THE UPTAKE IN VITRO OF C^{14}-LABELED GLYCINE, L-LEUCINE, AND L-LYSINE BY DIFFERENT COMPONENTS OF GUINEA PIG LIVER HOMOGENATE*

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We have reported (1) that L-lysine labeled with C^{14} can be incorporated into the proteins of guinea pig liver homogenate under two different conditions. In the one case the enzyme used was the whole homogenate, the optimum pH was near 6.2, there was an obligatory requirement of calcium, and the incorporation was independent of oxygen. This set of conditions is designated below as the "acid calcium" condition. In the other case the enzyme system was the precipitate obtained by centrifuging the homogenate diluted 15-fold with Ringer's solution at 2500 $\times$ g, the optimum pH was near to 7.3, the reaction was accelerated a little by calcium but the presence of calcium was not obligatory, and the incorporation was a little less under nitrogen than under oxygen. This set of conditions is designated below as the "alkaline" condition.

We have carried the study further. The homogenate was separated into four fractions by differential centrifugation, which are designated below as Sediments I, II, and III, and supernatant, and we investigated the ability of each of these fractions to incorporate lysine into the proteins under the two sets of conditions. It was found that all three sedimented fractions incorporated lysine into the proteins under the "alkaline" condition, and that the incorporation under the "acid calcium" condition was confined to the supernatant fraction.

We have investigated also and report here the incorporation of C^{14}-labeled glycine and leucine into the proteins by the three sedimented fractions, the absence of interaction of amino acids on their individual uptake, and the effects of certain metal ions.

Preparation of Liver Fractions

The animals were commercial adult guinea pigs of both sexes, in normal nutrition. They were killed by a blow on the head and bled. The livers,

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as soon as they were removed, were chilled and washed in ice water. We then employed a combination of the procedures of Schneider (2) and of Hogeboom *et al.* (3) to obtain by differential centrifugation three particulate fractions and a supernatant fraction of the homogenate. Hogeboom *et al.* found that the mitochondria are thrown down as small discrete particles in 30 per cent sucrose and as clumped aggregates in isotonic saline. Accordingly we carried out the differential centrifugation in both sucrose and in two saline solutions and tested the particulate fractions obtained. The two saline solutions were KCl solution whose composition is described below and Krebs-Henseleit Ringer's solution (4).

The sucrose solution contained 300 gm. of sucrose, 0.735 gm. of L-glutamic acid, 0.203 gm. of $MgCl_2 \cdot 6H_2O$, and 0.174 gm. of K_2HPO_4 per liter. The KCl solution was the same as the sucrose solution except that it contained 0.9 gm. of KCl per liter instead of the sucrose. The pH of the sucrose and KCl solutions was adjusted to 7.8. The glutamic acid was added because of an impression among workers that activity with respect to coupling of oxidation and phosphorylation is better preserved in mitochondria in the presence of an oxidizable metabolite.

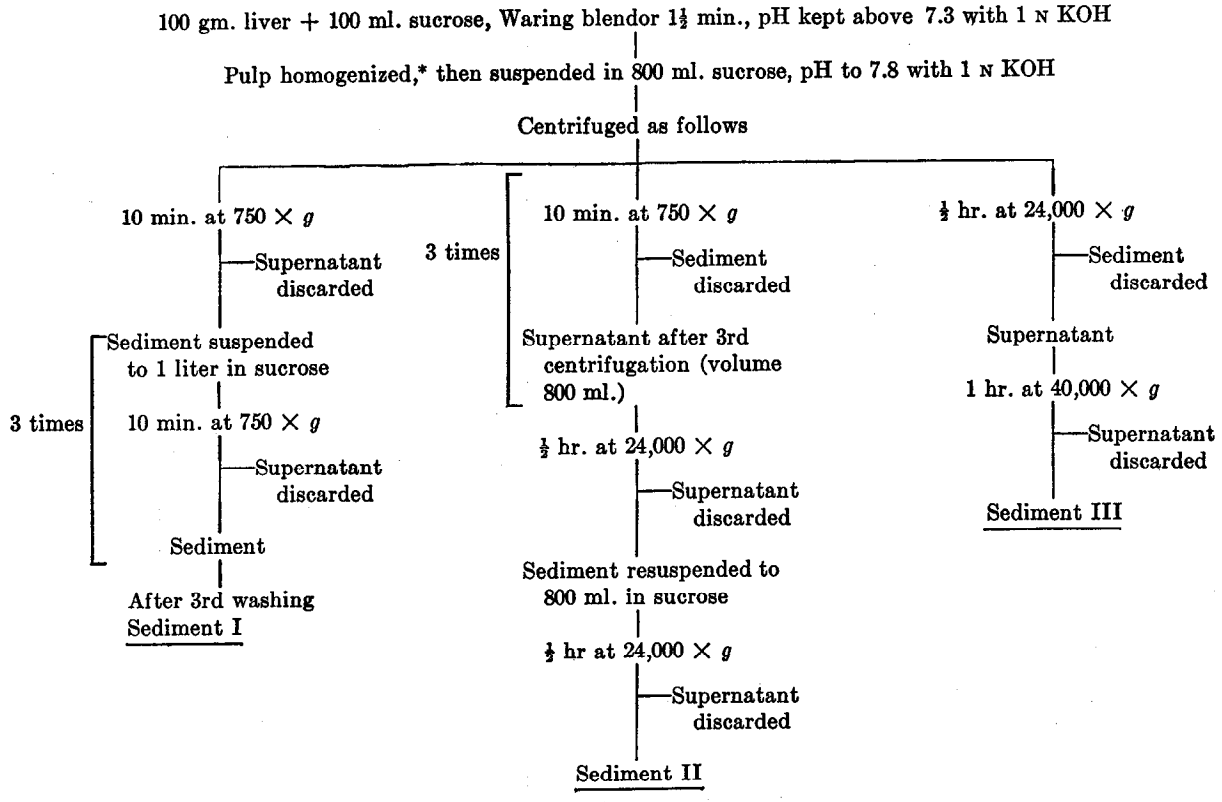
Diagrams 1 and 2 summarize our fractionation procedure in the sucrose and in the saline solutions.

