Metabolism of Ascites Tumor Cells

I. Rate of Glycolysis and Competitive Utilization of Fructose, Mannose, and Glucose

W. D. YUSHOK

(Biochemical Research Foundation, Newark, Del.)

The effects of carbohydrates and other chemical agents on the glycolysis of ascites tumors have been under extensive investigation in this laboratory (21). As part of the program, the three known glycolyzable sugars, D-glucose, D-mannose, and D-fructose, have been studied to help unravel some of the complex factors involved in the regulation of the rate of glycolysis in the intact cells. It was considered important to determine the influence of concentration of each hexose on the rate of anaerobic glycolysis. It was also considered relevant to study the glycolyzable sugars in combination to ascertain whether their effects on anaerobic hexose utilization and lactic acid production are competitive or additive. The metabolism of the sugars has been investigated under aerobic conditions also, so that the influence of the intricate interactions of the respiratory and glycolytic enzyme systems could be determined.

The rate of anaerobic glucolysis was reported to be dependent on the concentration of glucose below 0.011 M in slices of Flexner-Jobling carcinoma (20, p. 135) and of Jensen rat sarcoma (5). In Ehrlich ascites cells, however, the rate of anaerobic glucolysis was shown by Kun (10) to be the same at concentrations of 0.005 and 0.055 M, whereas the rate of anaerobic fructolysis at the lower concentration was 40 per cent of that at the higher level. Under aerobic conditions, the rate of lactic acid production by TA3 ascites tumor cells was found by Bloch-Frankenthal and Weinhouse (1) to be independent of glucose concentration between 0.0005 and 0.0025 M, whereas the rate of anaerobic fructolysis of this ascites tumor, however, has been shown to be dependent upon fructose concentrations below 0.015 M and to be at a maximum at 0.015 M and higher. Studies with the glycolyzable hexoses in combination have demonstrated the capacity of each hexose to inhibit the utilization of the other under both anaerobic and aerobic conditions and to control the glycolytic rate of the intact ascites cells.

MATERIALS AND METHODS

The Krebs-2 ascites carcinoma, which had been obtained from Dr. T. S. Hauschka, was propagated in female white Swiss mice by weekly intraperitoneal injection of the ascitic tumor fluid. For the metabolic experiments, ascitic fluid was usually withdrawn from the animals on the 7th day following inoculation, although occasionally it was withdrawn on the 6th or 8th day after injection. From 8 to 5 ml. of ascitic fluid were added to each of several centrifuge tubes held in ice water. Cold Krebs-Henseleit bicarbonate (KH) buffer, which had been previously equilibrated with 5 per cent carbon dioxide, was added to bring the total fluid volume in each tube to 14 or 15 ml. It was not necessary to add an anti-coagulating agent, since ascitic tumor fluid containing Krebs-2 cells displayed no tendency to coagulate, in contrast to ascitic fluid containing Ehrlich cells (10).

The tumor cells were separated from extracellular ascitic fluid and blood cells by a centrifugation procedure designed to keep to a minimum the time of cell preparation. The diluted cell suspension was spun for a 1-minute period which included the time of acceleration but not that of gradual deceleration. The maximum speed of centrifugation was 1800 r.p.m. (500 X g at midpoint of tube) in head 283 of the International centrifuge. After the supernatant fluid was removed by aspiration, the tumor cells were resuspended in KH buffer and centrifuged again under the same conditions as before. The cells were again resuspended in buffer and
centrifuged for a 3-minute period to assure gentle but firm packing of the cells. The pooled packed cells were diluted with KH buffer in the proportion of 1.5 ml. cells to a total volume of 10 ml. For the determination of packed cell volume, 2 ml. of the cell suspension were pipetted into a 3-ml. Bauer-Schenk tube in duplicate. The cells were spun down in a cooled centrifuge at 2000 X g for a 30-minute period. The dry weight in milligrams was calculated by multiplying the packed cell volume by 0.178 (25). For the manometric determinations, a 5-ml. aliquot portion of the cell suspension was added to 10 ml. of cold KH buffer. In the anaerobic experiments, exactly 1.0 ml. of this diluted cell suspension, equivalent to 30 ± 5 µlitters of packed cells, was pipetted into the main chamber of each standard-size Warburg vessel after the other solutions had been added. In the experiments with low concentrations of hexoses, Warburg microvessels of approximately 7 ml. volume were used. The volume of cells added to each microvessel was about one half that added to each larger vessel.

