THE IN VITRO INCORPORATION OF C\textsuperscript{14}-GLYCINE INTO ANTIBODY AND OTHER PROTEIN FRACTIONS BY POPLITEAL LYMPH NODES OF RABBITS FOLLOWING THE LOCAL INJECTION OF CRYSTALLINE OVALBUMIN

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Since the study of Ehrlich and Harris (1-4), it has been generally accepted that the popliteal lymph nodes are the active sites in the formation of antibody. Recently M. Ogata and Y. Mochizuki (5) (authors of the present paper) demonstrated, employing the quantitative precipitin method of Heidelberger (6-8), that precipitable antibody nitrogen per gram tissue of the popliteal lymph nodes were greater than that of serum in rabbits immunized by the foot-pad injection of ovalbumin, and confirmed the above fact.

On the other hand, Heidelberger et al. (9) demonstrated that the N\textsuperscript{15}-glycine was incorporated \textit{in vivo} into the newly formed antibody protein.

However, there have been only few reports concerning the formation of antibody in the tissue preparation \textit{in vitro}. Roberts et al. (10) demonstrated that minced lymphoid tissue from rats and mice immunized to sheep erythrocytes, released antibodies during the incubation with suitable medium and suggested that antibody production might occur \textit{in vitro}. Recently, Ranney and London (11) reported briefly that slices of the liver and spleen from rabbits immunized with type III pneumococci incorporated C\textsuperscript{14}-glycine into antibody, and Askonas and Humphrey (12) reported similar results using the slices of granulomata produced in rabbits by injection of Freud’s adjuvant containing ovalbumin.

But there have been neither reports on the rate of the incorporation of C\textsuperscript{14}-amino acid into antibody and other protein fractions of the immunized tissue preparation, nor reports concerning various metabolic
factors governing the rate of the incorporation.

For these reasons, the authors have undertaken a fundamental study in which the rate of incorporation of C\textsuperscript{14}-glycine into antibody and other protein fractions were determined and further various metabolic factors affecting the incorporation rate were investigated in detail, using cell suspensions or homogenates of popliteal lymph nodes of rabbits after the local immunization with crystalline ovalbumin.

**EXPERIMENTAL**

*Treatment of Animals*—The rabbits used for immunization were albino ranging in weight from 2 to 3 kg. They were given the subcutaneous injection of alum-precipitated crystalline ovalbumin in 8 mg. dose at foot pads every other day at least for 4 weeks. Thereafter the popliteal lymph nodes from two or three animals were removed and used for each isotopic experiment. The ovalbumin was prepared by the method of Kekwick (30).

*Tissue Preparation*—The following manipulation was performed in the cold room (−2° to +2°). To obtain cell suspensions, two kinds of procedures were employed, the one, in which the popliteal lymph nodes were ground with a mortar and pestle, then passed through a tissue masher fitted with a screen containing holes 0.5 mm. in size (cell suspension-1), the other, in which the lymphatic tissues were cut into very small pieces (0.5–1 mm. diameter or more) with curved ophthalmic scissors (cell suspension-2). They were transferred into a conical centrifuge tube with the aid of the modified Krebs-Ringer-Phosphate (m-K.R.P.) solution described below. After centrifugation the packed cells were washed twice with m-K.R.P. solution and diluted to the appropriate volume with the same solution. The aliquot of this suspension (usually 1 ml.) were added to each main compartment of the Warburg vessel.

To obtain cell free homogenate the following method was employed, in which the tissue was ground with a mortar and pestle after the addition of small amounts of powdered glass and homogenization medium described below. The homogenate was centrifuged at 1500 r.p.m. for five minutes, then the supernatant was used for the experiment.

