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Abstract

1. The DNA contents in mature lymphocytes of the mouse, rat and man are kept almost constant. 2. The variety in the DNA contents in tumor cells is attributed to the rapid DNA synthesis taking place at the interphase, though the degenerating cells and the cells in abnormal mitosis can not be discarded as the source of the variety in DNA content. 3. The RNA content in AH-130 (ascites hepatoma) is less than that in normal liver cells.

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QUANTITATIVE STUDIES OF NUCLEIC ACID IN THE CELL BY MICROSPECTROPHOTOMETRY

III. NUCLEIC ACID CONTENTS IN THE CANCER CELLS

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In the biological research field of development and genetics the most interesting theme may be nucleic acids, DNA. The constancy of the DNA contents per cell in each cell species have been reported recently by many investigators[1-19]. This fact seems to be a strong evidence to support the opinion that DNA is the bearer of genetic characteristics of an individual cell. However, there are several papers contradictory to the DNA constancy[20-27], which are mainly reported by French and Belgian scholars including Lison and Brachet. Consequently, it will be necessary that the problems concering the DNA constancy per cell must be reconfirmed before the bearer of gene is assumed to be DNA itself, because there is a great possibility that a fairly considerable error may be expected by the methods used, especially in the case of microspectrophotometry. From this view point the author tried to minimize the errors which might be introduced in the course of estimation, the errors by refractory, reflectory and SV-effects[28,29] and by the staining technique and the errors in the mathematical calculation as reported precisely in the previous report[32,33]. Using this method SENO and the author[30-32] succeeded in estimating the hemoglobin contents of a red cell exactly, the value of which coincides with that calculated by the chemical method on the same sample, and the DNA constancy per cell has been elucidated in the mature red cells of the frog and hen[33].

In this report the author presents the DNA contents of the lymphocytes in human, rat and mouse and spermatocytes in rat by the same method, showing the DNA contents also to be almost constant in mature lymphocytes and closely correlated to the chromosome number. Besides these, the author describes the variety in the DNA contents in cancer

cells, whose chromosome number is known to be seriously deviated from that of the host cells. The results show the DNA contents are also closely correlated to the chromosome number even in cancer cells and the irregularity in the DNA contents is mainly due to the accelerated DNA synthesis accompanied by the rapid cell division.

MATERIALS AND METHODS

The lymphocytes of mouse, rat and human in the circulating blood and spermatocytes of rat are used for the estimation of DNA as the representatives of the normal cells in each strain. As for the tumor cells the ascites hepatoma cells (AH-130), Ehrlich's ascites tumor cells, Yoshida sarcoma cells and H-cells[34] (cultured human amnion cells, which have the property of tumor cells in rat) served as materials. These tumor cells are used at a certain period after the transplantation; 8 to 10 days by AH-130 in hybrid rats; Ehrlich's ascites tumor cells 10 to 12 days in hybrid mice; Yoshida sarcoma cells 7 to 8 days in hybrid rats; and H-cells at 3 to 4 days' culture in Dulbecoo culture media.

The estimation of DNA has been carried out on the cells smeard or stamped, dried and fixed with acetic alcohol, with Feulgen reaction at 5,600 A or without staining at 2,600 A (AH-130 only are measured at 2,600 A without staining). Acetic alcohol is prepared by mixing gracial acetic acid with 3 volumes of ethanol[38]. For the observation of lymphocytes the peripharal venous blood is smeard and the spermatocytes are stamped on the glass cover slide $24 \times 50\,mm^2$ by the routine method and dried quickly by the gentle air current of fan, with acetic alcohol for 5 minutes, hydrolyzed with 1 N HCl at 60°C for 5 minutes, and stained by Shibatani's method[36] for 3 hours. The DNA contents in each cell have been measured with the light at 5,600 A under the microspectrophotometer[32].

The cancerous ascites is drawn out by glass capillary, and smeared on glass cover-slide $24 \times 50\ mm^2$, making two smears in each sample. These are dried quickly and fixed as in the case of the smears of blood. In the case of AH-130 cells, besides the smears on the glass cover-slides, smears are made on the quartz cover-slides $25 \times 50\,mm^2$. The smears on the quartz slides are used for the estimation of DNA at 5,600 A by staining with the Feulgen reaction. The H-cells cultured on the strips of the cover-slides $10 \times 50\,mm^2$ in the culture tube, TD15 are taken out after 3 to 4 days' culture, and washed 3 times with freshly prepared physiologic saline solution and dried at room temperature. These are

fixed with acetic alcohol, and stained with the Feulgen reaction by Shibatani's method; and the DNA contents of the cells have been measured at 5600 A under microspectrophotometer.