*d-Glucose, *d-mannose, and *d-fructose, each C.P. grade obtained from Pfannstiehl Labs., were dissolved in KH buffer and added to either the main chamber or sidearm depending upon the experiment. KH buffer was added to each vessel to bring the total fluid volume to 8.0 ml. To establish anaerobic conditions, the manometric system was flushed for 8 minutes with a mixture of 5 per cent carbon dioxide and 95 per cent nitrogen (prepurified). After the system was equilibrated at 37.5°C. for a 7-minute period, manometric measurements were usually taken at 10-minute intervals for a 3-hour period.

In the aerobic manometric experiments, the system was flushed with 5 per cent carbon dioxide and 95 per cent oxygen. The Warburg manometric method for aerobic glycolysis (20, p. 89) was considered to be useful and sufficiently reliable for following the changing rates of acid formation with time, although it is presumably subject to some error if the balance of oxygen uptake and carbon dioxide production by the cells is not the same in both the presence and absence of glucose and of lactate.

In experiments dealing with sugar utilization, each combination of sugar and tumor cells was incubated in duplicate vessels. For estimation of sugar used, the vessels were placed in an ice bath immediately after the final manometric reading. The fluid content of each vessel was transferred to a centrifuge tube held in an ice bath, and then the cells were spun down at 750 X g for 5 minutes. An aliquot portion of the supernatant solution was taken for the determination of sugar by Roe’s enzymatic colorimetric method (12). Glucose was determined colorimetrically by the specific glucose oxidase method (12). The presence of mannose or fructose had a very slight effect on the quantitative analysis of glucose by this specific enzymatic method. The absorbancies of mannose and fructose calculated on a basis of equal concentration were 0.6 and 0.025 per cent, respectively, of that of glucose.

**RESULTS**

**Anaerobic glycolysis.**—Under strictly anaerobic conditions, the rate of lactic acid production by the washed Krebs-2 cells did not immediately reach its maximum rate after glucose was tipped in from the sidearm at zero time, as shown by representative data plotted in Chart 1. Usually a 10- to 20-minute period was required for the glycolytic reaction to accelerate to its maximum steady-state rate. When 0.001 M pyruvate was added to the washed Krebs-2 cells prior to the equilibration period, lactic acid formation reached a maximum immediately after glucose was added.

At a glucose level of 0.015 M the rate remained essentially linear for 160 minutes of incubation following the lag period (Chart 1). When the initial glucose concentration was either 0.001 or 0.002 M, the apparent maximum rate was the same as with 0.015 M. However, with the low levels of glucose, the reaction stopped suddenly before all the glucose was converted to acid. Only 89 and 88 per cent, respectively, of the 3 and 6 µmoles glucose added was accounted for as acid in the typical experiment illustrated in Chart 1. If the added glucose was not accounted for as acid were still present in the extracellular medium, its concentration would be 0.00016 and 0.00042 M, respectively, which should be easily detected by sensitive methods of glucose assay. In another experiment performed under similar conditions, no glucose was detected in the extracellular medium by the sensitive glucose oxidase method immediately after the first 10-minute period in which the rate approached zero. This suggested that the extracellular glucose concentration supporting maximum glycolysis may be even lower than that indicated by the values presented above.
That the maximum rate of anaerobic glucolysis was independent of the glucose concentration within the range of 0.0005 to 0.1 M, the highest concentration tested, is shown in Chart 2, which includes the results of many separate experiments. In a given experiment with the same cell suspension, the rates at different concentrations were compared with that of glucose at 0.015 M given the index number of 100. When glucose was present at initial concentrations of less than 0.0005 M, the maximum rate could not be measured by the manometric methods employed, since the limited supply of glucose was used up before a steady-state maximum rate could have been attained. The lack of endogenous glycolyzable substrate in the washed Krebs-2 cells was shown by the negligible acid production of the cell controls to which no sugar was added.

