THE UTILIZATION OF LABELED GLYCINE IN THE PROCESS OF AMINO ACID INCORPORATION BY THE PROTEIN OF LIVER HOMOGENATE*

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The incubation of rat liver slices in an oxygenated medium in the presence of either S35-labeled methionine (1) or C14-labeled alanine (2) results in an uptake of the radioactive amino acid into the protein of the slices. A similar reaction has been noted between the protein of intestinal slices and C14-labeled glycine (glycine*) (3). Presumably the residues of the labeled amino acids become components of the protein molecules. The inhibition of the C14 incorporation by heat or sodium azide suggests that the process is mediated by enzymes (3).

Recently (4) it was reported that glycine* is incorporated into the proteins of homogenized preparations of spleen and liver upon incubation under suitable conditions. Further work has shown that the process involved is not simply one of glycine* incorporation into protein. Instead, the major portion of the C14 found in the homogenate protein can be attributed to other amino acids, particularly serine, derived apparently from glycine. These results are reported separately (5).

The present paper describes the effect of various metabolites, inhibitors, and certain experimental conditions on the process of C14 incorporation by liver homogenate protein in the presence of glycine*.

Of several different organs tested, rat spleen yielded the most active homogenate preparations. However, spleen tissue is complex histologically, in that it consists of several types of small cells, and the destruction of the latter by mechanical grinding is difficult to gage. By contrast, liver consists mainly of cells of one type. These cells are rather large (about 25 to 30 μ in diameter in the rat) and are readily ruptured in a glass homogenizer. The small fraction of the hepatic cells which escapes disintegration can be removed by low speed centrifugation. Homogenates prepared in this manner are free from intact liver cells. They contain abundant nuclei, as well as cytoplasmic material, leucocytes, and erythrocytes.

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In the presence of glycine* these cell-free preparations can incorporate C\textsuperscript{14} into their protein substance at rates comparable to those observed with liver slices.

As in p-aminohippuric acid (PAH) synthesis (6), the enzyme activity is found to be associated with the insoluble particles of the rat liver cells. However, as might be expected, PAH synthesis (from glycine and hippuric acid) and glycine* utilization by homogenate protein differ markedly in certain respects. One difference which may be mentioned at this point is that PAH synthesis was observed only in liver and kidney preparations, while glycine* utilization occurs in homogenates of virtually all of the several organs.

**Table I**

**Composition of Standard Medium**

The solution was prepared with sterile distilled water and saturated with a mixture of 95 per cent oxygen-5 per cent CO\textsubscript{2} gas. Its final pH was 7.4. It was found advisable to prepare fresh solutions about twice monthly and to store these in the refrigerator to prevent spoilage.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (gm. per 100 ml. solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.72</td>
</tr>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>0.28</td>
</tr>
<tr>
<td>K\textsubscript{2}HPO\textsubscript{4}</td>
<td>0.10</td>
</tr>
<tr>
<td>MgSO\textsubscript{4}·7H\textsubscript{2}O</td>
<td>0.06</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.10</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL**

**Radioactive Glycine**—The glycine* employed was labeled with C\textsuperscript{14} on the methyl carbon position (7). It had a specific activity of 3.1 microcuries (approximately 400,000 counts per minute per mg., as measured with the mica window counter tube employed in these experiments). Sterile solutions containing 1 mg. of glycine* per ml. of distilled water were prepared and stored in the refrigerator. Aliquots, generally containing 0.2 mg. (80,000 counts), were used for individual experiments.

**Standard Medium**—A modified Krebs-Henseleit medium, of the composition given in Table I, was used in the preparation of the liver homogenates. 3 ml. of 1 per cent calcium chloride were added to 100 ml. of medium just prior to use.\(^1\)

**Preparation of Homogenate**—Rats (150 to 200 gm.) were killed and the

\(^1\) A precipitate or turbidity due to calcium carbonate develops slowly on standing.
livers removed. 1 gm. portions of liver were homogenized thoroughly with 15 ml. quantities of cold standard medium in a glass homogenizer equipped with a close fitting, rapidly revolving piston. The resulting suspension was then centrifuged for 2 minutes at 500 to 600 R.P.M. (in an angle head centrifuge). The supernatant liquid was removed by siphoning and employed in the experiments to be described. The small sediment of surviving liver cells was discarded. The pH of the homogenate was 7.5 to 7.6.