Each fraction had the same appearance whether prepared in the sucrose or either saline solution. Sediment I was pearly white with a small amount of buff-colored material layered on top. Staining with methylene blue showed the sediment to consist mainly of nuclei; there were smaller clumps (presumably aggregated mitochondria), a few red cells, liver cells, and some débris. Sediment II was buff-colored; the staining properties of the fraction prepared with sucrose corresponded to those Hogeboom *et al.* ascribe to mitochondria. Sediment II prepared with saline solution had the same color as that prepared in the sucrose solution; approximately the same amount was obtained from a given weight of liver; the particles were much smaller than nuclei and stained faintly with methylene blue, deeply with Janus green B (3). From the centrifugal force required to throw down Sediment III the particulate matter would appear to correspond to the microsomes of Claude (6). Schneider *et al.* (7) found that most of the cytochrome *c* of liver cells appeared to be in the microsomes; Sediment III was reddish brown, quite distinct from the buff color of Sediment II, and resembled microsomes in this respect also. The supernatant fraction was a deep red transparent solution under a turbid surface layer; the fraction used was removed by careful pipetting.

Two refrigerated centrifuges were used in the foregoing preparations. One, for centrifugal forces up to 2500 $\times$ g, was an International centrifuge maintained at 0–4°; the other, for higher centrifugal forces, was a Servall

DIAGRAM 1

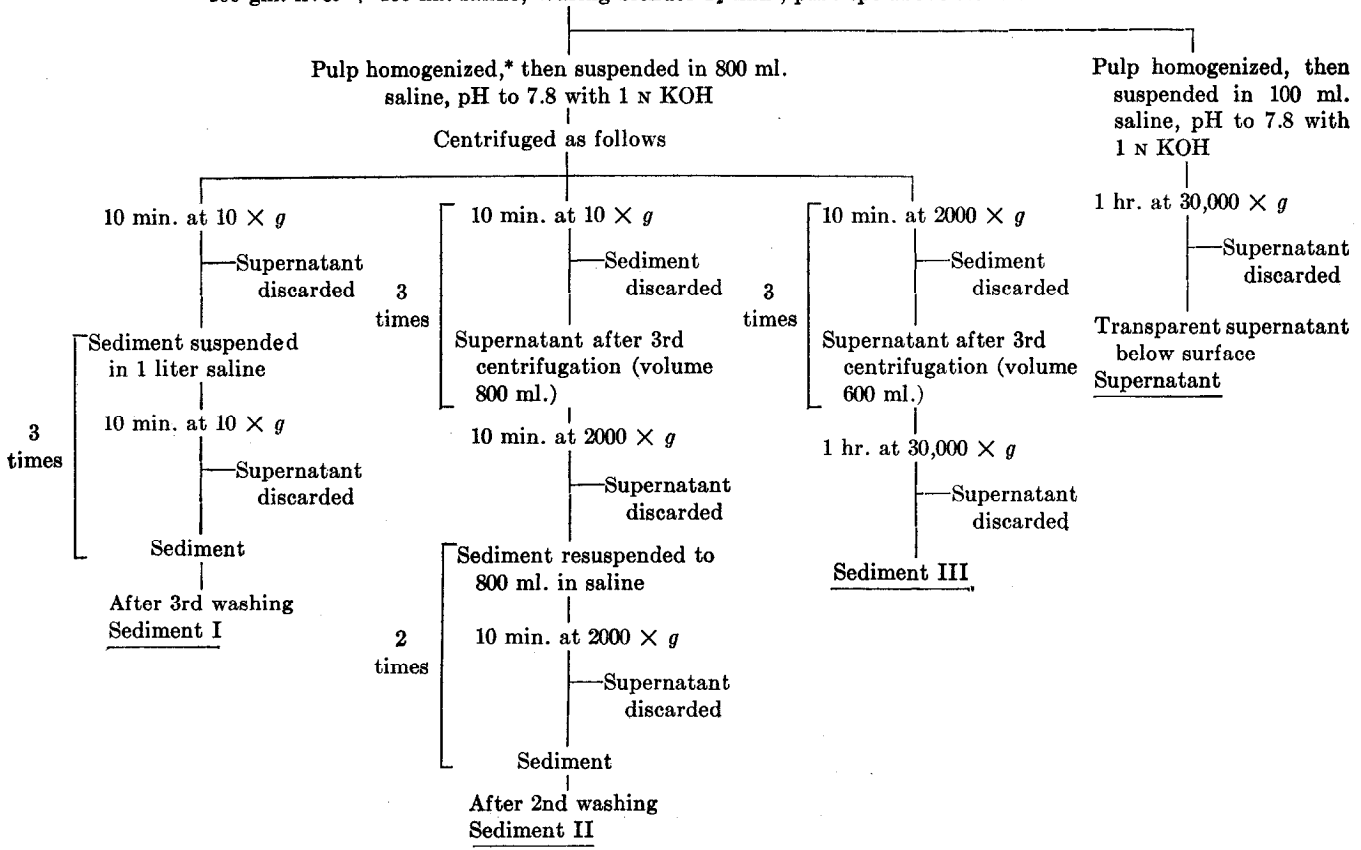
Preparation of Fractions in Sucrose Solution