For the calculation of DNA and RNA contents in the unstained cells the measurements at 2,600 A have been repeated 3 times on the same sample, i. e. the first measurement on the cells after the fixation, the second after treating with RNase and the third after treating with perchloric acid. The RNA contents are calculated by subtracting the values obtained by the second measurement from those obtained by the first measurement, and the DNA contents by subtracting the values obtained by the third measurement from those of the second measurement. For the treatment with RNase the fixed smears are exposed to a 0.1 % solution of RNase from cow pancreas, pH 6.78 at 60°C for one hour. For the treatment with perchloric acid the smears are exposed to a 10 % pechloric acid solution at 70°C for 30 minutes[37].

For the microspectrophotometry the absorption on the whole area of the smeared cell are obtained according to the following formula; $K_2\int |x| f(x)dx$ or $K_2\pi\int |x| f(x)dx$. The $f(x)$ means the area surrounded by the curve obtained by connecting the points showing the absorption maxima at the points ranging on the nuclear diameter. For the precise method see the first and second reports[32,33].

In Ehrlich's ascites tumor cells the period of the mitotic cycle has been calculated. The cells in mitosis are numbered on the smears stained with Giemsa or hematoxylin by the routine methods. On the other hand, the time required for mitosis has been estimated by observing the cells floated in liquid paraffin under a phasecontrast microscope according to Makino's method[38]. From these two values the mitotic period of the cells has been calculated in the manner to be described later.

The volumes of nuclei of living cells (V) are calculated on all strains, excepting Yoshida sarcoma cells, from the radius (r) measured on the smeared cells by the follwing formula;

$$V = \frac{4}{3}\pi(kr)^3$$

(k) is the constant derived from the rate r_1/r_2 where r_1 is the mean value of the radius of 30 living rat lymphocytes, which are estimated in lymphocytes found in the buffy coat of blood obtained by centrifugation of 2,000 rpm for 15 minutes, and floated in homologous serum. (r_2) is also the mean value of the radius of 30 lymphocytes from the same strain estimated on the smeared and stained cells.

EXPERIMENTAL RESULTS

The DNA contents of the lymphocytes have been found to be almost constant showing nearly the same values in each cell species, as is demonstrated in Table 1 : i. e. the mean values of the DNA contents calculated from 30 cells, having Feulgen reaction, are 3.76 in the mouse, 3.72 in the rat and 3.74 in man in arbitrary unit, and correspond to the absolute amount of the DNA contents of 5.64×10^{-9} mg, 5.58×10^{-9} mg and 5.61×10^{-9} mg respectively. But there are some lymphocytes having double the amount of DNA, probably of the tetraploidy (Fig. 1), These cells are found in about 4 per cent of the lymphocytes in the circulating blood of the rat and mouse but not of man. The volumes of nuclei show some deviation in each cell species, 46.1 μ^3 in the mouse, 37.6 μ^3 in the rat and 79.1 μ^3 in man, in mean value from 20 to 29 cells in each species. The DNA contents in the mature sperms are estimated only in the rat and show almost constant value, 1.93 in arbitrary unit and 2.89×10^{-9} mg per cell in the mean value from 30

Table 1. Amount of DNA in nuclei of various cell

Cell	Number of nuclei estimated	Mean value of nucleus diameter	DNA in Feulgen unit (standard deviation)	DNA in absolute amount (10^{-9}mg)
Ascites hepatoma cell (AH130)	mean value 29	12.5	9.85	14.80
	11	10.1	5.46 (1.69)	8.18
	16	13.7	11.24 (3.34)	16.84
	2	15.2	17.30	25.95
Ehrlich's ascites tumor cell	mean value 132	12.1	9.97	14.95
	74	10.5	6.74 (1.23)	10.11
	54	13.6	13.47 (1.15)	20.24
	3	15.8	20.21	30.32
	1	19.4	28.84	43.26
H-cell	mean value 33	12.9	9.96	14.93
	21	11.6	7.68 (2.1)	10.92
	12	14.7	14.03 (1.0)	21.05
Rat lymphocyte	30	5.0	3.72 (0.48)	5.58
Mouse lymphocyte	58	4.9	3.76 (0.57)	5.64
Human lymphocyte	30	6.8	3.74 (0.24)	5.61
Rat spermatocyte	30	1.9	1.93 (0.05)	2.89

