The Effect of Methotrexate on Enzymes Induced following Partial Hepatectomy

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SUMMARY

The synthesis of thymidine kinase, thymidylate kinase, thymidylate synthetase, DNA polymerase, and deoxythymydylate deaminase was initiated at about 12 hr following partial hepatectomy of rats. The intraperitoneal injection of Methotrexate inhibited the synthesis of all of the above enzymes with the exception of thymidylate synthetase, which was markedly stimulated. The synthetase activity was fourfold greater than that of the uninjected controls at 48 hr following partial hepatectomy, and at 72 hr it was 10- to 15-fold higher. At the latter time interval, the synthetase was still increasing in activity in the treated rats, while the enzyme activity in the control rat livers was decreasing. Puromycin had no effect on the synthetase elevation, while actinomycin inhibited the increase partially. However, cycloheximide, at levels that did not inhibit the normal rise in deoxythymydylate deaminase activity following partial hepatectomy, greatly inhibited the normal and Methotrexate-stimulated increase in synthetase activity. To emphasize the possibility that the Methotrexate stimulation may be an indirect effect, folate was found to cause a similar, though less dramatic, increase in thymidylate synthetase activity.

INTRODUCTION

In a recent report (20), we demonstrated that dUMP was converted to dUTP in regenerating liver extracts at a greater rate than dTMP was converted to dTTP. This result was unexpected, since all previous reports by other investigators had indicated that dUMP could not be phosphorylated in animal tissues, thus accounting for the apparent absence of dUMP in DNA. Although Escherichia coli contain a dUMP kinase, dUMP appears to be excluded from coli DNA by the presence of a deoxyuridine triphosphatase (13).

It was reasoned that if dTTP synthesis could be blocked in regenerating liver, dUMP might be incorporated in place of dTMP, in spite of the presence of some deoxyuridine triphosphatase activity in the liver extracts (20). To effect such a block, Methotrexate was employed but with little apparent effect on the incorporation of dUMP into DNA. Since it was possible that this result was related to an impairment in the synthesis of those enzymes normally induced after partial hepatectomy, the effect of Methotrexate on the inductive process was investigated. Included in this study were thymidylate synthetase, thymidine kinase, thymidylate kinase, deoxythymydylate deaminase, and DNA polymerase. An unexpected finding was that Methotrexate inhibited the induction of all of the above enzymes with the exception of thymidylate synthetase, which was markedly stimulated. In retrospect, this finding was not too surprising as thymidylate synthetase was shown previously by us (24) not to respond as other enzymes to the protein synthesis inhibitors, actinomycin and puromycin. These studies demonstrated that, while deoxythymydylate deaminase and thymidine kinase were reduced to their levels prior to partial hepatectomy by the inhibitors, the synthetase was, if anything, enhanced in activity. Because of these anamolous effects, an extensive study was undertaken, related to the temporal order of induction of the DNA-associated enzymes and the effect of various protein synthesis inhibitors on this process, with particular emphasis on Methotrexate.

MATERIALS AND METHODS

Hepatectomy. Albino rats (germ-free, weighing 100—125 gm) were partially hepatectomized according to the procedure of Higgins and Anderson (17) and were sacrificed between 9 and 10 A.M. at indicated time intervals. A cell-free supernatant fraction of the liver was prepared from a 30% homogenate in isotonic KCl by centrifuging at 144,000 X g for 1 hr in a Spinco model L ultracentrifuge.

Enzyme Assays. A unit of enzyme was defined as that amount capable of synthesizing 1 nmole of product in 30 min at 37°C and specific activity as units per mg of protein. The protein concentration was determined by the method of Lowry et al. (22).

Thymidine Kinase. The reaction mixture, incubated at 37°C for 15 min, contained thymidine-2,14C (Schwarz BioResearch, Inc.), 1.0 μmole (277,000 dpm/μmole); ATP, 5.0 μmoles;
MgCl₂, 6 μmoles; Tris buffer, pH 8.0, 30 μmoles; NaF, 150 μmoles; and 0.1 ml of the 144,000 X g liver supernatant fraction in a total volume of 0.35 ml. The reaction was stopped by heating the mixture in a boiling water bath for 2 min, after which it was centrifuged. A 20-μl aliquot from the deproteinized reaction mixture was spotted on a DEAE paper disc (DE 81, Whatman), 1.5 cm in diameter, and treated as described by Breitman (7).