*Potter and Elvehjem (5).

DIAGRAM 2

Preparation of Fractions in KCl Solution or in Krebs-Henseleit Ringer's Solution (4)



* Potter and Elvehjem (5).

model SS-2 centrifuge; the rotor was cooled in ice before use, the bearing was kept cool with continually circulating ice water, and at the end of the centrifugation the solution was usually about 10°.

Though the fractions were not pure they exhibited functional differences and it was unnecessary for present purposes to purify them further.

The yields of the four fractions per 100 gm. of liver were approximately as follows: Sediment I 20 ml., 300 mg. of protein (dry weight); Sediment II 40 ml., 3000 mg. of protein; Sediment III 10 ml., 300 mg. of protein; supernatant fraction 150 ml., 2500 mg. of protein.

Preparation of C^{14}-Labeled Amino Acids

Glycine—Glycine labeled in the carboxyl group was prepared by the method of Sakami *et al.* (8). It gave 55,000 c.p.m. per mg.

Synthesis and Resolution of Carboxyl-C^{14}-DL-leucine—The Strecker synthesis with $HC^{14}N$ and isovaleraldehyde yields DL-leucine which was isolated as the carbobenzoxy derivative and resolved with papain and aniline. Isovaleraldehyde free from isomers was obtained by oxidation of pure leucine.

Isovaleraldehyde—10 gm. of pure leucine were dissolved in 380 ml. of water and 8.4 ml. of 4 N NaOH; the solution was cooled in ice and mixed with 76 ml. of 1 N NaOCl solution (prepared according to Raschig (9)) and 16.8 ml. of 2 N acetic acid.

The mixture was steam-distilled in the apparatus described by Langheld (10). The distillate (volume 100 to 200 ml.) was acidified with sulfuric acid. By distillation at 76–95° one obtains 4 to 5 gm. of oil with an aqueous bottom layer of about 1 gm. After shaking with solid $KHCO_3$, the water phase was removed, and the top layer washed three times with concentrated sodium chloride solution. The isovaleraldehyde was dried over fused $CaCl_2$ and distilled twice; the fraction boiling at 90–93° was collected in a receiver containing a few crystals of hydroquinone. It was redistilled immediately before use.

Carbobenzoxy-DL-leucine—The strongly alkaline solution of $KC^{14}N$ obtained from $BaC^{14}O_3$ (11) was diluted with non-radioactive KCN to give a total of 135 mg. of KCN. The solution was acidified with 10 per cent H_2SO_4 and the $HC^{14}N$ distilled into a receiver kept at −20°. To the distillate (about 1 ml.) was added 0.8 ml. of concentrated ammonia while cold, then 530 mg. of freshly distilled isovaleraldehyde at room temperature. The mixture was shaken for 18 hours in a closed container. It was then transferred to a 50 ml. Erlenmeyer flask with 10 ml. of 48 per cent HBr, boiled for 30 minutes, heated on a steam bath for 4 hours, and evaporated with a stream of air at the end.