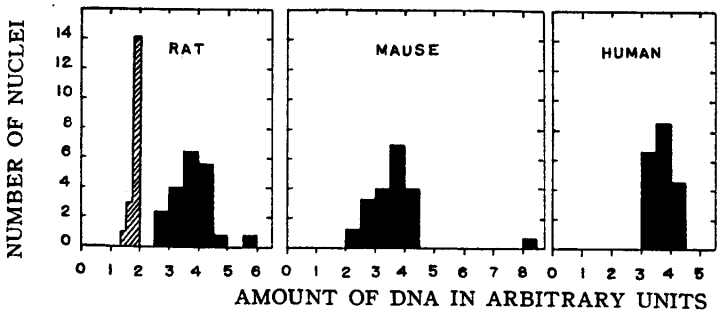

Fig. 1. Individual DNA contents in the nucleus of the lymphocytes in rat, mouse and man and of spermatocytes in rat. DNA contents of spermatocyte is about half that of lymphocyte.
■ lymphocyte ▨ spermatocyte

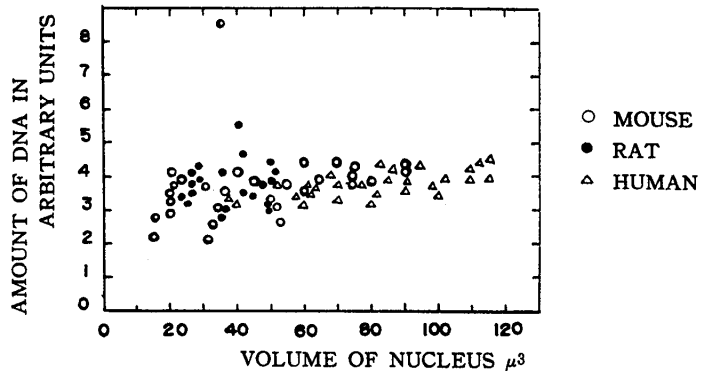

Fig. 2. The relation between the DNA contents and volumes of individual nuclei of rat, mouse and man.

cells (Table 1). These values are nearly half the mean value of the DNA contents in rat lymphocytes and correspond to the values of haploid.

In tumor cells, the DNA contents per cell show a marked variety in every stain, but in the distribution curves many cells are distributed nearly at the values of two and 4 times that of lymphocytes of each host animal. In Ehrlich's ascites tumor cells many cells are distributed nearly 6.5 and 13.5 in arbitrary unit, though there are some cells having an abnormally high content of DNA (Fig. 3). In the case of Yoshida sarcoma cells, AH-130 and H-cells almost the same tendency as in Ehrlich's ascites tumor cells can be observed (Figs. 4, 5, 6). But as the common characteristics of tumor cells, the DNA content in each cell presents a marked variety in distribution, and the distribution is superposed on two points aforementioned. This will be due to the active mitosis of the tumor cell as

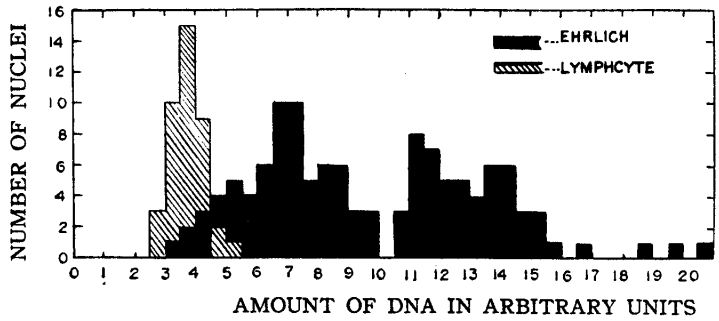

Fig. 3. Individual DNA contents in the nuclei of lymphocytes of mouse and of Ehrlich's ascites tumor cells.