**Thymidylate Kinase.** The reaction mixture, incubated at 37°C for 30 min, contained dTMP-2-14C (Schwarz BioResearch, Inc.), 0.25 μmoles (580,000 dpm/μmole); ATP, 3.0 μmoles; MgCl₂, 3.0 μmoles; dithiothreitol, 5.0 μmoles; potassium phosphate buffer, pH 7.5, 25 μmoles; 0.2 ml of the 144,000 X g liver supernatant fraction in a total volume of 0.41 ml. The reaction was stopped by spotting a 10-μl aliquot on a DEAE paper strip, which was chromatographed according to the procedure described previously (20).

**Thymidylate Synthetase.** The reaction mixture, incubated at 37°C for 30 min, contained dUMP, 1.5 μmoles; 14CH₂0 (New England Nuclear Corporation), 1.25 μmoles (3 X 10⁶ cmp/μmole); potassium phosphate buffer, pH 6.5, 50 μmoles; 0.1 ml of a mixture consisting of 0.04 ml of 0.02 M 2,3-dimercaptopropanol, 0.03 ml of 0.015 M H₄-folate, and 0.03 ml of water; 0.1 ml of the 144,000 X g liver supernatant fraction in a total volume of 0.37 ml. The reaction was stopped by heating the mixture in a boiling water bath for 2 min. The dTMP formed was estimated from the radioactivity that passed through a column of Dowex 50-H⁺ over Dowex 1-formate, after treatment of the reaction mixture with snake venom as described previously (21). The 14C-labeled thymidine was isolated from selected reaction mixtures, as described in Chart 3, by chromatography in sec-butanol (9), and the quantity of thymidine formed was determined by isotope dilution (21). The radioactivity in this region in the absence of dUMP could exceed 30% of that formed in the presence of dUMP, depending on the extent of dTMP formation.

**dCMP Deaminase.** The reaction mixture, incubated at 37°C for 30 min, contained 0.1 ml of a solution of 0.01 M dCMP-2-14C (190,000 dpm/μmole), in 0.1 M Tris, pH 8.0; NaF, 15 μmoles; 0.2 ml of the 144,000 X g liver supernatant fraction in a total volume of 0.5 ml. The reaction was stopped by heating the mixture in a boiling water bath for 2 min. The dUMP formed was converted to deoxyuridine by treatment of the heated solution with snake venom (21), and the deoxyuridine was separated from deoxycytidine on a Dowex 50-H⁺ column (23).

**DNA Polymerase.** DNA polymerase was assayed as described by Bollum (5) with 0.2 ml of the 144,000 X g liver supernatant fraction.

**Protein Synthesis Inhibitors and Dosage.** Puromycin and DL-p-fluorophenylalanine were purchased from Nutritional Biochemicals Corporation. Methotrexate, cycloheximide, and actinomycin D were generous gifts from Lederle Laboratories, the Upjohn Company, and Merck, Sharp and Dohme Research Laboratories, Inc. respectively. All compounds were injected i.p. Puromycin (10 mg), Methotrexate (0.5 mg), 5-formylltetrahydrofolate (4 mg), and folate (10 mg) were injected at the time of the operation and repeated 24 hr later. Actinomycin (45 μg) was injected at the time of operation. Cycloheximide (50 μg/100 gm body weight) was injected at 12, 24, and 36 hr after partial hepatectomy.