*Reaction Mixture*—Two kinds of modified Krebs-Ringer-Phosphate solutions were used as suspending media of the tissue slices. The one (m-K.R.P.-1) was prepared by mixing stock solutions of 110 ml. of 0.9 per cent NaCl, 4 ml. of 1.15 per cent KCl, 1 ml. of 3.82 per cent MgSO\textsubscript{4} \textsubscript{7} aq. and 6 ml. of 0.1 M sodium phosphate buffer (pH 7.8). The other (m-K.R.P.-2) was prepared by mixing stock solutions of 100 ml. 0.9 per cent NaCl, 1 ml of 3.82 per cent MgSO\textsubscript{4} \textsubscript{7} aq., 4 ml. of 1.15 per cent KCl, 6 ml. of 0.1 M sodium phosphate buffer (pH 7.8) and 3 ml. of 1.22 per cent CaCl\textsubscript{2}, which was added immediately before the experiments.

*Composition of Homogenization Medium*—The usual constituents were as follows: KCl 0.120 M, potassium phosphate buffer (pH 7.4) 0.04 M, and MgCl\textsubscript{2} 0.004 M.

*C\textsuperscript{14}-Glycine*—Three kinds of 1-C\textsuperscript{14}-glycine were used in the experiment, their speci-
fic activities being 8.5, 19.3 and 38.5 μC. per mg., respectively.

**Incubation Procedure**—The lymphatic cell suspensions were incubated with C\(^{14}\)-glycine in the main compartments of the Warburg vessels. Each of their center cups contained 0.2 ml. of 10 per cent KOH and a folded filter paper. The gas phase was air or oxygen. The incubation was carried out by shaking at 37°, and the stopcocks were closed after 10 minutes equilibration period. For the short time experiment C\(^{14}\)-glycine was added in the reaction mixture from the side arm of the vessel after the equilibration period. At the end of the incubation, the flask content was poured into an ice cold tubes of Potter and Elvehjem's glass homogenizer (13), washed twice with 1 ml. of 0.9 per cent NaCl solution and homogenized in the cold room, transferred into a centrifuge tube, lyophilized twice, and then the separation of antibody and the fractionation of other tissue proteins were carried out as follows.

**Separation of Antibody and Fractionation of Tissue Proteins**—For the separation of antibody, Heidelberger's quantitative precipitin method (6–8) was employed and the quantity of precipitated nitrogen was determined by Folin's tyrosine method (14). The fractionation of tissue protein was carried out employing the method of Griffin (15). The detailed procedure was as follow: after the extraction of soluble protein and ribonucleoprotein by lyophilizing the cells, the extract was centrifuged for 15 minutes at 20,000 r.p.m. in the No.40 Rotor of the Spinco Ultracentrifuge. The precipitate which contained desoxyribonucleoprotein was fractionated as described below.

The supernatant was taken into a centrifuge tube, adjusted to pH 4.8–5.0 by adding 0.2 N acetic acid and centrifuged. The precipitate was ribonucleoprotein fraction and washed once with 0.4 M NaCl. An appropriate amount of egg albumin, which was determined previously by Heidelberger's method, was added to the supernatant, incubated for 15 minutes at 37°, then placed in the refrigerator overnight. After the precipitation was complete, the centrifugation was carried out at 3000 r.p.m. in the cold room. The specific precipitate (antigen-antibody complex) was washed twice with ice cold 0.9 per cent NaCl solution. The residual supernatant was designated as soluble protein fraction.

The precipitate, which contained desoxyribonucleoprotein, was ground completely with a glass rod and extracted with 2 ml. of 1 M NaCl with stirring, and centrifuged for 15 minutes at 20,000 r.p.m. To the supernatant, 1.5 volume of cold distilled water was added and centrifuged at 20,000 r.p.m. for 10 minutes. The precipitate thus prepared was desoxyribonucleoprotein fraction, which was obtained as typical gel form.

Fractionation of cellular components of the lymphatic tissue was carried out by the Schneider centrifugal fractionation procedure using an isotonic sucrose solution (26). From nuclear fraction, desoxyribonucleoprotein was fractionated by the procedure as described above.