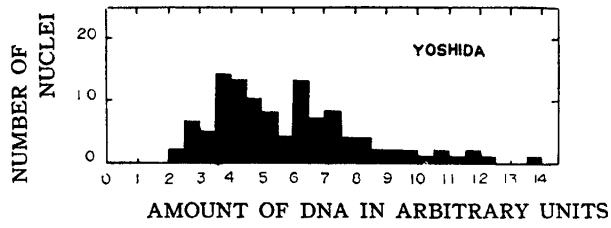

Fig. 4. Individual DNA contents in the nuclei of Yoshida tumor cells.

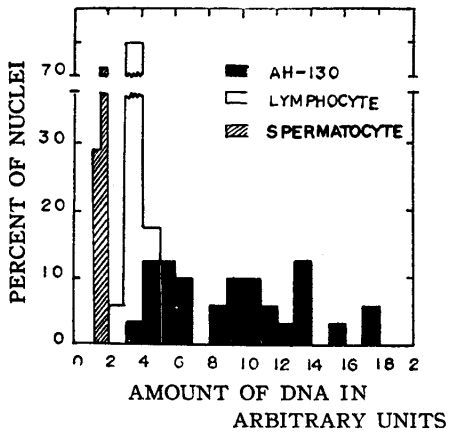

Fig. 5. Individual DNA contents in the nuclei of the spermatocytes of rat and of ascites hepatoma cells.

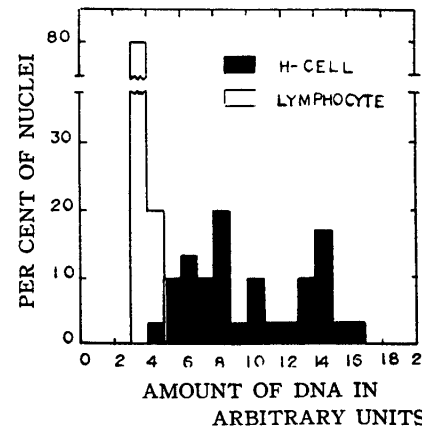

Fig. 6. Individual DNA contents in the nuclei of lymphocytes of rat and of H-cells.

will be discussed later.

The RNA contents, supposed to be closely correlated to the mitotic activity of tumor cell with respect to the protein synthesis or to the

material for DNA synthesis, are calculted only on the AH-130. As indicated in Table 2 the RNA content is 18.55×10^{-9} mg per cell in mean value from 30 cells.

Table. 2. Amounts of DNA and RNA in Ascites Hepatoma Cells

	Number of nuclei estimated	Mean value of nuclear diameters	NA/cell 10^{-9}mg.	Standard deviation
Values obtained without treatment Total nucleic acid	30	17.3	35.4 33.35	9.65
Values after RNase treatment Contents of RNA	30	16.0	16.88 18.55	3.85
Values after PCA treatment Contents of DNA	30	16.0	2.05 12.75	0.15

The volume of the nucleus in cancer cells also show a marked variety in each strain with an increasing tendency in volume with the increased content of DNA (Figs. 7, 8, 9).

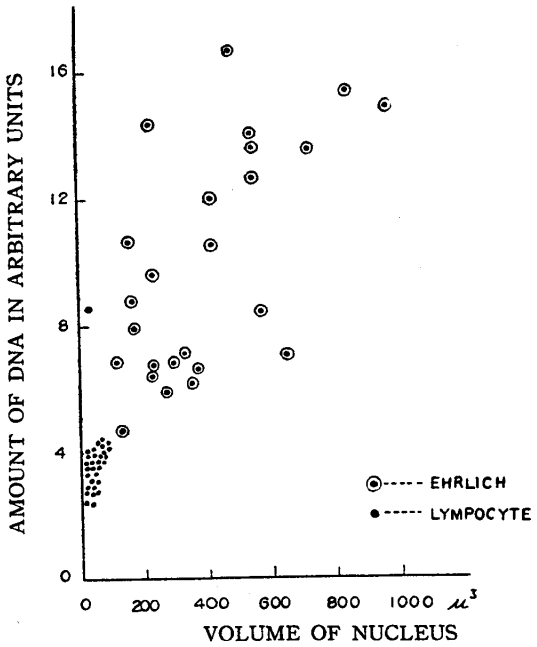

Fig. 7. Relation between the DNA contents and volumes of the nucleus of mouse lymphocytes and of Ehrlich's ascites tumour cells.