The residue was dried *in vacuo* over H_2SO_4 and KOH and boiled with 10 ml. of 1 N NaOH (glass rod to prevent bumping) until the vapor was

almost neutral to litmus. Filtration and washing with water furnished a clear colorless solution. The filtrate was acidified with 20 per cent HCl (about 0.1 ml.), concentrated to about 2 ml., and made strongly alkaline with 0.3 ml. of 4 N NaOH. After the solution was cooled in ice, the carbobenzoxylation was carried out with 0.6 ml. of carbobenzoxychloride and 0.9 ml. of 4 N NaOH in four equal portions. The alkaline reaction mixture was extracted with ether; the ether extract of the acidified mixture yielded 410 mg. of carbobenzoxy-DL-leucine. In a parallel run with non-radioactive HCN the carbobenzoxy-DL-leucine was hydrolyzed and the resulting DL-leucine isolated.

Analysis—$C_6H_{13}O_2N$. Calculated. C 54.94, H 9.99, N 10.68
Found. " 54.81, " 10.08, " 10.86

L-*Leucine*—The carbobenzoxy-DL-leucine was resolved according to Bergmann and Fraenkel-Conrat (12). From 410 mg. of carbobenzoxy-DL-leucine 253 mg. of L-anilide were obtained. It was refluxed with 5 ml. of 6 N HCl for 3½ hours, diluted with 15 ml. of water, and extracted with ether. The clear aqueous phase was evaporated to dryness *in vacuo* over H_2SO_4 and KOH.

The mixture of L-leucine hydrochloride and aniline hydrochloride was dissolved in 1.75 ml. of water. 495 mg. of non-radioactive L-leucine (Merck, methionine-free) dissolved in 2.75 ml. of 1 N HCl were added. The mixture was brought to pH 6 with concentrated ammonia and allowed to stand at room temperature for 2 hours after adding 1 volume of absolute alcohol. The leucine was filtered off and washed three times with absolute alcohol, then with ether. The yield was 461 mg. of carboxyl-C^{14}-L-leucine.

L-Leucine obtained by the foregoing procedure from carbobenzoxy-DL-leucine before dilution with non-radioactive leucine gave a rotation of $[\alpha] = +15.7°$ ($C = 3.6$ in N HCl). Dunn and Rockland (13) give a value of $[\alpha]_D^{26} = +15.5°$ in 21 per cent HCl.

The over-all yield of radioactive L-leucine based on the $KC^{14}N$ used was 28 per cent. The preparation used gave 27,500 c.p.m. per mg.

We have increased the yield of L-leucine by recovering the D-leucine remaining in the mother liquor of the L-anilide, racemizing it by heating in a sealed tube with $Ba(OH)_2$ at 160–170° (14), resolving, and isolating the L form as described above.

Carboxyl-C^{14}*-*L*-lysine*—Gaudry's synthesis of DL-lysine (15) provides a convenient method of preparing the amino acid labeled with C^{14} in the carboxyl group. The method was modified to adapt it to small scale work. Dihydropyran[1] was redistilled and hydrolyzed according to the method

[1] Kindly supplied by E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

of Schniepp and Geller (16). 10 gm. (0.119 mole) of dihydropyran and 40 ml. of 0.2 N HCl were refluxed for 15 to 20 minutes; the homogeneous solution thus obtained was cooled quickly and neutralized with NaOH, and 11.4 gm. of dry $Na_2S_2O_5$ were added to it with vigorous stirring. An aliquot corresponding to 0.015 mole of dihydropyran was transferred to a flask containing 0.01 mole of $NaC^{14}N$. DL-Lysine dipicrate was obtained by Gaudry's procedure via the hydroxy- and bromobutylhydantoin. The dipicrate was converted to the monopicrate. The yield based on the $NaC^{14}N$ used was 10 to 15 per cent. The monopicrate after recrystallization from water gave the following analysis.

Analysis—$C_{12}H_{17}O_9N_5$. Calculated. C 38.40, H 4.56, N 18.65
Found. " 38.54, " 4.68, " 18.56

The DL-lysine monopicrate was resolved enzymatically by the method used to resolve ϵ-C^{14}-lysine (17). As used the lysine gave 19,000 c.p.m. per mg.

Procedure

The reaction mixtures are given with Tables I to IV; their volume in every case was 1.01 ml. They were made up in 20 ml. Pyrex beakers and incubated at 38° in the apparatus of Dubnoff (18), under either 95 per cent O_2 and 5 per cent CO_2 or 95 per cent N_2 and 5 per cent CO_2. At the end of the incubation the proteins were precipitated by 7 per cent trichloroacetic acid, washed, dried, and their radioactivity measured as previously described (1) The pH values given in Tables I to IV were those at the end of the incubation after the addition of 4 ml. of water.