**Washing, Plating and Counting the Protein**—Antibody (antigen-antibody complex) and each protein fraction were precipitated with trichloroacetic acid (TCA) (final concentration, 10 per cent), washed three times successively with 5 ml.-portion of 5 per cent TCA with centrifugation. Thereafter each precipitate was washed with
cold 95 per cent ethanol, twice with hot ethanol-ether, then dispersed in 3 ml. of 5 per cent TCA and heated for 15 minutes at 90° to remove nucleic acid. The residual precipitate was washed twice with 5 per cent TCA and then ethanol. The residual pellets of protein were homogenized in a petroleum ether-ether-acetone solution (6:3:1). This suspension was poured onto stainless steel disc and evaporated to dryness under the infrared rays. After the dried protein was equilibrated in air at least for 1 hour, the weight of the protein on the disc was determined and its radioactivity was estimated either with a thin mica end-window Geiger-Müller Counter or with a Lauritsen Electroscope. All samples were counted long enough to reduce the counting error. The specific activity of the protein\(^1\) was corrected for self-adsorption from an empirically determined curve.

The incorporation data were expressed as \(\mu\)M of glycine per g. protein or \(\mu\)g. C\(^{14}\) per g. protein, which was calculated according to Farber, Kit and Greenberg (16).

**Analytical Method**—Lactic acid was determined by the method of Barker and Summerson (17).

**RESULTS**

**Experiments with Cell Suspension**—As a preliminary experiment, the cell suspensions-l were incubated with C\(^{14}\)-glycine in the m-K.R.P.-l solution containing glucose in a gas phase of air at 37°. The time course of the incorporation of glycine into antibody and soluble protein was presented in Fig. 1. As shown in this figure, the incorporation proceeded for 2 hours and the rate of uptake of glycine into antibody was greater than that into the soluble-protein, but the differences were not so prominent. Next, under the same experimental conditions, the rate of incorporation of glycine into antibody and the proteins of cellular components, fractionated by Schneider’s procedure (26), were compared with each other at different time intervals up to 2 hours. As shown in Fig. 2, the glycine uptake into antibody was the most rapid, followed by microsome and soluble proteins, and that into nucleus (desoxyribonucleoprotein) was the slowest.

The effect of the addition of various metabolic inhibitors on the glycine uptake was presented in Table I. It was observed that azide (5 \(\times\) 10\(^{-3}\) \(M\)), arsenate (10\(^{-3}\) \(M\)), monooiodoacetate (2 \(\times\) 10\(^{-3}\) \(M\)) and 2,4-dinitrophenol (5 \(\times\) 10\(^{-4}\) \(M\)) inhibited markedly the incorporation of glycine into antibody as well as that into each cellular protein.

\(^1\) The specific activity of antibody may be about 10 per cent higher than the value presented in this paper, since in the antigen-antibody precipitate the ratio of the two proteins was about 1:10).
Fig. 1. Rate of the incorporation of glycine into antibody and soluble protein by cell suspensions of the immunized lymph nodes.

○, represents antibody; ×, soluble protein.

Incubation mixture; 1.6 ml of lymphatic cell suspensions-1 (contained 300 mg tissue), 0.2 ml of 0.25 M glucose, 0.1 ml of C\textsuperscript{14}-glycine in 0.9 per cent NaCl. The concentration of glycine (specific activity, 38.5 µC./mg.) was 0.66 mM.

Incubation at 37°, in air.

But because under the experimental conditions described above the oxygen uptake by the cellular suspensions was rather low, examinations to arrive at suitable conditions of the cellular respiration were carried out. The best conditions which could be attained, were to use cell suspensions-2 and oxygen as gas phase. In this case, the m-K.R.P.-2 was used as a suspension medium. The results was presented in Fig. 3. The oxygen and amino acid uptake proceeded almost constantly for 2 hours. Especially the incorporation of glycine into antibody increased linearly after a short lag period, which was not observed in other protein fractions. It was remarkable that the specific activity of antibody was much higher than those of the other protein fractions, in which that of the soluble protein showed the next higher value and that
Fig. 2. Rate of the incorporation of glycine into antibody and the proteins of the various cellular fractions by cell suspensions of the immunized lymph nodes.

