carbon dioxide. It was found in separate trials that the pH found after incubation at 38° for 2 hours had been attained within half an hour.

The data in Table I show that when the whole homogenate was used the proteins gave the highest number of counts when the final pH of the re-

TABLE II

Influence of Hydrogen Ion Concentration on Incorporation of Labeled Lysine into Proteins of Sedimented Fraction of Guinea Pig Liver Homogenate

Buffer in which sedimented fraction was suspended	Initial pH of diluted sedimented fraction	Gas mixture	Final pH	Counts per min. per mg. protein
Succinate, 0.025 M	5.5	O_2 + CO_2	5.3	0.3
" 0.025 "	6.0	" + "	5.8	0.5
Ringer's solution without $NaHCO_3$	6.7	" + "	6.3	1.5
" " containing 0.23% $NaHCO_3$	7.4	" + "	7.2	3.9
Same	7.5	" + "	7.3	4.4
Ringer's solution containing 0.38% $NaHCO_3$	7.9	" + "	7.8	3.6
Same	8.5	" + "	8.1	3.4
"	8.85	"	8.8	3.0

Reaction mixtures incubated at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.2 M $CaCl_2$; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.5 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.4 ml. of the sedimented fraction diluted with half its volume of buffer solution, its initial pH adjusted, and incubated under the gas mixture indicated. The pH values at the end of the run were after the addition of 4 ml. of water.

action mixture was near 6.1. Whether bicarbonate, phosphate, fumarate, or succinate was used as a buffer, or whether the reaction was carried out under 100 per cent oxygen or 95 per cent oxygen plus 5 per cent carbon dioxide, the number of counts in the protein at any given pH was the same.

The optimum pH with the sedimented fraction was at, or near, 7.3 (Table II). At the optimum pH nearly twice as many counts per mg. of protein were found in the proteins of the sedimented fraction as in those of the whole homogenate. Some of the counts obtained in the whole homogenate near or above pH 7 undoubtedly came from components of the sedimented fraction.

Influence of Calcium Concentration—Table III shows the influence of the concentration of calcium added to the reaction mixture. With the whole homogenate the counts in the protein were greater the greater the concentration of added calcium chloride up to a final concentration of 0.004 M; this was the case at hydrogen ion concentrations below and above the

TABLE III

Influence of Concentration of Calcium on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Homogenate fraction used	Concentration of added $CaCl_2$ in reaction mixture	Initial pH of phosphate buffer	pH at end of run	Counts per min. per mg. protein
		molal			
1	Whole, initial pH 6.9	None	4.5	6.1	0.9
	" " " 6.9	0.001	4.5	6.1	1.8
	" " " 6.9	0.002	4.5	6.1	2.0
	" " " 6.9	0.003	4.5	6.0	2.3
	" " " 6.9	0.004	4.5	6.0	2.5
	" " " 6.9	0.005	4.5	6.0	2.6
	" " " 6.9	0.01	4.5	6.0	2.6
2	" " " 5.9	0.003	6.4	6.4	1.2
	" " " 5.9	0.0006	6.4	6.4	0.4
	" " " 5.9	0.003	6.4	6.1	1.7
	" " " 5.9	0.0006	6.4	6.2	0.5
	" " " 7.1	0.003	4.5	5.5	1.1
	" " " 7.1	0.0006	4.5	5.6	0.5
3	Sedimented fraction, initial pH 7.8	0.004	No buffer used	7.6	4.0
	Same	None	" " "	7.5	3.3

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the final concentration given; 0.1 ml. of 0.05 M phosphate buffer; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.3 ml. of undiluted homogenate or 0.4 ml. of sedimented fraction after dilution with an equal volume of Ringer's solution containing 0.38 per cent sodium bicarbonate. The pH values at the end of the run were after the addition of 4 ml. of water.

optimum. There is practically no further augmenting effect of calcium above 0.004 M; 0.01 M calcium chloride is not inhibitory; we have not explored the effect of higher concentrations.