**RESULTS**

**Induction of DNA-related Enzymes in Partially Hepatectomized Rats.** An integral part of this study was concerned with determining the time sequence of initiation of thymidine kinase, thymidylate kinase, deoxycytidylate deaminase, thymidylate synthetase, and DNA polymerase. Although this type of study has been reported previously by ourselves (23) and others (6, 14, 16, 30, 31), an accurate assessment of the earliest times of detection and of the order of appearance of these enzymes has to our knowledge never been reported. The reason for this relates to the number of animals that must be employed and the low levels of enzyme activity present. Even with 10 animals at each time period, wide ranges in the standard deviations were obtained, as shown in Chart 1. The reason for the high standard deviation in the case of thymidylate synthetase is related to the fact that some of the enzyme values were zero or close to it. Thus, if those animals with negligible synthetase activity were excluded from the calculations, the following enzyme specific activities would be obtained: basal level or zero hr, 0.20 ± 0.03 (7); 16 hr, 0.21 ± 0.08 (6); 18 hr, 0.76 ± 0.05 (5); 20 hr, 0.60 ± 0.04 (9). Exclusion of the minimal enzyme values does not, however, present a true picture of the variability usually encountered in such experiments. Therefore, all the values were included in estimating the mean synthetase specific activity. It is not surprising that the greatest variation was encountered during the earliest part of the curve, since it is here that the synthetase assay is least reliable (as determined by difference in substrate blank and reaction sample). It is seen that in some instances the standard deviations are quite low, but whether this is fortuitous is not known. Despite the variation, the trend is definitely toward an elevation in activity from the 12th hour on, following partial hepatectomy. Most of the earlier studies emphasized that the increase occurred mainly between 18 and 24 hr, but usually the number of animals and time periods at which the enzymes were measured were less. Thus, it was not particularly surprising to find measurable increases after 12 hr, increases that occurred subsequent to the first elevated wave of messenger RNA synthesis (4–6 hr) in regenerating liver (12) and that appeared to coincide with the increased template efficiency of chromatin (26). While it was not possible to determine if there was a temporal order of appearance of these enzymes, because of the variability in activities, the rates of increase and the time at which maximal activities were achieved, differed among them. What bearing the latter results have on determining which of the enzymes are the most rate-limiting is not known, but if specific activity is any criterion, thymidylate synthetase and DNA polymerase should be considered in this light. Even these cases are circumstantial, as all of the enzymes are measured under optimal conditions in vitro, a condition which may not apply in vivo.

A basal value is indicated on Chart 1 for thymidylate synthetase, an enzyme not usually detectable in livers from older rats. This value is believed to be valid, as F-dUMP eliminated the activity.
Effect of Methotrexate on Enzyme Induction. Since, as indicated in the Introduction, Methotrexate may have prevented the incorporation of dUMP into DNA by impairing enzyme induction, its effect on the enzymes described in Chart 1 was investigated. As shown in Table 1, administration of Methotrexate reduced the level of all the enzymes, with the exception of thymidylate synthetase, by at least 50 percent. In the latter case, however, a fourfold elevation in activity was noted. A small reversal of the inhibition, without an apparent effect on the synthetase increase, was obtained with 5-formyltetrahydrofolate. Measurement of the synthetase at times subsequent to 48 hr revealed that, in contrast to un-
treated animals, the synthetase kept increasing in the Methotrexate-injected rats (Chart 2). Thus, at 72 hr following partial hepatectomy, the synthetase activity was about 10- to 15-fold higher than the level in the untreated controls. Following this period, the mortality rate among the treated animals was quite high and it would be difficult to determine if this was, in part, related to the apparent decrease in enzyme activity (broken line, Chart 2). Perhaps if less toxic doses of Methotrexate were given, the enzyme rise might be sustained past the 96-hr period. To rule out the possibility of artifacts contributing to the apparent increase in thymidylate synthetase activity, the products of the reaction following snake venom treatment and passage through a mixed resin ion-exchange column (see Methods), were chromatographed on Whatman 3MM paper, with water-saturated sec-butanol (9). The radioactivity patterns obtained following passage through a strip-scanner (Chart 3) revealed the presence of unknown components I and II in all of the Methotrexate samples. That Peak III, which coincides with marker thymidine, represents this compound is suggested by the marked impairment of its formation by F-dUMP. It was of interest to note that even in reaction mixtures not containing substrate, small amounts of