●, represents antibody ○, microsome, ×, soluble protein, △, mitochondria, ○, nucleus. The experimental conditions were the same as those in Fig. 1, except that the concentration of glycine (specific activity, 8.5 μC./mg.) was 2.7 M.

Incubation at 37°, in air.

of the protein moiety of the desoxyribonucleoprotein was the lowest. Thus it was indicated that the oxygen uptake by the cell suspension was related closely to the glycine uptake into antibody. The rate of in-
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TABLE I(1)

**Effects of Various Metabolic Inhibitors on the Incorporation by Cell Suspensions of the Immunized Lymph Nodes**
(Incorporation expressed as per cent of that without inhibitor)

<table>
<thead>
<tr>
<th>Experimental No.</th>
<th>Protein fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sol-P</td>
</tr>
<tr>
<td>Addition</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100 (70§)</td>
</tr>
<tr>
<td>Arsenate (10⁻³ M)</td>
<td>11</td>
</tr>
<tr>
<td>Azide (5×10⁻³ M)</td>
<td>6</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (5×10⁻⁴ M)</td>
<td>18</td>
</tr>
<tr>
<td>Monoiodoacetate (2×10⁻³ M)</td>
<td>17</td>
</tr>
</tbody>
</table>

The experimental conditions were the same as those in Fig. 2.
§ The figure in parenthesis represents the value of the incorporation expressed μg. C¹⁴ per g. protein per 2 hours.
† represents the negligible value.

TABLE I(2)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Protein fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody</td>
</tr>
<tr>
<td>None</td>
<td>100 (90§)</td>
</tr>
<tr>
<td>Arsenate (10⁻³ M)</td>
<td>12</td>
</tr>
<tr>
<td>Azide (5×10⁻³ M)</td>
<td>12</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (5×10⁻⁴ M)</td>
<td>24</td>
</tr>
<tr>
<td>Monoiodoacetate (2×10⁻³ M)</td>
<td>14</td>
</tr>
</tbody>
</table>

The experimental conditions were the same as those in Fig. 2.
§ The figure in parenthesis represents the value of the incorporation expressed μg. C¹⁴ per g. protein per 2 hours.
† represents the negligible value.

* Sol-P, soluble protein RN-P, protein moiety of ribonucleoprotein,
  DN-P, protein moiety of desoxyribonucleoprotein.

The effect of anaerobiosis was then examined, which was represented...
in Table IV. From this Table it can be seen anaerobiosis inhibits almost completely the glycine uptake into antibody as well as those into other protein fractions. The addition of glucose or/and ATP, phosphocreatine and hexosediphosphate in the anaerobic condition increased the lactic acid formation by cell suspensions, while restored only slightly the glycine uptake.

Experiments with Tissue Homogenate—When cell free homogenate was used, the C\textsuperscript{14}-glycine uptake into antibody as well as each protein fraction was greatly reduced, as compared with the value obtained by using intact cell suspensions.

But, as shown in Fig. 4, even in this case the rate of the incorporation of C\textsuperscript{14}-glycine into antibody was observed to be much higher than those of the other protein fractions. The azide (10\textsuperscript{-3} M), monooiodoacetate (10\textsuperscript{-3} M), 2,4-dinitrophenol (10\textsuperscript{-3} M) or malonate (1.2\times10\textsuperscript{-3} M) inhibited the glycine uptake as shown in Table V.

![Fig. 3 (1)](image-url)
Fig. 3. Rate of oxygen uptake and the incorporation of glycine into antibody and other tissue proteins by cell suspensions of immunized lymph nodes.

