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THE INTRACELLULAR DISTRIBUTION OF MERCURY IN RAT LIVER AFTER METHOXYETHYLMERCURY INTOXICATION

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Abstract—The intracellular distribution of mercury in the liver has been investigated in rats after methoxyethylmercury intoxication. The applied centrifugal technique for the cell fractionation is based on marker enzymes for characterizing the distribution of cellular particles in the fractions. Mercury content is reported in the different fractions. Estimates are presented of the mercury content in mitochondria, in lysosomes and peroximes, and in microsomes (membrane fragments). Some aspects are given of mercury distribution in relation to protein content of fractions, and in relation to thiol group distribution. The usefulness is pointed out, of the method for evaluating different biochemical aspects of mercury intoxication.

THE BIOCHEMICAL lesions underlying the clinical picture of mercury intoxication are unknown. Considering the affinity of mercury for thiol groups in organic compounds,[1] the binding of mercury to such radicals must be assumed. Mercury sulfide is also found in the tissues after mercury intoxication.[2] Mercury may, however, combine with organic radicals other than thiol groups.[1, 3] It is unknown to what extent this reaction takes place in the cells.[2] Enzyme inhibition[3, 4] or stimulation[5] and structural changes of proteins[4, 6] or membranes[4] are among the possible results of mercury and tissue interaction. The resulting clinical symptoms, if any, may thus show a considerable variation.

The uptake, retention and elimination of different mercury compounds from various organs in experimentally intoxicated animals have previously been tested and partially evaluated.[7] There seem to be no essential differences in mercury distribution or symptoms of mercury intoxication between animals (rabbits, rats and mice) and man,[7, 8] and obviously some correlation exists between functional disturbances and the distribution of mercury.

The assumption of characteristic compartments within the cells is necessary to explain the dynamics of cell metabolism. Knowledge of the distribution of different mercury compounds within the cells might thus supply additional information about biochemical lesion in mercury intoxication. Histochemical methods are applied in order to evaluate this problem in a series of investigations by Jonek *et al.*[9] and some reports on subcellular distribution of mercury, using electronic microscopy, histochemical and autoradiographic methods have also been published.[10] Cell fractionation by centrifugation has been applied to a very limited degree, but some results are given.[10, 11]

The purpose of this paper is to present a study of intracellular mercury distribution with a centrifugal cell fractionation technique based on marker enzymes to characterize particle distribution within the cells.[12] Inasmuch rat liver has been extensively investigated by similar technique,[12] it was found reasonable to adopt this organ as a model system. Even though the symptoms of mercury intoxication are most likely not related to the liver, it seemed useful as a model for the intracellular distribution of mercury in relation to different compounds, route of absorption and excretion, as well as retention rate and treatment. The liver, in addition to the kidneys, is also the excretory organ for some mercury compounds.[13]

MATERIALS AND METHODS

The seed dressing agent methoxyethylmercury acetate was a gift from A/B Casco, Stockholm, Sweden. This mercury compound was chosen because of its toxicological importance, and because it is partially excreted through the liver.[13] All other chemicals were obtained through commercial sources in the purest form obtainable.

Ten female rats weighing about 300 g were kept on an ordinary laboratory diet, but without milk, and with free access to drinking water containing methoxyethylmercury acetate. During the first five weeks of treatment the mercury content was 3·5 mg/l., followed by 0·75 mg/l. for 4–6 weeks, because the animals were killed successively during the last two weeks of the treatment. The rats were fasted for 24 hr before being killed. During this period, they had access to water without mercury.

The animals were killed by a blow on the head and subsequently bled. The livers were removed immediately and chilled on ice, 5 g of tissue from each of two rats were pooled and homogenized as described by de Duve *et al.*[14] with slight modifications. After being cut to pieces, the liver tissue was homogenized in 3 vol. ice-cold 0·25 M sucrose with 1 mM EDTA. The tube, which was kept on ice, was given a single run upward against the pestil which rotated about 1200 rpm, until the latter had reached the tube bottom. The resulting slurry was centrifuged at 4° by 6000 *g*-min*.[15] The sediment was rehomogenized with 3 vol. of sucrose–EDTA with a single run of the pestil. A centrifugation by 4100 *g*-min was followed by a third homogenization and centrifugation by 4100 *g*-min. The final sediment was taken as the nuclear fraction, and the combined supernatants were the cytoplasmic extract.