Chart 3. Reaction products from the thymidylate synthetase assay. The assay was conducted as described in Materials and Methods, except for the following additions or deletions: Curve A, the reaction mixture contained no dUMP, 1.85 microles of F-dUMP, and liver extract from a Methotrexate-injected rat; Curve B, reaction mixture minus dUMP and liver extract from a Methotrexate-injected rat; Curve C, same as B but with dUMP; Curve D, same as C, but with liver extract from an untreated rat. The enzyme was assayed as described in Materials and Methods, but after passage through the mixed ion-exchange columns, 0.50 microle of carrier thymidine was added to each of the column eluates (A-D). They were then concentrated to about 1 ml and lyophilized in conical centrifuge tubes, after which they were applied to Whatman 3MM paper and chromatographed ascending in water-saturated sec-butanol. The developed chromatograms were cut into strips which were passed through a Nuclear Chicago Actigraph III strip counter. See Footnote 3 for abbreviations.
dTMP were formed (Chart 3B), as evidenced by the complete elimination of this region by F-dUMP (Chart 3A). It is apparent from Chart 3C and D that much higher levels of dTMP are produced by liver extracts from Methotrexate-treated rats than by those from normal rats.

Effect of Folate on Thymidylate Synthetase Induction. Since Methotrexate should increase intracellular folate levels as a result of its impairment of folate reduction, the latter compound may be more directly involved in the synthetase stimulation than is Methotrexate. The activity of the synthetase following folate injection was therefore examined. As shown in Table 2, an increase in synthetase activity similar to that promoted by Methotrexate was induced by the folate but with little or no effect on the appearance of the other enzymes.

Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Folate injected</th>
<th>Uninjected</th>
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<tbody>
<tr>
<td>dCMP deaminase</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>dTMP kinase</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>dTMP synthetase</td>
<td>3.5</td>
<td></td>
</tr>
</tbody>
</table>

Effect of folate on the enzymes induced following partial hepatectomy. Five rat livers were used to determine each value. The dosage of folate is presented in Materials and Methods. See Footnote 3 for abbreviations.

Inhibition of Protein Synthesis and Its Effect on the Methotrexate-stimulated Rise in Thymidylate Synthetase. In a previous study (23), we demonstrated that the rise in thymidylate synthetase induced by partial hepatectomy was practically unaffected by actinomycin and puromycin, whereas the increase in thymidine kinase and deoxycytidylate deaminase was markedly impaired. To determine if the elevation in thymidylate synthetase was in fact due to an enhanced rate of protein synthesis above that normally obtained (Chart 2), the effects of these potent inhibitors on the thymidylate synthetase increase were measured. The results, presented in Table 3, reveal the Methotrexate stimulation of the synthetase to be as refractory to puromycin as in the normal increase (24). However, the fourfold increase usually obtained was decreased about twofold by actinomycin. In contrast to the previously described sensitivity of the synthetase to p-fluorophenylalanine, little or no impairment in the increase was elicited by this amino acid analog. The most striking effects, however, were obtained with cycloheximide, which, as seen in Table 4, completely prevented the Methotrexate-stimulated rise in the synthetase. Of interest was the finding that, although the increase in thymidine kinase and thymidylate kinase were also impaired at 24 hr, their subsequent increase at 48 hr, as well as that of the synthetase, was not prevented. It thus appears that the cycloheximide delays the onset of protein synthesis in this case, and on detoxification or elimination of the drug, the inhibition is reversed. Unlike the above enzymes, the increase in deoxycytidylate deaminase was unaffected by cycloheximide at 24 hr, but the normally obtained two-fold increase in enzyme activity at 48 hr was prevented.

Table 3

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Units/mg protein at 48 hr</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>1.1 ± 0.05 (10)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>4.2 ± 1.3 (20)</td>
</tr>
<tr>
<td>Methotrexate plus puromycin</td>
<td>4.6 ± 0.3 (2)</td>
</tr>
<tr>
<td>Methotrexate plus actinomycin</td>
<td>2.4 ± 0.89 (9)</td>
</tr>
<tr>
<td>Methotrexate plus p-fluorophenylalanine</td>
<td>4.4 ± 0.6 (5)</td>
</tr>
<tr>
<td>Methotrexate plus cycloheximide</td>
<td>1.02 ± 0.9 (4)</td>
</tr>
</tbody>
</table>

The effect of protein synthesis inhibitors on the Methotrexate stimulation of deoxothymidine 5'-monophosphate synthetase.