The calcium content of guinea pig liver reported in the literature ranges from 0.5 to 14.9 mg. per cent (15–17). The lowest concentration of added calcium which permitted a maximum number of counts in the protein was near 0.004 M or 16 mg. per cent. Changes in calcium content, and es-

pecially in the portion not combined with fatty acids, may, therefore, affect significantly the incorporation of lysine in the proteins of the liver.

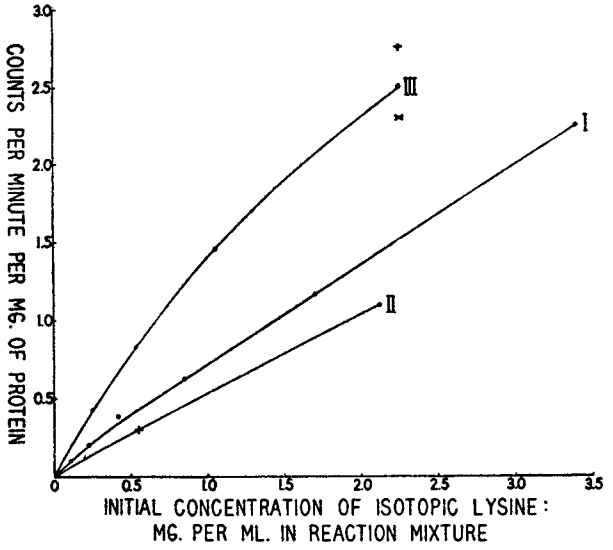

FIG. 1. Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours. Curve I, 1.01 ml. of the reaction mixtures containing 0.3 ml. of Ringer's solution without bicarbonate; 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.06 M $CaCl_2$; 0.1 ml. of Ringer's solution, adjusted to pH 6.0, without bicarbonate, containing the radioactive L-lysine (18,300 counts per minute per mg.); 0.6 ml. of undiluted homogenate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.6. Curve II, 0.51 ml. containing 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.05 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0; 0.1 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate, containing the radioactive L-lysine (18,300 counts per minute per mg.); and (●) 0.3 ml. of undiluted homogenate, (+) 0.3 ml. of homogenate diluted 1:1 with Ringer's solution without bicarbonate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.4. Curve III, 0.51 ml. containing 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.15 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer, pH 4.5; 0.1 ml. of Ringer's solution without bicarbonate, adjusted to pH 6.0, containing the radioactive L-lysine (9150 counts per minute per mg.); and (●) 0.3 ml. of undiluted homogenate, (+) 0.3 ml. of homogenate diluted 1:1 with Ringer's solution without bicarbonate, and (×) 0.3 ml. of homogenate diluted 1:2 with Ringer's solution without bicarbonate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.2.

Table III shows that the effect of added calcium was much less on the sedimented fraction than on the whole homogenate. Dounce and Beyer (18) give the concentration of calcium in rat liver nuclei as 2.3×10^{-4} gm. per 100 gm., wet weight, or approximately 6×10^{-5} M. On the assump-

tion that the concentration in guinea pig liver nuclei is similar, and in view of the results with the whole homogenate, it seems unlikely that the lesser effect of added calcium on the sedimented fraction was because its calcium concentration was high initially.

Influence of Concentration of Lysine—The count per mg. of the proteins of the whole homogenate was close to a linear function of the initial concentration of labeled lysine (Fig. 1).

This result was obtained at pH 6.2, 6.4, and 6.6, and with 0.00049, 0.00059, and 0.0029 molal final concentrations of added $CaCl_2$. Provided the lysine, the $CaCl_2$ concentration, and the pH were kept constant, the subsequent count per mg. of the proteins was independent of the concentration of the homogenate.