Anaerobic mannolysis.—After an initial lag period, mannolysis at three different concentrations of mannose proceeded at the same steady-state rate as shown in Chart 1. When the quantity of mannose added was 3 or 6 μmoles, the rate continued at its maximum until the total acid production accounted for, respectively, 90 and 85 per cent of the mannose; then the rate suddenly decelerated to zero.

Mannose, like glucose, was converted to lactic acid at its apparent maximum rate at all mannose levels tested between 0.0005 and 0.1 M (Chart 2). The average rate of mannolysis was 72 per cent of that of glucolysis. The concentrations at which the rates were lower than the maximum could not be determined, just as in the case of glucose.

Anaerobic fructolysis.—Fructose was converted to lactic acid at a maximum rate at concentrations of between 0.015 and 0.1 M (Chart 2), after an initial lag period. The maximum rate of fructolysis was the same as that of glucolysis. At concentrations below 0.01 M, the rate of fructolysis was proportional to the concentration. During incubation in the presence of low concentrations of fructose, the rate of fructolysis gradually decreased but did not decelerate to zero within a 3-hour period, in contrast to that of glucolysis and of mannolysis.

The reciprocal of the average relative rate of anaerobic fructolysis plotted against the reciprocal of the concentration of fructose according to the method of Lineweaver and Burk (11) is shown in Chart 3. The reciprocal velocities for the concentrations at which the rates were lower than the apparent maximum formed a straight line which, on extrapolation, intersected the origin, whereas those for concentrations of 0.015 M and higher formed a line parallel to the abscissa. The concentration of fructose at which the rate was one half the apparent maximum was 0.005 M, which is indicated by the point at which the reciprocal of the relative velocity is equal to 2. When the total volume of Krebs-2 cells added to each Warburg vessel was doubled, the concentration of fructose for one half the maximum rate was found to be unaltered. Therefore, the mutarotation of fructopyranose to fructofuranose, the form phosphorylated by hexokinase (18), did not limit the rate of fructolysis.
Combination of fructose and mannose.—When the concentration of fructose was the same as or twice that of mannose, the rate of anaerobic glycolysis was approximately the same as that with mannose alone (Table 1). The results also indicate that, when mannose was present either at the same or at one half the concentration of fructose, the inhibition of fructose utilization was essentially complete.

Combination of fructose and glucose.—In the presence of the combined substrates, fructose and glucose, the anaerobic glycolytic rate was essentially the same as with either hexose alone, as shown in Table 1. The \( Q_{\text{cyt}} \) values with each substrate in Experiments 1 and 2 were within the range of rates obtained with different Krebs-2 cell preparations. The utilization of fructose was completely inhibited by glucose with both sugars at an equimolar concentration (Table 1). In contrast, the conversion of glucose to acid was not affected by fructose at this concentration, since the glycolytic rate was essentially the same both with and without fructose. When the initial concentration of fructose was twice that of glucose, metabolism of fructose was inhibited 92 and 96 per cent in the two experiments.

The results presented in Table 2 show that when fructose was present in a concentration 25–100%

### Table 1

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<th>Fructose</th>
<th>Mannose</th>
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### Table 2

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times greater than that of glucose, it inhibited the anaerobic disappearance of glucose to a degree dependent upon the fructose concentration. An approximation of the relative concentrations of fructose and glucose for 50 per cent inhibition of glucose consumption was obtained by plotting the concentration of fructose as an inhibitor against the maximum rate of glucose utilization divided by its inhibited rate. The approximate concentration of each sugar was calculated for the mid-point of the incubation period. The ratios of fructose to glucose concentrations for 50 per cent inhibition of glucose utilization were found to be 53 and 54 in Experiments 3 and 4.

**DISCUSSION**

The delay in the attainment of the maximum rate of anaerobic glycolysis by washed Krebs-2 cells was similar in duration to that reported in 1932 for slices of Jensen rat sarcoma by Dickens and Greville (6). The complete abolition by pyruvate of this lag period, observed in the Krebs-2

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**Chart 4.** Rates of aerobic glucolysis (G), mannolysis (M), and fructolysis (F). Each vessel contained 68.3 μl of Krebs-2 cells.