...□□□□... represents the oxygen uptake, --- antibody; −−x−−, soluble protein; −−Δ−−, protein moiety of ribonucleoprotein; −−O−−, that of deoxyribonucleoprotein. The incubation mixture: 1.2 ml. of the cell suspension (containing 500 mg. tissue), 0.4 ml. of 0.9 per cent NaCl and 0.1 ml. of C¹⁴-glycine (specific activity, 19.3 μC./mg.). The concentration of glycine was 0.68 mM. Incubation at 37°, in oxygen.
TABLE II

Rate of the Incorporation of $^{14}$C-Glycine into Antibody and Other Cellular Proteins

<table>
<thead>
<tr>
<th>Experimental No.</th>
<th>Glycine in $\mu$M per g. protein per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody</td>
</tr>
<tr>
<td>1</td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>15.0</td>
</tr>
<tr>
<td>4</td>
<td>17.8</td>
</tr>
<tr>
<td>5</td>
<td>8.1</td>
</tr>
<tr>
<td>Mean</td>
<td>11.9</td>
</tr>
</tbody>
</table>

The experimental conditions were the same as in Fig. 3.
* The same as in Table I.

TABLE III

Effect of Glycine Concentration on the Incorporation

<table>
<thead>
<tr>
<th>Glycine concentration (mM)</th>
<th>Glycine in $\mu$M per g. protein per 1.5 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0†</td>
</tr>
<tr>
<td>Antibody</td>
<td>32.0</td>
</tr>
<tr>
<td>Sol-P*</td>
<td>7.2</td>
</tr>
<tr>
<td>RN-P*</td>
<td>6.2</td>
</tr>
<tr>
<td>DN-P*</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The experimental conditions were the same as in Fig. 3, except that various concentrations of glycine were used.
* The same as in Table I.

DISCUSSION

In recent years it has become possible to approach the problem of the synthesis of tissue protein by the use of suitable isotopic amino acids and since then a great number of reports concerning this problem have been published. But only few investigations were made concerning the incorporation of labeled amino acids into the protein of lymphoid tissue. Using the cell suspension of the lymphosarcoma and spleen,
The final concentration of glucose, ATP, creatine phosphate and hexose-diphosphate were 47 mM, 1 mM, 11.7 mM and 1.12 mM, respectively.

Other experimental conditions were the same as in Fig. 3.

The figures represents the incorporation expressed as $\mu$M per g. protein per 90 minutes.

* The same as in Table I.

Farber, Kit and Greenberg (16, 18) studied the incorporation of C$^{14}$-amino acid under various conditions, and Kit and Barron (31) demonstrated the effects of corticoid upon the incorporation of C$^{14}$-glycine into the protein of lymphoid tissues.

From the data represented in Fig. 3 and Table II, it can be seen...
TABLE V

The Effect of Metabolic Inhibitors on the Rate of Incorporation by Cell Free Homogenate

<table>
<thead>
<tr>
<th>Addition</th>
<th>Antibody</th>
<th>Sol-P</th>
<th>RN-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Arsenate ((10^{-3} M))</td>
<td>24</td>
<td>51</td>
<td>36</td>
</tr>
<tr>
<td>Monoiodoacetate ((10^{-3} M))</td>
<td>51</td>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2,4-Dinitrophenol ((10^{-3} M))</td>
<td>58</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Malonate ((1.2 \times 10^{-3} M))</td>
<td>33</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

Incorporation expressed as per cent of that without inhibitor. The experimental conditions were the same as those in Fig. 4.

that the rate of incorporation of C\(^{14}\)-glycine into protein fraction of immunized tissue, especially that into antibody is very high, as compared with the figures of the rate of the incorporation of C\(^{14}\)-amino acids into the lymphosarcoma and spleen \((16, 18)\) or into other tissue proteins reported by other investigators using intact tissue slices or suspensions \((21-23, 28, 29)\)). This result may clearly demonstrate that the antibody is rapidly synthesized in the tissue of popliteal lymph nodes of the locally immunized animal, and agrees with the immunological investigations of Ogata and Mochizuki \((5)\).

The fact that the rate of C\(^{14}\)-glycine uptake into antibody is much higher than that into other protein fractions, suggests that the other tissue proteins are not direct precursors of antibody, though the further fractionation of the soluble protein was not carried out in this experiment.