TABLE IV

Effect of Total Amount of Labeled Lysine Available on Rate of Its Incorporation into Proteins of Sedimented Fraction of Guinea Pig Liver Homogenate

0.4 M $CaCl_2$	L-Lysine dihydrochloride solution, 16 mg. per ml.	Ringer's solution	Total volume of reaction mixture	Counts per min. per mg. protein
ml.	*ml.*	*ml.*	*ml.*	
0.01	0.1	0	0.51	3.3
0.02	0.2	0.4	1.02	6.7
0.04	0.4	1.2	2.04	10.5

The sedimented fraction (0.4 ml.) was used undiluted in this experiment. The $CaCl_2$ and lysine solutions were made up in Ringer's solutions without $NaHCO_3$ and adjusted to pH 6.0. The specific activity of the L-lysine dihydrochloride was 6100 counts per minute per mg. The Ringer's solution used for dilution contained 0.38 per cent $NaHCO_3$. The reaction mixtures were incubated under a mixture of 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours. The pH at the end of the run after the addition of 4 ml. of water was in every case 7.55.

When the sedimented fraction was used, the count per mg. of protein was dependent on the total amount of labeled lysine in the whole volume of the solution and not on its concentration (Table IV). Thus with a 4-fold increase in the volume of the reaction mixture with the initial labeled lysine concentration constant and the amount (but not the concentration) of sedimented fraction the same, the count per mg. of the protein was greater the greater the volume of the reaction mixture, *i.e.* the more labeled lysine made available to the sedimented fraction.

Progress of Reaction with Time in Whole Homogenate—The count per mg. of the protein attains, at 38°, its maximum in about 2 hours (Fig. 2). One factor which may be responsible for the rapid slowing down of the reaction is that a large fraction of the added lysine disappears. The major product (we have not yet identified it) is neither α-aminoadipic acid nor an immediate metabolic product of the latter.

The evidence for an additional factor which may be responsible for the cessation of the reaction was obtained in other experiments. After 2 hours incubation with labeled lysine, when the reaction had come nearly to a stop, the protein gave 2.4 counts per minute per mg. At this point the concentration of the isotope was increased to 4 times the initial value.

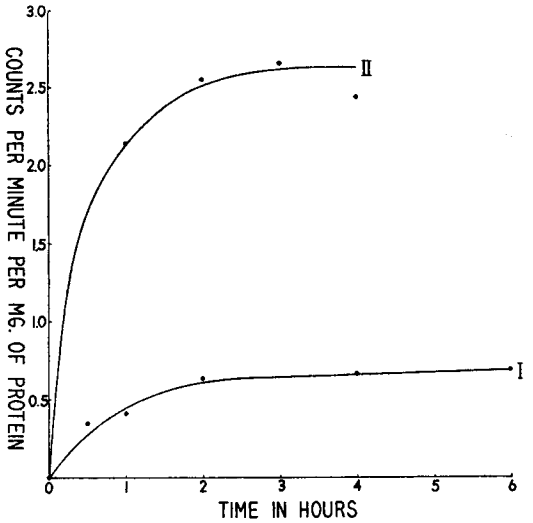

FIG. 2. Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38°. Curve I, 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.03 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0; 0.1 ml. of Ringer's solution without bicarbonate adjusted to pH 6.0 containing 0.26 mg. of radioactive L-lysine dihydrochloride (12,200 counts per minute per mg.); 0.3 ml. of undiluted homogenate. After incubation, following addition of 4 ml. of water, all mixtures were at pH 6.4. Curve II, 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.25 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 4.5; 0.1 ml. of Ringer's solution adjusted to pH 6.0, with bicarbonate, containing 2.13 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.3 ml. of undiluted homogenate. After incubation, following addition of 4 ml. of water, all mixtures were at pH 6.2. The zero counts shown at zero time in Curves I and II are experimental determinations.

After another 2 hours incubation the count would be expected to be increased 4-fold in view of the almost linear relation between protein count and initial concentration of labeled lysine (Fig. 1), if the reaction system were in the same state after 2 hours as at the beginning. The count found, however, was only 3.7 per minute per mg. The increase was only 33 instead of the expected 400 per cent. Among the possible explanations, one is that most of the enzymatic activity is lost after 2 hours incubation; another is that the reaction is not an exchange of lysine in the protein but a combination to saturation at loci on the protein capable of combining

with lysine, and that after 2 hours incubation with the initial addition of lysine it is nearly saturated. The almost linear relation in Fig. 1 argues against the latter interpretation, but it is not decisive.