Preparation of Washed Insoluble Cell Particles—Liver homogenate was prepared with either the standard medium or an isotonic saline-bicarbonate solution, in the manner already described. About 200 ml. of this homogenate were centrifuged for 15 minutes at 4000 R.P.M. in a refrigerated (angle head) centrifuge. The supernatant solution was discarded. The sediment was mixed thoroughly with 75 ml. of standard medium, or saline-bicarbonate solution, and centrifuged as before. This last step was repeated a specified number of times. Finally, the sediment was suspended in 10 ml. of either standard medium or saline-bicarbonate and used without delay in the incubation procedure.

Incubation of Homogenates in Presence of Glycine*—3 ml. aliquots of freshly prepared homogenate were pipetted into 50 ml. glass-stoppered flasks containing measured amounts of glycine. Each flask was flushed thoroughly with 95 per cent oxygen-5 per cent carbon dioxide mixture, stoppered, and then agitated in a 37° water bath for a definite time, generally 90 minutes.

In certain specified cases, 0.5 ml. aliquots of the preparation of washed insoluble particles were used instead of 3 ml. of whole homogenate. In these instances, 2.5 ml. of nutrient medium were added to each flask.

Separation of Radioactive Protein—At the conclusion of the reaction period, the protein of the homogenates was precipitated by the addition of 10 ml. of 12 per cent trichloroacetic acid to each flask. The precipitates were collected by centrifugation, washed three times with 12 ml. quantities of 5 per cent trichloroacetic acid, and then twice with acetone, being centrifuged after each washing.

Measurement of Radioactivity of Protein—The protein material in each tube was suspended in acetone and transferred to a weighed 5.5 cm. No. 50 Whatman filter paper, with the aid of a Tarver type filter. The precipitates (12.5 cm. in area, generally 22 to 26 mg. in weight) were dried for

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2 Composed of 8.0 gm. of NaCl and 2.5 gm. of NaHCO₃ per liter of water, saturated with 95 per cent O₂-5 per cent CO₂ gas; pH approximately 7.3.

3 It consisted of two layers: a light brown upper layer, rich in nuclei and mitochondria, and a smaller bottom layer of erythrocytes. It was not considered important to separate these layers.

Each contained about 25 mg. of protein.
an hour at 100° and then equilibrated in air. The papers were reweighed, and the radioactivity of the layers determined with the Autoscaler model Geiger-Müller counter.

In order to compensate for the self-absorption of radiation, the radioactivity of each protein sample was compared to that of a glycine standard, prepared by diluting a definite amount of glycine with a quantity of inactive organic material (glycine) of the same weight and layer thickness as the protein. In this way the concentration of labeled carbon in the protein could be ascertained. This concentration was generally expressed as counts of C\textsuperscript{14} per minute per mg. of protein per hour of incubation.

### Table II

Effect of Repeated Washing with Standard Medium on Activity of Insoluble Homogenate Particles

The results were corrected for a background of 51 counts per minute. Errors due to self-absorption of radiation were compensated by comparison with glycine standards, as indicated in the experimental section. The triplicate values in the second column pertain to separate incubations of aliquots taken from the homogenate preparation at different stages. The quantity of protein in the aliquots was in the range of 20 to 30 mg.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Radioactivity of protein, counts per min. per mg. per hr. of incubation</th>
<th>Average per cent deviation of individual results from mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original homogenate</td>
<td>7.0, 7.1, 8.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Homogenate centrifuged 15 min. at 4000 R.P.M.; supernatant assayed</td>
<td>1.2, 1.4, 1.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Sediment from preceding experiment assayed</td>
<td>14.7, 15.9, 16.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Sediment washed once</td>
<td>12.3, 13.7, 14.6</td>
<td>6.3</td>
</tr>
<tr>
<td>Sediment washed once “ 3 times”</td>
<td>11.1, 11.4, 11.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Sediment washed once “ 5 times”</td>
<td>8.1, 9.1, 9.5</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Accuracy of Radioactivity Measurements and Reproducibility of Results—The protein samples usually had activities of the order of 5 to 10 times the background strength. They were counted for time intervals (generally 5 to 20 minutes) sufficient to reduce the counting error to less than 3 per cent.