The data of the comparison of the rate of the incorporation into protein of different cellular fractions show clearly that the microsome protein incorporates amino acids at a much higher rate than any other cellular fractions. This result is in agreement with that of Hultin’s \((19)\) in vivo experiments and Siekevitz’s \((20)\) in vitro experiments using liver homogenates, though in the latter case the incorporation into the supernatant exhibits the lowest value. Very recently, after the finish-

2) For the incorporation rate in various tissue proteins readers are refered to Tarver \((1954)\) \((27)\).
C\textsuperscript{14}-GLYCINE INCORPORATION INTO PROTEIN

Fig. 4. Incorporation of glycine into antibody and other tissue proteins by the cell free homogenate versus time.

The homogenization medium contained MgCl\textsubscript{2} 0.004 M, K phosphate buffer (pH 7.4) 0.04 M, KCl 0.12 M. Nicotinamide (0.01 M) was added further in the case of Fig. 4 (1).

Each manometric flask contained 1.0 ml. of cell free homogenate, 0.2 ml. of 0.9 per cent NaCl, 0.1 ml. of C\textsuperscript{14}-glycine (specific activity, 38.5 μC/mg.). The concentration of C\textsuperscript{14}-glycine was 1.27 μM. Incubation at 37°, in oxygen.
ment of this work, Ziegler and Melchior (25) obtained similar results with ours, by cellular fractionation of liver slices after the incubation with $^{35}S$-methionine.

The incorporation of $^{14}C$-glycine into antibody and other protein fractions was shown to be greatly inhibited by anaerobiosis. Anaerobiosis has been reported to produce complete or variable inhibition of $^{14}C$-amino acid uptake into several tissue proteins (18, 21). But recently Rabinowitz et al. (24) reported that the incorporation of $^{14}C$-amino acids into the protein of Ehrlich ascites carcinomatosa was supported anaerobically when sufficient glucose presented and active glycolysis occurred. In the present experiment the addition of glucose and ATP, and other P-compounds stimulated remarkably the production of lactic acid by cell suspensions, while restored the $^{14}C$-glycine incorporation only slightly as compared with the results of Rabinowitz et al. This discrepancy may be due to the difference between cell types studied. Further, using cell suspension or cell free homogenate system the detrimental effects of the inhibitors of respiration or phosphorylation were demonstrated. In addition it was indicated that the oxygen uptake stood in very intimate relation to the most suitable incorporation of glycine into antibody. Thus it may be concluded that in the tissue of the popliteal lymph nodes the aerobic energy yielding reaction may be absolutely necessary for the amino acid uptake into tissue protein and especially into antibody.

**SUMMARY**

Following the injection of crystalline ovalbumin into the foot-pads of rabbits, the popliteal lymph nodes were removed and the incorporation of $^{14}C$-glycine into antibody and other cellular protein fractions was studied, using cell suspensions or cell free homogenates.

The following results were obtained.

1. Under suitable aerobic conditions the cell suspensions of the lymph nodes from immunized rabbits incorporated very rapidly $^{14}C$-glycine into antibody. The rates of incorporation of glycine into antibody, soluble protein, the protein moiety of ribonucleoprotein and that of deoxyribonucleoprotein, expressed as $\mu$M glycine per g. protein per hour, were 11.9, 3.5, 2.1, and 0.9, respectively, when glycine concentration was 0.68 mM.

2. The rate of the $^{14}C$-glycine uptake into antibody was higher than the proteins of the cellular components, fractionated by the pro-
C14-Glycine Incorporation Into Protein

procedure of Schneider, of which that of the microsome fraction showed the highest value, that of the soluble fraction the next, and protein moiety of desoxyribonucleoprotein the lowest value.

3. Arsenate, azide, dinitrophenol, monooiodoacetate and anaerobiosis inhibited the incorporation of glycine into antibody as well as into other cellular proteins. Under anaerobic conditions, the addition of glucose, ATP, and other P-compounds restored only slightly the C14-glycine uptake.

4. When the cell free homogenate was used, the rate of the incorporation was greatly depressed. But even in this condition, the isotope uptake into antibody was more rapid than that into other protein fractions. The arsenate, monooiodoacetate, 2,4-dinitrophenol, or malonate inhibited the amino acid uptake in the cell free homogenate system.

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