Controls and Inhibitors—Boiling destroys most of the enzymatic activity in both the whole homogenate and in the sedimented fraction (Table V).

TABLE V

Effect of Boiling Homogenate Fraction and of D- *instead of* L-*Lysine on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate*

Experiment No.	Homogenate fraction used	Treatment of homogenate fraction before incubation	Lysine isomer used	Concentration of added $CaCl_2$ in reaction mixture	pH at end of run	Counts per min. per mg. protein
				molal		
1	Whole homogenate	None	L	0.0029	6.1	2.5
	" "	Boiled	"	0.0029	6.1	0.04
2	" "	None	"	0.00059	6.5	1.2
	" "	Boiled	"	0.00059	6.5	0.08
3	" "	None	"	0.0029	6.1	2.5
	" "	"	D	0.0029	6.1	0.6
4	" "	"	L	0.00059	6.5	1.2
	" "	"	D	0.00059	6.5	0.5
5	Sedimented fraction	"	L	0.0039	6.8	3.0
	" "	Boiled	"	0.0039	6.8	0.1
6	" "	None	"	0.0039	7.8	3.65
	" "	Boiled	"	0.0039	7.8	0.2

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the molalities given; 0.1 ml. of 0.05 M phosphate at pH 4.5 in Experiments 1 and 3, and at pH 6.0 in Experiments 2 and 4; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.5 containing 1.6 mg. of L- or D-lysine dihydrochloride (12,200 counts per minute per mg. in Experiments 1 and 4, and 6100 counts per minute per mg. in the others); 0.3 ml. of undiluted homogenate in Experiments 1 to 4, 0.4 ml. of sedimented fraction after dilution with half its volume of Ringer's solution without bicarbonate and adjusted to pH 7.0 in Experiment 5, but with 0.38 per cent sodium bicarbonate and adjusted to pH 7.9 in Experiment 6. The pH values at the end of the run were after the addition of 4 ml. of water.

The procedure in this test was to transfer the whole homogenate or the sedimented fraction to the reaction vessel, immerse it in a boiling water bath for 15 minutes, cool, add the other components of the reaction system, stir them together thoroughly with a stirring rod, and then incubate. It will be noted that Table V ascribes some residual activity to the heated preparations. In the case of the whole homogenate the values given were not significantly above the background count;[1] they were significantly above the background in the boiled sedimented fractions.

[1] A sample is usually counted for 30 minutes. The background count in this interval is about 330. We do not consider a sample to have a count significantly above

The proteins gave some counts when D-lysine was used instead of L-lysine (Table V). The count was always much less than in the corresponding L experiment, and could be accounted for largely by the 7 to 15 per cent[2] of the L form in the D preparation used. From the results of feeding experiments (19) it would be expected that isotopic D-lysine would not be incorporated, if the sole reason for the indispensability of lysine is its incorporation into protein.

The experiments with boiled whole homogenate and sedimented fractions were also a check on the efficacy of the procedure used to wash out labeled lysine, which was not combined with protein. The zero counts at zero time shown in Fig. 1 provided evidence to the same effect. The experiments with labeled D-lysine served this purpose also to some extent.

Table VI summarizes experiments on the effects of anaerobiosis and of some oxidation inhibitors. Anaerobiosis does not inhibit the incorporation of lysine into the proteins of the whole homogenate; it does so, but only incompletely, in the sedimented fraction. 0.02 M fluoride inhibits almost completely in the whole homogenate and only slightly in the sedimented fraction. Arsenate, arsenite, azide, cyanide, and dinitrophenol (all in 0.001 M concentration) inhibit in the whole homogenate to varying degrees, all short of completion, and less so or not at all in the sedimented fraction. Similar results were obtained with the whole homogenate with the different inhibitors whether the concentrations of calcium and of hydrogen ion were optimum or suboptimum.