All experiments were performed at least in duplicate. Table II illustrates the degree of reproducibility of replicate assays. In Tables III and IV the averages of duplicate determinations are given. The average agreement between these duplicate determinations was 6 per cent.

Inasmuch as homogenates prepared on different days (from different

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*These preparations contained 14 per cent nitrogen.

*Manufactured by Tracerlab, Inc., 55 Oliver Street, Boston.
livers) sometimes differed as much as 30 per cent in activity, it was simplest to express certain of the results (Tables III and IV) in terms of relative, rather than absolute, radioactivity units. In these instances, values are given for the radioactivities of the reference homogenates.

RESULTS AND DISCUSSION

Rate of $^{14}C$ Incorporation into Protein—Curve 2 of Fig. 1 indicates that the quantity of $^{14}C$ incorporated into protein increased uniformly with time. From the initial slope of the curve, it may be calculated that 0.004 $\gamma$ of labeled carbon was contained in each mg. of protein after 1 hour. 1 $\gamma$ of this carbon was equivalent to 2000 counts per minute. The radioactivity

![Graph showing rates of oxygen consumption and $^{14}C$ utilization by liver homogenates.](http://www.jbc.org/)

incorporated into the 25 mg. of protein of the average homogenate in this period was equivalent to 0.1 $\gamma$ of labeled carbon, or 0.25 per cent of the total amount employed (40 $\gamma$ of carbon in 0.2 mg. of glycine*).

The heated homogenates (Curve 4) exhibited virtually zero activity.

The rate of $^{14}C$ uptake by homogenates was approximately one-third that found with liver slices under almost identical conditions.7

It is of interest that the homogenate utilized oxygen (Curve 1). The initial rate of oxygen consumption was approximately one-fourth that with liver slices.7  Heated homogenate showed a very low consumption (Curve 3). That the oxygen consumption was related to the glycine* utilization

7 The reaction rate with slices was determined by Mr. P. Siekevitz of our department.
by protein was further indicated by the observation that the latter process was drastically inhibited when the incubations were conducted in a nitrogen atmosphere. Frantz and coworkers (2) found that liver slices do not take up labeled alanine under anaerobic conditions.

Homogenates, evidently, afford particularly favorable conditions for the growth of microorganisms. In some preliminary experiments, in which undue contamination apparently occurred, Curve 2 of Fig. 1 assumed an autocatalytic character, with a steep upward inflection after 3 to 5 hours.

Influence of Glycine* Concentration—Fig. 2 indicates that the absolute amounts of C^1^4 incorporated into protein increased with increasing concentration of glycine* in the homogenate system. However, the efficiency of the process was greater at lower concentrations. For example, at a glycine* concentration of 2 × 10^{-4} M, about 0.3 per cent of the total C^1^4 in the system was taken up by the protein per hour, while at a concentration of 35 × 10^{-4} M, the corresponding percentage was 0.06. Similar results were obtained by Melchior and Tarver (1) with liver slices and labeled methionine.

Effect of pH—The preparation of washed insoluble particles (rather than the whole homogenate) was employed here to facilitate pH adjustments. The rate of glycine* utilization was found to have a wide optimum pH range, centering about pH 7.5 (Fig. 3). By contrast, the curve for PAH synthesis exhibited a very sharp peak at pH 7.5 and relatively low activity above pH 7.9 (9).