The dosage is presented in Materials and Methods.

Table 4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Cycloheximide*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unis/mg protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>dTMP synthetase</td>
<td>1.1 ± 0.6 (10)</td>
<td>1.1 ± 0.1 (10)</td>
</tr>
<tr>
<td>dTMP kinase</td>
<td>3.4 ± 0.9 (6)</td>
<td>3.3 ± 1.1 (6)</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>15.2 ± 6.0 (10)</td>
<td>9.6 ± 4.3 (10)</td>
</tr>
<tr>
<td>dCMP deaminase</td>
<td>20.9 ± 3.7 (10)</td>
<td>40.8 ± 5.2 (10)</td>
</tr>
</tbody>
</table>

Effect of cycloheximide on the enzymes induced following partial hepatectomy. Basal values are given in Table 1. The numbers in parentheses refer to the number of rat livers used to determine the enzyme activity, which is given as the mean ± S.D. See Footnote 3 for abbreviations.

The dosage is presented in Materials and Methods.

Differential Effect of Cycloheximide on Enzyme Induction. The differential response of the enzymes to cycloheximide suggested in Table 4 is demonstrated more dramatically in Chart 4 of the four enzymes measured, the synthetase and thymidine kinase appear to be the most sensitive, while the deaminase is unaffected at levels of cycloheximide that completely prevent the induction of the other enzymes. The results suggest that the locus of action of this inhibitor may not be the same for all the enzymes or that some protein synthesis sites are less accessible than others.
**Elevation of Thymidylate Synthetase by Methotrexate**

**DISCUSSION**

By analogy to the operon hypothesis (19), it may be considered that the rise in enzyme synthesis following partial hepatectomy results from either a derepression or an induction of closely linked genes. It is not possible to determine if these genes are part of a single polycistronic unit, but the almost simultaneous increase in the enzymes suggests this to be the case. Attempts to determine if a temporal sequence of transcription was detectable, as described in bacterial systems for the histidine and lactose operons (2, 4), were not successful, due to the wide range in responses obtained at the time message translation was initiated (approximately 12 hr). However, it does appear that the increase in enzyme activity can be extrapolated to a period earlier than previously reported (approximately 18 hr). These data would be consistent with the finding that the first wave of messenger RNA synthesis is completed at this period, following partial hepatectomy (12). While derepression of messenger RNA synthesis resulting from alterations in histone structure (1, 26) appears to be an attractive hypothesis to explain the onset of new enzyme synthesis, this area is still open for speculation and experimentation.

Any attempt to rationalize the elevation in thymidylate synthetase must be considered in a similar light but must be modified somewhat to encompass the unusual behavior of the synthetase to protein synthesis inhibitors. The following proposals can be considered responsible for the increase in synthetase activity:

(a) A decrease in the rate of degradation of messenger RNA coding for thymidylate synthetase or of the enzyme protein. The latter possibility is believed responsible for the elevated levels of dihydrofolate reductase found in Methotrexate-treated lymphoblast cells (18). It was proposed, in this case, that the enzyme containing bound Methotrexate was less susceptible to degradation. Although Methotrexate inhibits the synthetase, its affinity for the synthetase is much lower than that for the reductase, and it is doubtful whether such a mechanism is applicable in this case.

(b) An enhanced rate of enzyme synthesis due to induction by Methotrexate or a product accumulating as a result of the presence of this drug. Included in such a category could be folate, dihydrofolate, or even dUMP.