**Combination of mannose and glucose.—**The anaerobic utilization of glucose was inhibited 52 and 54 per cent by mannose at an equimolar concentration of the two hexoses, while the rate of glycolysis was not significantly different from that with glucose alone (Experiments 5 and 6, Table 2). At higher mannose concentrations, the inhibition of glucose consumption was greater, and the glycolytic rate was intermediate between that of glycolysis and mannolysis.

**Aerobic glycolysis, mannolysis, and fructolysis.—** Under aerobic conditions, glycolysis attained its maximum rate immediately after glucose was tipped in from the sidearm. When 45 μmoles of glucose was added, the rate remained constant for 1 hour, after which interval it decreased slightly, as indicated in Chart 4. The maximum rate of aerobic glycolysis, expressed on a basis of mg. dry weight (Q%), was 21.6 during the 1st hour, slightly less than one half the average maximum anaerobic rate. When a limited amount of glucose (9 μmoles) was added, the rate remained high for 110 minutes, after which it abruptly decelerated to zero with 86 per cent of the glucose accounted for as acid.

After an initial lag period, the rate of aerobic mannolysis stabilized slightly lower than that of glycolysis. When 9 μmoles of mannose was added, mannolysis proceeded at a high rate for 20 minutes longer than glycolysis and then suddenly stopped, when the same total acid was produced as in glycolysis. After the acid production from glucose and mannose ceased, the gas volume of the manometric system decreased more rapidly than that of the endogenous control and was essentially the same as that of the l-lactate control.

The rate of fructolysis in the presence of 45 μmoles of fructose reached almost the same maximum as glycolysis after an initial lag period (Chart 4). At an initial fructose concentration of 0.003 M, the rate was about one half that at 0.015 M during the 1st hour of incubation. It gradually decreased during subsequent incubation, but in contrast to glycolysis and mannolysis at the same low hexose concentration it did not reach zero during the entire 8-hour period.

**Aerobic utilization of glucose inhibited by mannose and fructose.—** Mannose was found to inhibit the aerobic uptake of glucose to a degree proportional to the concentration of mannose (Table 3). The inhibitory effect of mannose was essentially the same with Krebs-Ringer phosphate (Exp. 7) and with Krebs-Henseleit bicarbonate (Exp. 8) buffers. Glucose utilization was inhibited 50 per cent at a mannose to glucose ratio of 1.0.

Fructose at an initial concentration 30–60 times greater than that of glucose inhibited the utilization of glucose from 44 to 62 per cent (Table 3). The molar ratio of fructose to glucose for 50 per cent inhibition, calculated in the same manner as indicated for Experiments 3 and 4, was found to be 48 and 49 in Experiments 9 and 10.
other chemical agents which substantially increased the rate of anaerobic glucolysis and fructolysis is necessary (17). This enzyme, which Embden-Meyerhof pathway, phosphomannose phosphorylation by this enzyme is only 40 per cent of that of glucose (19). This low rate of phosphorylation may limit the over-all rate of its aerobic conversion to lactic acid in Krebs-2 cells. The rate of anaerobic mannolysis of Krebs-2 cells was probably at its absolute maximum, because it was not stimulated by dinitrophenol or the other chemical agents which substantially increased the rate of anaerobic glucolysis and fructolysis (22).

Since the apparent maximum rate of anaerobic fructolysis of Krebs-2 cells was essentially the same as that of glucolysis, both processes appear to be limited by the same pacemaker. The apparent maximum rate of fructolysis is probably controlled by a different limiting factor than its submaximum rates, since the Lineweaver-Burk plot for low concentrations of fructose formed a straight line differing in slope from that for high concentrations. The rate of penetration of fructose at low concentrations to the site of hexokinase in the cell is suggested as a possible limiting factor in its anaerobic metabolism, although fructose at the high extracellular concentration of 0.088 M was found by Crane, Field, and Cori (4) to penetrate through the cell membrane of Ehrlich cells at a rate which does not limit its utilization at 30°C.

The metabolism of fructose by the Krebs-2 cells probably does not involve a specific fructokinase, such as is active in liver and muscle. Phosphorylation of fructose by this enzyme was reported not to be inhibited by mannose or glucose (8, 18). In the tumor cells, however, mannose not only had a potent inhibitory effect on the utilization of fructose but also was able to control the glycolytic rate at its lower level even in the presence of excess fructose. The combination of glucose with fructose did not have an additive effect on the metabolism, although glucose at the high extracellular concentration of 0.088 M was found by Crane, Field, and Cori (4) to penetrate through the cell membrane of Ehrlich cells at a rate which does not limit its utilization at 30°C.