Association of Protein Activity with Insoluble Particles of Homogenate—

Fig. 2. Effect of variations in glycine* concentration on the rate of C^1^4 incorporation into the protein of homogenate. The reaction time was 90 minutes. Each of a series of flasks contained 3 ml. of homogenate and a quantity of glycine* ranging from 0.07 to 0.85 mg.
The enzyme system which regulated the C\textsuperscript{14} incorporation into protein was found to be associated with the insoluble particles of the homogenate, since it sedimented together with these particles (Table II). The particles were about twice as active (per mg. of protein) as the whole homogenate, or about two-thirds as active as slices. The supernatant solution had a relatively low activity. Repeated washing of the particles with standard medium resulted in a partial loss of activity. The third column in Table II indicates the degree of reproducibility with replicate experiments.

**Effect of Various Preliminary Treatments (Table III)—**The importance
of tonicity is shown by the fact that homogenizing in the presence of distilled water destroyed most of the activity. The need for structural integrity is further indicated by the markedly lowered rate of C\textsuperscript{14} uptake following lyophilizing or repeated freezing and thawing. Similar results were obtained in connection with PAH synthesis (6) and were interpreted in terms of changes in the state of aggregation of nucleoprotein.

Table IV  

*Influence of Various Substances on Uptake of C\textsuperscript{14} by Insoluble Particles of Liver Homogenate*

The particles were washed three times with saline-bicarbonate solution.

<table>
<thead>
<tr>
<th>Change in composition of standard medium</th>
<th>Relative radio-activity of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (standard medium)</td>
<td>100*†</td>
</tr>
<tr>
<td>Medium replaced by saline-bicarbonate solution</td>
<td>35</td>
</tr>
<tr>
<td>Potassium omitted from standard medium</td>
<td>80</td>
</tr>
<tr>
<td>Phosphate “”</td>
<td>87</td>
</tr>
<tr>
<td>Magnesium “”</td>
<td>85</td>
</tr>
<tr>
<td>Calcium omitted</td>
<td>43</td>
</tr>
<tr>
<td>Glucose “”</td>
<td>79</td>
</tr>
<tr>
<td>Citrate “”</td>
<td>77</td>
</tr>
<tr>
<td>Both glucose and citrate omitted</td>
<td>78</td>
</tr>
<tr>
<td>Citrate replaced by 0.002 M fumarate</td>
<td>87</td>
</tr>
<tr>
<td>5 × 10\textsuperscript{-4} M cytochrome C added to medium</td>
<td>88</td>
</tr>
<tr>
<td>5 × 10\textsuperscript{-4} M cozymase added</td>
<td>88</td>
</tr>
<tr>
<td>0.002 M pyridoxal added</td>
<td>98</td>
</tr>
<tr>
<td>O\textsubscript{2} replaced by N\textsubscript{2} atmosphere</td>
<td>10</td>
</tr>
<tr>
<td>0.001 M ATP added; N\textsubscript{2} atmosphere</td>
<td>15</td>
</tr>
<tr>
<td>0.002 M L-phosphoglyceric acid added; N\textsubscript{2} atmosphere</td>
<td>15</td>
</tr>
<tr>
<td>Cytochrome + ATP + fumarate + cozymase added</td>
<td>67</td>
</tr>
<tr>
<td>0.005 M cyanide added</td>
<td>5</td>
</tr>
<tr>
<td>0.005 M azide added</td>
<td>5</td>
</tr>
<tr>
<td>0.1% amino acid mixture added</td>
<td>65</td>
</tr>
</tbody>
</table>

† This activity averaged 17.1 counts per minute per mg. of protein per hour of reaction time with homogenates prepared on different days.

The homogenate lost activity slowly at refrigerator temperatures and retained only 18 per cent of its original activity after 24 hours at 3°.

Since intact blood cells were present in the homogenates, the possibility existed that these cells were responsible for the C\textsuperscript{14} uptake. However, the activity was not lowered when *perfused* liver was used in the preparation of the homogenate. In addition, it was found that neither rat (non-nucleated) nor chicken (nucleated) erythrocytes had significant activity when incubated with glycine* in the standard nutrient solution.