The fluoride inhibition in the whole homogenate was examined in more detail (Table VII). The results with 0.01 M fluoride show that fluoride was more effective at pH 6.2 than at 6.6. Rothschild (20) observed that fluoride inhibition of lipase was greater the lower the hydrogen ion concentration. Runnström and Sperber (21) found the same on the fermentation and respiration of yeast, and Warburg and Christian (22) on enolase.

Experiment 2 in Table VII was designed to test whether calcium and fluoride bear the same relation to the enzyme system in the whole homogenate as do magnesium and fluoride to enolase. Warburg and Christian showed that magnesium ion activates enolase and also increases the inhibition by fluoride. Their interpretation was that magnesium ion forms an enzymatically active complex with enolase protein and that magnesium, fluoride, and enolase protein form an inactive complex. In a quantitative study of the inhibition they found, in support of their interpretation, an equilibrium constant between the concentration of mag-

the background unless its excess over the background is more than 3 times the standard deviation of the latter, in practice, 14 counts per minute or more.

[2] The optical rotation of the L preparation was $[\alpha]_D^{22} = +15.95°$, in water, $c = 5.46$; and of the D preparation, $[\alpha]_D^{22} = -13.5°$, in water, $c = 4.47$. The latter figure was the less reliable, as we had only a little of the D preparation.

TABLE VI

Effect of Oxidation Inhibitors on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	pH at end of run	Inhibitor, concentration in reaction mixture	Counts per min. per mg. protein	Degree of inhibition*
	molal				*per cent*
1	0.00059	6.5	None	1.2	
	0.00059	6.5	Anaerobiosis	1.25	
2	0.0049	6.6	None	0.81	
	0.0049	6.6	Anaerobiosis	0.77	5
	0.0049	6.6	Sodium arsenate (0.001 M)	0.59	27
	0.0049	6.6	" arsenite (0.001 ")	0.27	67
	0.0049	6.6	" azide (0.001 ")	0.46	43
	0.0049	6.6	Potassium citrate (0.005 ")	0.82	0
	0.0049	6.6	Sodium cyanide (0.001 ")	0.70	14
	0.0049	6.6	" fluoride (0.02 M)	0.04	95
3	0.0049	6.2	None	2.5	
	0.0049	6.2	Anaerobiosis	2.4	4
	0.0049	6.2	Sodium arsenate (0.001 M)	1.9	24
	0.0049	6.2	" arsenite (0.001 ")	1.2	52
	0.0049	6.2	" azide (0.001 ")	2.0	20
	0.0049	6.2	" fluoride (0.02 M)	0.1	96
	0.0049	6.2	Dinitrophenol (0.001 M)	1.8	28
4	0.0039	7.7	None	5.1	
	0.0039	7.7	Anaerobiosis	3.9	24
	0.0039	7.7	Sodium arsenate (0.001 M)	5.0	2
	0.0039	7.7	" arsenite (0.001 ")	5.1	0
	0.0039	7.7	" azide (0.001 ")	4.7	8
	0.0039	7.7	" fluoride (0.02 M)	4.4	14
	0.0039	7.7	Dinitrophenol (0.001 M)	5.4	0

Reaction mixtures incubated at 38° for 2 hours. The aerobic experiments were incubated under 95 per cent O_2 and 5 per cent CO_2; the anaerobic experiments under 95 per cent N_2 and 5 per cent CO_2. 0.52 ml. of the reaction mixtures containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the concentration given; 0.1 ml. of 0.1 M phosphate buffer at pH 6.0 in Experiments 1 and 2 and at pH 4.5 in Experiment 3; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (12,200 counts per minute per mg. in Experiment 1, and 6100 counts in Experiments 2 to 4); 0.3 ml. of undiluted whole homogenate in Experiments 1 to 3, and 0.4 ml. of sedimented fraction after dilution with half its volume of Ringer's solution containing 0.38 per cent sodium bicarbonate and its pH adjusted to 7.9 in Experiment 4; 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with the inhibitors 51 times the molalities given. The pH values at the end of the run were after the addition of 4 ml. of water.