Results

Table I gives representative results of the incorporation of L-lysine into the proteins of the four fractions in acid and alkaline pH, with and without added calcium under oxygen and under nitrogen. In the three particulate fractions more lysine was incorporated on the alkaline than on the acid side of neutrality; the addition of calcium had practically no effect; and the uptake of lysine under nitrogen was as great as under oxygen, except possibly in Sediment II where a slightly greater uptake of lysine was obtained consistently under oxygen than under nitrogen. It may be concluded, then, as a first approximation, that the three particulate fractions incorporated lysine into their proteins mainly under the "alkaline" condition. Sediments I and II were more active than Sediment III. As one obtains approximately 10 times as much of Sediment II as of Sediment I from liver homogenate, it may be said that in the whole homogenate the uptake of lysine under the "alkaline" condition occurs mostly in the particles of Sediment II.

In the supernatant fraction the uptake of lysine was greater on the acid than on the alkaline side of neutrality; there was very little without added calcium, and it was as great under nitrogen as under oxygen. The uptake in this fraction on the alkaline side of neutrality was, evidently, the same process as on the acid side, but at a less favorable pH. It may be concluded, then, that the uptake of lysine observed in the whole homogenate under the "acid calcium" condition was largely, if not entirely, confined to the supernatant fraction.

TABLE I

Incorporation of C^{14}-Labeled L-Lysine into Proteins by Different Fractions of Guinea Pig Liver Homogenate

Homogenate fraction	pH	Calcium	Gas mixture	Counts per min. per mg. protein	Homogenate fraction	pH	Calcium	Gas mixture	Counts per min. per mg. protein
Sediment I	6.2	−	$O_2 + CO_2$	0.6	Sediment III	6.0	−	$O_2 + CO_2$	0.4
	6.2	+	" + "	1.0		6.0	+	" + "	0.5
	6.2	−	$N_2 + CO_2$	0.3		6.0	−	$N_2 + CO_2$	0.5
	6.2	+	" + "	0.7		6.0	+	" + "	0.6
	7.8	−	$O_2 + CO_2$	2.8		7.6	−	$O_2 + CO_2$	1.1
	7.8	+	" + "	4.3		7.6	+	" + "	1.2
	7.8	−	$N_2 + CO_2$	3.2		7.6	−	$N_2 + CO_2$	0.8
	7.8	+	" + "	4.2		7.6	+	" + "	1.0
" II	6.4	−	$O_2 + CO_2$	0.6	Supernatant	6.2	−	$O_2 + CO_2$	0.09
	6.4	+	" + "	0.8		6.2	+	" + "	3.45
	6.4	−	$N_2 + CO_2$	0.4		6.2	−	$N_2 + CO_2$	0.11
	6.4	+	" + "	0.5		6.2	+	" + "	3.38
	7.5	−	$O_2 + CO_2$	3.3		7.4	−	$O_2 + CO_2$	0.10
	7.5	+	" + "	3.3		7.4	+	" + "	1.8
	7.5	−	$N_2 + CO_2$	3.0		7.4	−	$N_2 + CO_2$	0.13
	7.5	+	" + "	3.0		7.4	+	" + "	1.7

All the fractions were prepared in the KCl solution. The acid reaction mixtures contained 0.7 ml. of the homogenate fraction indicated, pH adjusted to 6.2; calcium, when added, 0.01 ml. of 0.4 M $CaCl_2$ in 0.9 per cent KCl, adjusted to pH 6.0; 0.2 ml. of 0.1 M succinate in the KCl solution used in the preparation of the fractions, adjusted to pH 5.9; and 0.1 ml. of 0.026 M carboxyl-C^{14}-L-lysine in the KCl solution, adjusted to pH 6.2. The alkaline reaction mixtures contained 0.7 ml. of the homogenate fraction indicated, its pH adjusted to 7.8; calcium, when added, 0.01 ml. of 0.4 M $CaCl_2$ in 0.9 per cent KCl adjusted to pH 6.0; 0.2 ml. of 0.2 M $NaHCO_3$ in the KCl solution; and 0.1 ml. of 0.26 M L-lysine-1-C^{14} in the KCl solution, adjusted to pH 7.5. All the reaction mixtures were incubated 2 hours.

All four fractions in the experiments of Table I were prepared with the KCl solution as diluent because, even with the highest centrifugal force we could obtain, nearly 40,000 $\times$ g, we could not get a sufficient amount of

the supernatant fraction to work with the sucrose solution as diluent. In separate experiments we prepared the particulate fractions from the same liver, on the one hand with the sucrose solution and on the other with the KCl solution as diluent; the amount of lysine incorporated into the proteins, and the conditions with respect to pH, calcium, and independence of oxygen were the same with each fraction prepared by either method.