Importance of Various Metabolites (Table IV)—The particles, freed of
soluble constituents by three washings with saline-bicarbonate solution, provided a favorable material for a study of the role of these same constituents in the process of C\textsuperscript{14} incorporation into the homogenate protein. When incubated in saline-bicarbonate solution, the particles had only a third as much activity as when tested in the presence of the standard medium.

Of the individual inorganic ions, the omission of calcium resulted in the greatest decrease in the rate of C\textsuperscript{14} uptake. In contrast to this observation, the omission of calcium led to a stimulation of PAH synthesis (9).

The omission of either glucose or citrate (or both) from the medium resulted in a decrease of approximately 20 per cent in the rate of C\textsuperscript{14} incorporation. Fumarate appeared to substitute only partially for citrate in the presence of glucose. In the case of PAH synthesis, fumarate was the most effective of a number of metabolites (chiefly components of the tricarboxylic acid cycle) in stimulating the reaction (9).

Other indications that the mechanism of C\textsuperscript{14} incorporation differs from that of PAH synthesis are findings that neither cytochrome c, under aerobic conditions, nor adenosine triphosphate (ATP), anaerobically, stimulated the former process significantly. Likewise, it was found that ATP failed to reactivation preparations which had previously stood for 24 hours in the refrigerator. Phosphoglyceraldehyde, which can act as an ATP generator in certain cases (10), did not promote C\textsuperscript{14} uptake anaerobically. Cohen and McGilvery (6) regard ATP as essential to PAH synthesis.

Pyridoxal, concerned with phosphate transfer in connection with decarboxylase and transaminase activity, had no effect on C\textsuperscript{14} utilization by homogenate protein, and had a slight depressant action at higher concentrations.

A combination of cytochrome, cozymase, ATP, and fumarate exerted a depressant effect on C\textsuperscript{14} utilization, whereas a like combination stimulated PAH synthesis.

The results obtained thus far permit no definite conclusions concerning the mechanism of the glycine\* utilization process. There is as yet no evidence that phosphate bond energy is required. However, some type of oxidative mechanism is certainly suggested by the fact that oxygen is required and that cyanide and azide are strong inhibitors of the reaction.

It is worthy of note that the addition of a mixture of all of the various amino acids (except glycine) depressed, rather than stimulated, the rate of C\textsuperscript{14} uptake. In this connection, it may be noted that C\textsuperscript{14} incorporation occurs with washed particles, presumably freed from amino acids. However, certain of the acids other than glycine can apparently be derived from glycine (5), while autolysis may supply others. The C\textsuperscript{14} of the homogenate protein is distributed among several amino acids (5). Nevertheless, it cannot be concluded that the incorporation of a given amino acid requires the participation of other amino acids.
SUMMARY

The incorporation of C\textsuperscript{14} into the protein of cell-free homogenates of rat liver has been observed, following incubation with labeled glycine at 37° in an oxygen atmosphere. The process appears to be enzymic in nature. It is inhibited by heat, cyanide, azide, and anaerobic conditions. The rate of incorporation of C\textsuperscript{14} is dependent upon the glycine concentration, time of reaction, and pH of the medium.

The enzyme system which promotes the utilization of the C\textsuperscript{14} of glycine is associated with the insoluble particles of the homogenate. The process is promoted by certain inorganic ions: magnesium, phosphate, potassium, and particularly calcium. Of a number of organic substances tested, glucose and citrate exerted a slight stimulatory effect. Adenosine triphosphate, cytochrome, pyridoxal, and cozymase, on the contrary, caused a slight inhibition.

Addendum—Very recently Mr. E. Peterson of our laboratory has obtained a several fold increase in the rate of C\textsuperscript{14} incorporation by reducing the volume of the incubation mixture to 0.33 ml. without altering the amount of homogenized liver or liver particles customarily employed. Concentrated particles are about 3 times as active as liver slices, and since smaller amounts of glycine* (0.03 mg.) are employed, the efficiency is such that approximately 10 per cent of the C\textsuperscript{14} is incorporated within 1 hour.

BIBLIOGRAPHY

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