* $\frac{\text{(Counts without inhibitor)} - \text{(counts with inhibitor)}}{\text{(Counts without inhibitor)}} \times 100.$

nesium and fluoride ions and the degree of inhibition. Najjar (23) found the same relations between magnesium, fluoride, and phosphoglucomutase

activity, and gave the same interpretation. The data in Experiment 2 of Table VII show that the degree of inhibition by 0.01 M fluoride was the same in 0.005 M as in 0.001 M calcium. A calcium-fluoride-enzyme complex, which is inactive, as an explanation of the fluoride inhibition appears, therefore, to be excluded.

The following metabolites and salts neither accelerated nor inhibited the reaction in the whole homogenate, at either pH 6.6 with 0.0006 M

TABLE VII

Inhibiting Effect of Fluoride on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	NaF concentration in reaction mixture	pH at end of run	Counts per min. per mg. protein	Degree of inhibition
	molal	*molal*			*per cent*
1	0.00059	None	6.6	1.21	
	0.00059	0.02	6.6	0.04	97
	0.00059	0.01	6.6	0.64	47
	0.00059	0.0025	6.6	0.94	22
	0.00059	0.001	6.6	1.22	0
2	0.0049	None	6.2	1.99	
	0.0049	0.02	6.2	0.12	94
	0.0049	0.01	6.2	0.69	65
	0.001	None	6.2	0.83	
	0.001	0.02	6.2	0.08	96
	0.001	0.01	6.2	0.30	64

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.52 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the molality given; 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with sodium fluoride 51 times the molality given; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0 in Experiment 1 and at pH 4.5 in Experiment 2; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (12,200 counts per minute per mg. in Experiment 1, and 6100 counts per minute per mg. in Experiment 2); 0.3 ml. of undiluted homogenate. The pH values at the end of the run were after the addition of 4 ml. of water.

added $CaCl_2$ or at pH 6.1 with 0.003 M added $CaCl_2$, nor did they relieve either the fluoride or arsenite inhibitions (the figures in parentheses are final concentrations in the reaction mixture): adenosine triphosphate (0.001 M), coenzyme I (20 mg. per cent), potassium citrate (0.005 M), creatine (200 mg. per cent), cysteine (0.005 M), cytochrome (65 mg. per cent), fumarate (0.005 M), D-glucose (100 mg. per cent), glucose-1-phosphate (75 mg. per cent), glutathione (300 mg. per cent), glycerophosphate (0.005 M), hexose diphosphate (180 mg. per cent), α-ketoglutarate (0.005 to 0.01 M), oxalacetate (0.005 M, slightly inhibitory), 2-phosphoenolypyruvate (0.005 M), 3-phosphoglycerate (0.005 M), pyridoxal phosphate (50 to 250

mg. per cent), pyridoxine (1 to 10 mg. per cent), pyruvate (0.005 M), $CoCl_2$ (0.00005 to 0.0005 M), $CuCl_2$ (0.0005 to 0.005 M), $MgCl_2$ (0.0005 to 0.005 M), $MgSO_4$ (0.00005 to 0.0005 M), $MnCl_2$ (0.0005 to 0.005 M), $ZnCl_2$ (0.0005 to 0.005 M). Replacement of all the sodium by potassium salts and *vice versa* in the Ringer's solution similarly neither accelerated nor inhibited the reaction.

We investigated whether the counts in the proteins of the whole homogenate were incorporated as lysine or in some other form. 300 mg. of the protein, giving a total of 360 counts per minute, were hydrolyzed by refluxing for 20 hours with 20 per cent hydrochloric acid. The lysine was isolated as the picrate by Block's modification (24) of the Kossel procedure: this is a specific and standard method for the isolation of lysine.

The lysine picrate isolated gave, after one recrystallization from water, the following elementary analysis.