Table II summarizes the results on the uptake of glycine, L-leucine, and L-lysine by the three particulate fractions. The results in the pH range 7.2 to 7.7 only are given, because with glycine and leucine the uptake was less on the acid and alkaline sides of this pH range in all three fractions.

TABLE II

Incorporation of C^{14}-Labeled Amino Acids into Proteins by Particulate Fractions of Guinea Pig Liver Homogenate

The results are expressed as millimoles $\times 10^{-3}$ per gm. of protein.

C^{14}-labeled amino acid	Sediment I	Sediment II	Sediment III
Glycine	0.52 ± 0.07*	0.40 ± 0.09	0.075 ± 0.004*
L-Leucine	0.61 ± 0.15*		
L-Lysine	4.1 ± 1.2	3.2 ± 0.14	0.9 ± 0.05

The reaction mixtures contained 0.7 ml. of the fraction indicated, pH adjusted to 7.5; 0.01 ml. of 0.4 M $CaCl_2$ in 0.9 per cent KCl, adjusted to pH 6.0; 0.2 ml. of 0.2 M $NaHCO_3$ in either the sucrose or KCl solution according to which was used in the preparation of the fraction; and 0.1 ml. of the labeled amino acid, 0.053 M in the KCl solution, pH adjusted to 7.5. The reaction mixtures containing glycine or leucine were run for 4 hours, those with lysine for 2 hours.

* Values for preparations made with KCl solution only.

In all these experiments $CaCl_2$ was added to a final concentration of 0.004 M because of earlier findings that the uptake of lysine (1) and of glycine (19) was slightly augmented by this concentration of added calcium. The gas mixture used was 95 per cent O_2 + 5 per cent CO_2, as although the uptake of lysine is not significantly inhibited by anaerobiosis that of glycine is (19). The experiments with lysine were run for 2 hours and those with glycine and leucine for 4 hours, because the reaction with lysine stops at the end of 2 hours (1) while that with glycine, though much slower, continues logarithmically up to 6 hours (19).

Table II gives the averages and standard deviations of the results from eight or more different preparations of the fractions; all three amino acids were tested on the same preparation of the fraction in each experimental run. The fractions prepared with sucrose took up much less glycine and leucine than did those with the KCl solution; in fact Sediments I and III prepared with sucrose solution took up no leucine. When

the results obtained with the preparations made with sucrose solution were consistently much lower than those with KCl, the former were not included in the averages given in Table II; those averages are marked with an asterisk. We used in these experiments lysine labeled in either the carboxyl or the ϵ-carbon atom (17) and obtained essentially the same results with both.

Each of the three amino acids was taken up most actively in Sediment I and least in Sediment III. The reason for the greatest reactivity in Sediment I may be that it contained clumps of intact liver cells; cell destruction reduced the rate of incorporation of amino acids in every case which has been tested (20).

Of the three amino acids the uptake of lysine was the greatest in all three fractions. The uptake of glycine in 4 hours in Sediments I and II of 0.52 to 0.40 $\times$ 10^{-3} mM per gm. of protein was of the same order of magnitude as that observed by Winnick *et al.* (19) in their rat liver fraction sedimented at about 2500 $\times$ g which is a composite of our Sediments I and II. They observed in their thrice washed fraction an average of 11.4 c.p.m. per mg. of protein per hour after incubation with glycine of 400,000 c.p.m. per mg. This corresponds to an uptake of 0.38 $\times$ 10^{-3} mM of glycine per gm. of protein per hour. Later they reported that 30 (21) to 80[2] per cent of the radioactivity in the protein was due to adsorbed phosphatidylserine. The radioactivity in the protein in our experiments with glycine could not have come from adsorbed phosphatidylserine formed from the labeled glycine added initially, because after treating the protein with ninhydrin the CO_2 liberated gave no counts (see Table IV below).

The uptake of leucine in Sediments II and III was irregular. In many experiments we observed no uptake; in some there was as much as 0.8 $\times$ 10^{-3} mM per gm. of protein. We have not found the cause of the irregular uptake of leucine in different preparations. For this reason no values are given in Table II for the uptake of leucine by Sediments II and III.

We found a synergistic action between Sediments II and III in the uptake of glycine. When the reaction mixture contained Sediments II and III, the glycine taken up varied from 120 to 250 per cent of the sum of the separate uptakes. No interaction was found between any of the other possible combinations of fractions on the uptake of glycine, nor with any combination of fractions on the uptake of leucine and lysine.