To emphasize the plausibility of this suggestion, the experiments presented in Table 2 provide evidence that folate alone can effect the elevation of thymidylate synthetase. Thus, the enhancement of synthetase activity by Methotrexate may be an indirect effect brought about by the accumulation of folate in the inhibited cells. Folate has been found to enhance DNA and protein synthesis in rat kidney (28), an effect impaired by actinomycin D and cycloheximide (27). But whether the increase in DNA synthesis in kidney is promoted by parameters similar to those effecting the increase in liver thymidylate synthetase is as yet undetermined. However, it should be emphasized that folate injected into unoperated rats does not elicit an increase in liver thymidylate synthetase to detectable levels, which would indicate a basic difference in the response of these two tissues. The assumption that folate may accumulate to a higher degree in Methotrexate-treated animals than in those untreated is not supported by the experiments of Brown et al. (8), who demonstrated that Methotrexate, when injected at 5–10 times the level used in our studies, did not inhibit liver dihydrofolate reductase. In contrast to their findings, we have found the reductase to be inhibited by about 50 percent, which is in essential agreement with the studies reported earlier by Ngu et al. (25).

(c) A derepression of enzyme synthesis as a consequence of diminished levels of S-adenosylmethionine, a side effect of the Methotrexate treatment. This effect could in turn result in a decrease in histone methylation, which has been observed to be associated with the enhancement of messenger RNA synthesis following partial hepatectomy (29).

As mentioned previously (21), the relative insensitivity of thymidylate synthetase to actinomycin and puromycin, as compared with cycloheximide, distinguishes this enzyme from the others (Tables 1, 3). It is possible that the synthetase cistron is located in a less accessible place than are the genes for the other enzymes, but it is of interest to note that conditions employed for inhibiting the translation of the lac operon messenger (2) impaired only the synthesis of those enzymes following β-galactosidase, the first enzyme in the operon. Extrapolation of these results to the case at hand is not possible, but the similarity cannot be dismissed as implausible.
To emphasize the complexities involved in providing a reasonable explanation and experimental approach, Eker (10) has recently shown Methotrexate to stimulate thymidine kinase in Chang liver cells. Similarly, an impairment in protein and RNA synthesis by deoxyadenosine was found to be associated with an increase in the kinase (11). These results are in contrast to the inhibition in thymidine kinase synthesis described here for Methotrexate and indicate that different cells may respond in an entirely unanticipated manner to this drug.

The commonly accepted mode of action of Methotrexate has been attributed to its potent inhibition of dihydrofolate reductase, which in turn results in the inhibition of thymidylate synthetase (15). However, in view of the rather involved reductase, which in turn results in the inhibition of thymidine kinase (11). These results are in contrast to the recent results of Methotrexate to stimulate thymidine kinase in Methotrexate and indicate that different cells may respond in the inhibition in thymidine kinase synthesis described here for a reasonable explanation and experimental approach, Eker (10) has shown that inhibition of protein synthesis (3) and on the impairment of RNA synthesis (15), the importance of such effects cannot be ignored in considering the extent of inhibition or resistance elicited by a cell in response to Methotrexate.

REFERENCES

Announcements

FELLOWSHIPS IN CANCER IMMUNOLOGY

The New York Cancer Research Institute, Inc., has established a number of fellowships for individuals holding an M.D. or Ph.D. degree and who wish to receive training and experience in cancer immunology. Applications will be considered three times a year and should be received by the 1st of February, June, or October.

For further information, write to Mrs. William B. Nauts, Executive Director, New York Cancer Research Institute, Inc., 1225 Park Avenue, New York, New York 10028.

Errata

The paper, entitled "The Effect of Methotrexate on Enzymes Induced Following Partial Hepatectomy," by Rosalind Labow, Gladys F. Maley, and Frank Maley, which appeared in the February 1969 issue of CANCER RESEARCH, was inadvertently omitted from the collected Table of Contents of Volume 29, which appeared in the December 1969 issue. The paper was, however, listed in the Table of Contents in the February 1969 issue.

In the article, entitled "Effects of Caffeine on L-Cells Exposed to Mitomycin C," by A. M. Rauth, B. Barton, and C. P. Y. Lee, which appeared in the November 1970 issue of CANCER RESEARCH, the material on pp. 2725 and 2726 should have followed the material on pp. 2727 and 2728 even though the page numbering is correct.
The Effect of Methotrexate on Enzymes Induced following Partial Hepatectomy

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