$C_{12}H_{17}O_9N_5$.	Calculated.	C 38.36, H 4.57, N 18.66
375.30	Found.	" 38.29, " 4.47, " 18.46

The picrate gave 20 counts per minute per mg. of lysine; further recrystallization did not change this count. According to Block and Bolling (24) the lysine content of liver proteins is 6.3 per cent. On the basis that all the counts in the protein came from the lysine, its 360 counts correspond to a "calculated" count of its lysine of 19 counts per minute per mg., which is the value found in the lysine isolated, within the accuracy of the measurement.

Another portion of the protein was hydrolyzed in the same manner and then precipitated with phosphotungstic acid. The precipitate gave 330 counts per minute; the protein before hydrolysis gave 360 counts; the filtrate from the phosphotungstic precipitate gave no counts.

On the basis of the foregoing data the lysine incorporated accounted for all the radioactivity of the protein.

A measure of the extent to which a labeled amino acid is incorporated into a protein is the fraction of that amino acid isolated from the protein found to be carrying the label. In the foregoing instance the lysine isolated gave 20 counts per minute per mg. The lysine added to the homogenate gave 9150 counts per minute per mg. $20/9150 \times 100$ or 0.22 per cent of the lysine in the protein was, therefore, labeled. Nearly all of this incorporation occurred in the 1st hour of incubation (Fig. 2).

DISCUSSION

Studies we have now in hand with labeled leucine and glycine caution against generalizing from the findings on labeled lysine to the incorporation of other amino acids into homogenate proteins. Leucine and glycine, under the best conditions we have found so far for lysine, are incorporated

far more slowly than lysine; this is the case both with the whole homogenate and the sedimented fractions of guinea pig and rat liver.

SUMMARY

1. Two sets of conditions were found in guinea pig liver homogenate in which L-lysine (labeled with C^{14} in the ϵ position) is incorporated into the proteins in relatively large amounts. In one case the enzyme system was the whole homogenate, in the other the precipitate obtained by centrifuging the 15-fold diluted homogenate at 2500*g*.

2. Characteristics of the reaction with the whole homogenate are as follows: its optimum pH is approximately 6.1; the presence of calcium is obligatory, the concentration of the latter for maximum activation being approximately 0.004 M; 0.01 M does not inhibit; the reaction proceeds as well in nitrogen as in oxygen; it is inhibited nearly completely by 0.02 M fluoride, 50 per cent by 0.001 M arsenite, and somewhat less by 0.001 M arsenate, azide, cyanide, and dinitrophenol; the concentration of L-lysine incorporated into the proteins is approximately a linear function of the lysine concentration and is independent of the concentration of the homogenate.

3. Characteristics which differentiate the reaction with the sedimented fractions from that with the whole homogenate are as follows: its optimum pH is near to 7.3; the reaction is accelerated only a little by calcium, and the presence of calcium is not obligatory; it proceeds more slowly under nitrogen than under oxygen, but is not completely inhibited by anaerobiosis; it is inhibited slightly by 0.02 M fluoride, and practically not at all by 0.001 M arsenate, arsenite, azide, or dinitrophenol; the concentration of lysine incorporated is a function of the amount of lysine in the total volume of the solution in which the sedimented fraction is suspended.

4. The rate of incorporation of labeled lysine into the proteins of the whole homogenate is approximately the same as that *in vivo;* after 1 hour's incubation at 38° about 0.15 per cent of the lysine in the proteins is labeled. This rate is 60 times faster than any hitherto reported in whole homogenates for other amino acids.

5. The rate of incorporation of labeled lysine into the proteins of the sedimented fraction, under the most favorable conditions for the latter, is several times that in the whole homogenate under its most favorable conditions.

6. All the radioactivity found in the proteins of the whole homogenate was accounted for as labeled lysine.

The microanalyses were carried out by Mr. G. Swinehart. The authors were assisted by A. A. Dvorsky, H. E. Jeffrey, M. Keighley, and A. Tollestrup.

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