It has been found in feeding experiments that an indispensible amino acid is ineffective for growth or for recovery from protein depletion unless

[2] Greenberg, D. M., personal communication.

it is fed within a few hours of other necessary amino acids (22). The possibility, which these findings suggested, that the uptake of one amino acid may be increased by the presence of others was examined by comparing the uptake of labeled glycine, leucine, and lysine when the three were incubated in the reaction mixture separately and together.

Table III is a summary of the result of such an experiment. When the three labeled amino acids were added together, the protein count was the sum of the three added separately. The addition of the non-radio-

TABLE III

Incorporation of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine into Proteins When Incubated with Sediment II Separately and Together*

Labeled amino acids	Unlabeled amino acids	Counts per min. per mg. protein	
		Observed	Calculated
Glycine		6.4	
"	Leucine + lysine	6.0	
Leucine		3.0	
"	Glycine + lysine	2.5	
Lysine		4.0	
"	Glycine + leucine	4.1	
Glycine + leucine + lysine		13.1	13.4

The reaction mixtures contained 0.7 ml. of Sediment II, pH adjusted to 7.85; 0.01 ml. of 0.4 M $CaCl_2$ in 0.9 per cent NaCl, pH adjusted to 6.0; 0.1 ml. of each of the amino acids indicated in Krebs-Henseleit Ringer's solution, pH adjusted to 7.5. Krebs-Henseleit Ringer's solution was added when necessary to bring the final volume of the reaction mixture to 1.01 ml. The glycine, leucine, both labeled in the carboxyl group, and ϵ-C^{14}-lysine concentrations were 4.0, 3.3, and 3.7 mg. per ml. respectively. The reaction mixtures were incubated 4 hours under 95 per cent O_2 and 5 per cent CO_2. The pH values at the end were in the range 7.3 to 7.4.

* Prepared with Krebs-Henseleit Ringer's solution (4) instead of the KCl solution as diluent.

active forms of two of the three did not affect the uptake of the radioactive amino acid added to the reaction mixture. In other experiments it was found that the uptake of each of these three labeled amino acids was the same in the presence or absence of a mixture of amino acids corresponding to the composition of casein. We may conclude, therefore, that glycine, L-leucine, and L-lysine were taken up independently under our experimental conditions.

We have surveyed the effects of added cobaltous, cupric, and manganous salts on the uptake of glycine, leucine, and lysine by Sediment II. With final concentrations in the reaction mixture of 0.005, 0.001, 0.0005,

and 0.0001 M $CuCl_2$, the uptakes of glycine, expressed as a per cent of that without added copper, were 13, 68, 93, and 100 respectively, and of lysine 33, 75, 94, and 100 respectively.

TABLE IV

Effect of Added $CoCl_2$ on Incorporation of Glycine and of Leucine into Proteins of Sediment II of Guinea Pig Liver Homogenate*

Concentration of added $CoCl_2$ in reaction mixture	Counts per min. per mg. protein			Inhibition of uptake
	Before ninhydrin treatment	In CO_2 liberated in ninhydrin treatment	Calculated remaining in protein after ninhydrin treatment	
Glycine				
M				*per cent*
0	7.4	0	7.4	
0.005	9.3	6.3	3.0	59
0.001	4.1	1.8	2.3	68
0.0005	7.7	1.3	6.4	13
0.0001	8.5	0.6	7.9	0
Leucine				
0	3.0	0	3.0	
0.005	10.0	9.5	0.5	83
0.001	2.5	1.4	1.1	63
0.0005	2.6	0.7	1.9	36
0.0001	4.2	0.1	4.1	0

The reaction mixtures contained 0.7 ml. of Sediment II, pH adjusted to 7.8; 0.01 ml. of 0.4 M $CaCl_2$ in 0.9 per cent NaCl, pH adjusted to 6.0; 0.1 ml. of a solution of $CoCl_2$ in Krebs-Henseleit Ringer's solution 10 times the concentration indicated, pH adjusted to 7.0; 0.1 ml. of amino acid solution in Krebs-Henseleit Ringer's solution, pH adjusted to 7.5; and 0.1 ml. of Krebs-Henseleit Ringer's solution, or 0.2 ml. when no cobalt was added. The glycine and leucine concentrations were 4.0 and 3.3 mg. per ml. respectively. The reaction mixtures were incubated 4 hours under 95 per cent O_2 and 5 per cent CO_2. The pH values at the end were in the range 7.3 to 7.4. In the treatment with ninhydrin 5 mg. of non-radioactive L-leucine were added to provide CO_2 as a carrier of the CO_2 from the protein. The CO_2 was trapped in $Ba(OH)_2$ solution, and the $BaCO_3$ filtered off, dried, and counted.

* Prepared with Krebs-Henseleit Ringer's solution (4).

Added $MnCl_2$ over a concentration range of 0.005 to 0.0001 M was without effect on the uptake of either glycine, leucine, or lysine.