For mannose to be metabolized through the Embden-Meyerhof pathway, phosphomannose isomerase is necessary (17). This enzyme, which does not participate in the breakdown of glucose and fructose, may be the rate-limiting enzyme in the Krebs-2 cells for the anaerobic metabolism of mannose, maintaining its maximum rate significantly below that of glucose and fructose. However, although mannose has the highest reported affinity with mammalian hexokinase, its maximum rate of phosphorylation by this enzyme is only 40 per cent of that of glucose (19). This low rate of phosphorylation may limit the over-all rate of its anaerobic conversion to lactic acid in Krebs-2 cells. The rate of anaerobic mannolysis of Krebs-2 cells was probably at its absolute maximum, because it was not stimulated by dinitrophenol or the other chemical agents which substantially increased the rate of anaerobic glucolysis and fructolysis (22).

The uniformly high rate of anaerobic glucolysis of Krebs-2 cells over a wide range of glucose concentration, in which the highest level was 200 times the lowest, is in agreement with the results obtained with Ehrlich ascites cells by Kun (10) over a more limited range. The abrupt cessation of glucolysis before the limited amount of glucose was completely converted to acid by the Krebs-2 cells was also similar to that observed in Ehrlich ascites cells by Kun. Since no glucose was detected in the extracellular fluid suspending Krebs-2 cells after acid formation stopped, it is possible that the glucose unaccounted for was used to resaturate depleted levels of intermediate glycolytic metabolites of the washed cells or that it was converted to some nonacidic end-product. That the difference between the uptake of glucose and its conversion to acid is probably not owing to accumulation of glucose in the cells was indicated by the finding of Crane, Field, and Cori (4) to penetrate through the cell membrane of Ehrlich cells at a rate which does not limit its utilization at 30°C.

TABLE 3

<table>
<thead>
<tr>
<th>EXP. NO.</th>
<th>KREBS-2 CELLS MANNOSE ADDED (umoles)</th>
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Each reaction mixture was incubated with 6 umoles glucose for 60 minutes at 37.5°C. Experiments 7, 9, and 10 were conducted in a modified Dubnoff apparatus with Krebs-Ringer phosphate buffer (pH 7.8, calcium omitted), air in the gas phase, total fluid volume of 3 ml. in each round-bottomed centrifuge tube of 40-ml. capacity, 1.0 ml. buffered cell suspension added at zero time. Experiment 8 was conducted in the Warburg apparatus, 95 per cent oxygen and 5 per cent carbon dioxide in the gas phase, hexoses tipped in from the sidearm at zero time.
rate of lactic acid formation. The reciprocal inhibition of utilization observed between glucose and fructose suggests that they are competing for the same enzyme system.

The capacity of each hexose to inhibit the utilization of the other appears to be related to its order of affinity with animal particulate hexokinase. The properties of particulate hexokinase of Krebs-2 cells (13) are in general similar to those of brain, so well described by Sols and Crane (19). However, the ratio of Michaelis constants of fructose to glucose is 200 for hexokinase of brain (19) and 90 for hexokinase of Krebs-2 ascites tumor. The fructose:glucose ratio for 50 per cent inhibition by fructose of glucose utilization is approximately 50 in Krebs-2 cells under both anaerobic and aerobic conditions. Therefore, the ability of fructose to inhibit glucose utilization by the Krebs-2 cells is evidently greater than is expected from their relative affinities with hexokinase. However, the maximum rates of phosphorylation of fructose by particulate hexokinase of brain (19) and of Krebs-2 tumor are 1.5 and 2.1 times higher than that of glucose and may influence the inhibitory effects of fructose at high concentrations on glucose utilization in the tumor cells. The degree of inhibition by fructose may also depend upon the fact that glycolysis requires one additional enzyme, phosphoglucoisomerase, which is not involved in fructolysis.