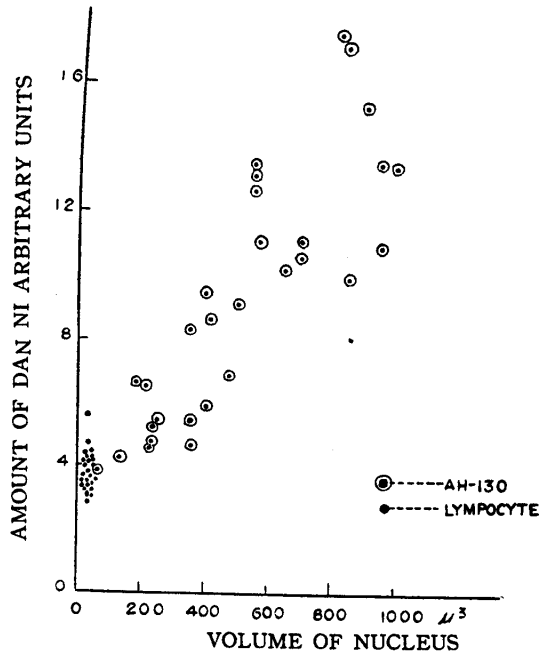

Fig. 8. The relation between the DNA contents and the volume of the nucleus of rat lymphocytes and of ascites hepatoma cell

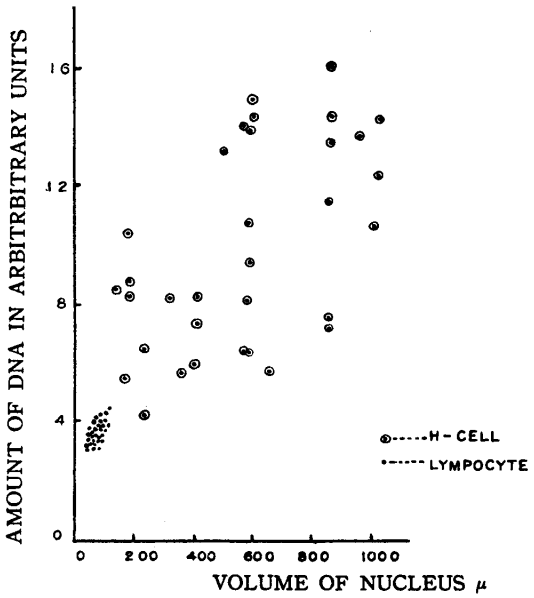

Fig. 9. The relation between the DNA contents and the volumes of the nucleus of human lymphocytes and of H-cells.

The time required for a whole cycle of mitosis that can be calculated from the length of the mitotic period is observed in Ehrlich's ascites tumor cells. The cells in mitosis are found to be about 7.7 per cent of the total cells counted and requires about 72 minutes from the beginning to the termination of mitosis in mean value of 10 cells, 52 minutes for prometaphase, 10 minutes for metaphase, 4 minutes for anaphase and 6 minutes for telophase.

COMMENT

As demonstrated first in lymphocytes and spermatocytes, the DNA contents of the completely matured cell will be kept almost constant, suggesting that DNA is closely correlated to the gene of each animal species though some deviation can be seen. This deviation, to some extent, may be due to an error in the estimating method, but there is a possibility that some variation actually exists in each nucleus as the result of an error occurring in estimation of same cell in which the error is less than 2 per cent by this method on lymphocytes. This point need be settled by the further observation, but the results seem to support the theory of the constancy in the DNA contents per cell destined in each strain of animal.