The results with added cobalt were different. The counts in the protein after exhaustive washing with trichloroacetic acid were very high, but they were also very high in the boiled controls and when the Sediment II fraction was first precipitated with acetone and dried. When the

radioactive protein was treated with the ninhydrin reagent (23) most of the counts appeared in the liberated CO_2. Evidently the radioactive amino acids which could not be washed from the protein with trichloroacetic acid were not bound in peptide bonds. Some typical results with glycine and leucine are given in Table IV; they show that, so far as the actual incorporation of the amino acids into the proteins is concerned, $CoCl_2$ added to a final concentration of 0.005 to 0.0005 M was inhibitory. Similar results were obtained with L-lysine, but as it was labeled in the ϵ position, it could not serve for the measurement of the non-peptide-bound amino acid by treatment with ninhydrin.

The degree of non-peptide-bound amino acid was greater the greater the concentration of added cobalt. The amount of amino acid so bound resulting from the addition of cobalt varied with the protein; the proteins of Sediment II after precipitation with acetone were more active than the mixture of proteins of the whole homogenate; the proteins of Sediment II were more active than diaphragm proteins; the latter were more active than bone marrow cell proteins; and with egg albumin there was very little combination with the amino acids. The combination of protein with amino acid in the presence of cobalt at 38° takes about 6 hours to reach completion; it does not proceed below pH 6.0 and the rate increases progressively with increasing pH up to 8.8, which was the highest pH we examined.

The preparations of Sediment II of the experiments of Tables III and IV were made with Krebs-Henseleit Ringer's solution (4). These experiments were carried out before those of Tables I and II, in which the fractions were prepared with the KCl solution as diluent. We found no consistent differences in the incorporation of glycine, leucine, and lysine between the two saline preparations.

In controls run simultaneously with all the foregoing experiments proteins precipitated at zero time gave no counts; the proteins in liver preparations boiled for 15 minutes at pH 6.0 before addition of labeled amino acids also gave no counts; in the preparations boiled at pH 7.5 the proteins gave about 10 per cent of the counts of those in the unboiled preparations.

DISCUSSION

All four fractions of liver homogenate, three particulate fractions and the superanatant solution, incorporated labeled amino acids into their proteins, but they differed in the rate at which they incorporated each amino acid, and in the case of lysine, the three particulate fractions on the one hand and the supernatant solution on the other had different optimal conditions. These findings indicate that there are functional differences among the components of liver homogenate with regard to in-

corporation of amino acids into their proteins. There are no doubt other functional differences; the finding of Schneider *et al.* (7) that the succinoxidase activity was associated almost exclusively with the larger granules (Sediment II) points in the same direction.

Of more general interest is the conclusion which must be drawn that the incorporation of amino acids into proteins does not, in the adult cell, necessarily depend on direct participation of the nucleus. While not all the nuclei may have been removed from Sediment II, the amount left must have been small, and it is highly improbable that there were any nuclei in the Sediment III or supernatant fractions.

Labeled glycine, leucine, and lysine were incorporated into the proteins to the same extent whether each was in the reaction mixture alone or when others were added with it, radioactive or non-radioactive. It is improbable that it is the uptake of only these three labeled amino acids which has this characteristic. They were the first three amino acids tested; many, if not all, amino acids probably would behave in the same way.

Of course it is possible that other amino acids are actually necessary for the incorporation of any one into the proteins, and that, although the homogenate fraction (Sediment II) used in these experiments was washed twice, it contained, nevertheless, amino acids sufficient for the uptake of the labeled amino acids tested.

SUMMARY

1. The synthesis and resolution of L-leucine and of L-lysine labeled with C^{14} in their carboxyl groups are described.

2. Three different particulate fractions and a supernatant fraction were prepared by differential centrifugation. The four fractions exhibited functional differences in the rates at which they incorporated glycine, L-leucine, or L-lysine.

3. L-Lysine was incorporated into the proteins of the particulate fractions of guinea pig liver homogenate under one set of conditions and into those of the supernatant fraction under another set of conditions.

4. When labeled glycine, L-leucine, and L-lysine together were incubated with a homogenate fraction, the uptake, as measured by the radioactivity of the protein, was the sum of that of the three amino acids incubated separately. Addition to the reaction mixture of a mixture of non-radioactive amino acids corresponding to the composition of casein did not affect the uptake of the labeled amino acids.

5. $CuCl_2$ and $CoCl_2$ in the concentration range 0.005 to 0.0005 M inhibited the incorporation into the proteins of glycine, L-leucine, and L-lysine. The inhibition was greater the higher the concentration of cobalt.

$MnCl_2$ exerted neither a stimulation nor an inhibitory effect when added in the range 0.005 to 0.0001 M.

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