It may also be observed that mannose, present at an equimolar concentration with glucose, inhibited glucose utilization by Krebs-2 cells about 50 per cent under both anaerobic and aerobic conditions. In contrast to the results obtained with fructose, this is less inhibition than would be expected from consideration only of the higher affinity of mannose than of glucose with hexokinase. The lower maximum rate of phosphorylation of mannose than of glucose by this enzyme also appears to have an effect on their relative utilization in the ascites tumor cell.

The ability of each glycolyzable sugar to compete with the other two is therefore best indicated by its coefficient of phosphorylation by hexokinase, which is equal to the maximum rate of phosphorylation of the sugar divided by its Michaelis constant relative to this ratio for glucose, as defined by Sols and Crane (19). When glucose is given the value of 1.0, the phosphorylation coefficients of mannose and fructose for Krebs-2 tumor hexokinase are 1.1 and 0.02. The molar ratios of glucose to mannose and to fructose for 50 per cent inhibition of glucose utilization in the Krebs-2 cells correspond closely to the phosphorylation coefficient of each inhibiting sugar.

Although the anaerobic metabolism of glucose by neoplastic tissues had been the subject of numerous investigations following the pioneering work of Warburg (20), that of mannose and of fructose had been studied only to a relatively limited extent. Anaerobic mannosylation of Flexner-Jobling carcinoma slices was found by Warburg (20, p. 137) to have nearly as high a rate as glycolysis, in contrast to the difference in their rates in Krebs-2 cells. Fructose was reported to be decomposed by Flexner-Jobling carcinoma slices to lactic acid equally with glucose (20, p. 112) and at 14 per cent of the rate of glucose (20, p. 137). Dickens and Greville (5, 7) observed that the rates of anaerobic fructolysis in a variety of malignant tumors varied from zero to about one half that of glycolysis, while the glycolytic rate was consistently high. At a concentration of 0.005 M and higher, fructose was reported by Clowes and Kelch (3) to be utilized and converted anaerobically to lactic acid by both Ehrlich and Sarcoma 180 ascites cells at less than one half the rate of glucose. In contrast, the fructolysis rates of Ehrlich ascites cells (10) and of rat Sarcoma 308 slices (14) at high fructose concentrations were found to be similar to those of glycolysis. The results with Krebs-2 ascites cells are in agreement with the latter findings.

The wide range of apparent maximum rates of fructolysis of different neoplastic tissues and cells is as yet unexplained. The conclusion reached by Dickens and Greville in 1932 (5) still applies: "The appearance of fructolysis in one tissue and its complete absence from another tissue of similar type, the great variability shown by the various tissues in their attack on fructose when their metabolism of glucose is similar, still await an explanation, which, when it is forthcoming, can hardly fail to clear up many obscure features of carbohydrate metabolism."

**SUMMARY**

The rates of anaerobic glycolysis and mannosylation of Krebs-2 ascites carcinoma cells were found to be independent of the hexose concentration between 0.0005 and 0.1 M. Anaerobic mannosylation proceeded at 72 per cent of the rate of anaerobic glycolysis. When small quantities of glucose or mannose were added, 85–90 per cent of each sugar was converted to acid at its apparent maximum anaerobic rate, after which the reaction stopped abruptly.

The rate of anaerobic fructolysis was independ-
ent of fructose concentration between 0.015 and 0.1 m and was the same as that of glucoaly. At lower levels of fructose, the rate was dependent on substrate concentration.

Mannose was shown to control the rate of anaerobic glycolysis when added together with fructose and also to inhibit completely the anaerobic utilization of fructose. Mannose suppressed utilization of glucose approximately 50 per cent under both anaerobic and aerobic conditions when both hexoses were present at equimolar concentrations.

In the presence of both glucose and fructose, the rate of anaerobic glycolysis was essentially the same as with either hexose alone. The utilization of glucose was inhibited 50 per cent when fructose was present at approximately 50 times the concentration of glucose under both anaerobic and aerobic conditions.

The competition between the glycolyzable hexoses in substrate utilization and in controlling the glycolytic rate can be partly explained by their relative affinities with and phosphorylation coefficients for hexokinase.

REFERENCES


Metabolism of Ascites Tumor Cells I. Rate of Glycolysis and Competitive Utilization of Fructose, Mannose, and Glucose

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