However, the results obtained on the volume of the nucleus of lymphocyte shows that the DNA content is not so closely correlated to the volume of the nucleus. In cancer cells the DNA contents show a marked variety from cell to cell even in the cell belonging to the same strain, though there is some tendency in which many cells contain as much as about 2 and 4 times of DNA as that of lymphocytes. However, in such event the accelerated mitotic activity must be taken into consideration, where an active DNA synthesis is postulated and accordingly the variation in the DNA content will result. The period of mitotic cycle (p) of Ehrlich's ascites tumor cell is calculated as 17 hours from the number per cent of the dividing cell ($a = 7.7$) and the period required for the termination of the whole course of mitosis ($b = 72$ minutes) as calculated by the following formula:

$$p = b/a \times 100$$

If it is assumed that the variation in the DNA content in each cell is due to only the difference in the stage of the DNA synthesis, the following calculation can be applied:

As the DNA synthesis is supposed to proceed during the interphase reaching the maximum content between the prophase to telophase, then the curve showing the rate of DNA synthesis in each stage of mitotic cycle will be demonstrated by arranging the number per cent of the cell

according to their DNA contents, from the least one to the largest. For example, as in the case of Ehrlich's ascites tumor cell which is supposed to be of hypotetraploid originally, the least one is the number per cent of the cell found at the first peak of the distribution curve and the maximum one is that of the second peak. As is well known the cancer cells generally show some abnormalities in mitosis and some of them are found in the course of degeneration. Therefore, those cells having smaller DNA contents than those found at the first peak in the distribution curve and larger ones than those in the second peak can be reasonably discarded.

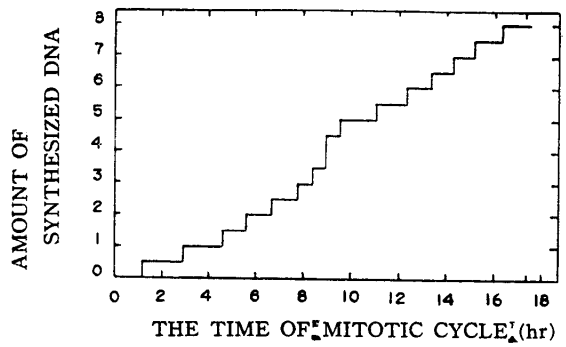

Fig. 10. The curve of DNA synthesis in mitotic cycle of Ehrlichs ascites tumour cell: Abscissa shows the number per cent of cells arranged according to the DNA contents in the time of mitotic cycle (17hrs) and ordinate shows the amount of synthesized DNA.

Thus a curve is obtained as indicated in Fig. 10. Abscissa shows the number per cent of cells arranged according to the DNA contents in the time of mitotic cycle (17 hours) and ordinate shows the amount of synthesized DNA. As the small distibution in some period of mitotic cycle is understood to show the accelerated DNA synthesis in this stage and the large one in distribution the delayed DNA synthesis. This curve will actually show the rate of DNA synthesis in each stage of mitotic cycle. If this conception is accepted, the DNA synthesis is proceeding rapidly between 8-10 hours and slowly during the period of 0 to 8 and 10 to 17 hours after the termination of mitosis. The DNA synthesis during mitosis is actually zero, because the lower value would be obtained at the terminal stage of interphase if DNA synthesis should occur in some stage of mitosis.

There are many reports claiming the DNA synthesis to occur during the interphase[19], and this claim will support that the inconstancy of DNA contents in tumor cell is due to the active synthesis of DNA, and con-

sequently the first peak appearing in distribution curve in DNA contents will show the DNA value fixed to the destined chromosome number in each cell, the stem line cell of Makino[38]. The fact that the volume of the nucleus increases with an increase in DNA content will also support this view, because it is generally observed that the volume of the nucleus increases before mitosis.

RNA is less in contents in AH-130 cells in comparison with those in normal liver cells observed by several authors. The result seems to be contradictory when the RNA is supposed to be closely correlated to the protein synthesis and DNA synthesis. But in the case of tumor cells it is supposed that the protein synthesis like albumin which is only secreted from the cell ceases and the protein used only for cell division will be produced and RNA for the albumin synthesis as in normal liver cells may be lost. This might be the reason why the tumor cells contain less RNA.

SUMMARY

1. The DNA contents in mature lymphocytes of the mouse, rat and man are kept almost constant.

2. The variety in the DNA contents in tumor cells is attributed to the rapid DNA synthesis taking place at the interphase, though the degenerating cells and the cells in abnormal mitosis can not be discarded as the source of the variety in DNA content.

3. The RNA content in AH-130 (ascites hepatoma) is less than that in normal liver cells.

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