The cytoplasmic extract was further fractionated according to the scheme outlined by Appelmans *et al.*[16] using the Spinco model L ultracentrifuge with rotor 50. The mitochondrial fraction was first isolated by integrated forces of 33×10^3 *g*-min, the light mitochrondrial fraction by 25×10^4 *g*-min, and the microsomal fraction by 3×10^6 *g*-min. As a rule 60–80 ml supernatant was first spun down, the sediments were collected in two tubes and given one washing. The sediments from the two tubes were then combined in sucrose with EDTA and diluted to give the tissue concentrations of 1/5 for the nuclear fraction, 1/2 for the mitochondrial fractions, and 1/4 for the microsomal fraction, all expressed as g wet liver tissue per ml suspension. The supernatants were combined and made up to a known volume for the next step. The dilution of the cytoplasmic extract was 1/10 and for the final supernatant usually about 1/15.

3–Hydroxybutyrate dehydrogenase (D–3–hydroxybutyrate: NAD oxidoreductase 1.1.1.30) was assayed at 25° by following the increase in optical density at 340 mμ on

* *g*-min = average centrifugal force in the tube at constant rpm × centrifugal time (min).

a Zeiss selfrecording spectrophotometer RPQ 20.[17] The enzyme suspensions were sonificated to release the activity, because detergents are usually strong inhibitors for this enzyme.

Total acid phosphatase (orthophosphoric monoester phosphohydrolase 3.1.3.2.) was assayed by measuring the amount of inorganic phosphor set free in the presence of 0·05 M β–glycerophosphate in 0·05 M acetate buffer with 0·1 % Triton X–100 as releasing agent.[18] The reaction was stopped by trichloroacetic acid. The proteins were removed by filtration, and the phosphor was assayed by the method of Fiske and Subarrow.[19]

The rate of hydrolysis of glucose–6–phosphate (D–glucose–6–phosphate phosphohydrolase 3.1.3.9) was measured by determining the amount of inorganic phosphor set free after an incubation of 30 min at 37° in 0·02 M histidine–HCl buffer pH 6·5.[14] The amount of phosphor was determined as for acid phosphatase.

Urate oxydase (urate: oxidoreductase 1.7.3.3) was measured by following the disappearance of urate spectrophotometrically at 292 mμ on a Zeiss selfrecording spectrophotometer RPQ 20 at 37° [14] with Triton X–100 as releasing agent.

Proteins were estimated by the method of Lowry *et al.*[20] and the thiol groups by the amperometric argentimetric method of Benesch *et al.*[21] with the modifications of Børresen.[22]

Mercury was determined after neutron irradiation followed by chemical separation and gamma spectroscopy by the method of Sjøstrand,[23] but mercury was isolated as mercury sulfide.

RESULTS

The total activities of the various enzymes are taken as the sum of activity in the nuclear fraction and in the cytoplasmic extract, and expressed in units per g wet liver tissue.[14] One unit of activity refers to one μmole of substrate decomposed per min under the conditions of the assay.[14] The total activities recorded in Table 1 resemble those recorded by others using similar methods, with some exceptions.[14, 17, 24] The

TABLE 1. ENZYMIC ACTIVITY AND CONTENT OF PROTEIN, MERCURY AND THIOL GROUP SIN RAT LIVER

	No. of expts.	Mean ± S.D.
Protein	5	198·3 ± 40·8 mg
3-Hydroxybutyrate dehydrogenase	5	4·63 ± 0·81 units
Acid phosphatase	5	7·06 ± 1·34 units
Urate oxydase	5	2·88 ± 0·29 units
Glucose-6-phosphatase	5	21·30 ± 5·36 units
Mercury	5	0·83 ± 0·22 μg
Thiol groups	1	23·67 m–mole

The values are given per g wet liver tissue.
* For definition see the text.

3–hydroxybutyrate dehydrogenase activity was about half that reported by Beaufay *et al.*[17] Greater activity has, however, been obtained later with male rats of another weight, indicating a sexual or age dependent variation.

For the distribution study, the enzymic activities in the different fractions are expressed as percentage of the total activities as shown in Table 2.[14] The distribution of enzymes is similar to that previously reported with some minor differences.[14, 17, 24] Because the two mitochondrial fractions are partially sedimented together, the changed distribution of acid phosphatase and urate oxydase must be related to the removal of the light mitochondrial fraction. This is a very delicate step, and the results tend to vary, even within the same laboratory.[14] The low accumulation of glucose–6–phosphate in the microsomal fraction is also probably related to a different technique when the sediments and supernatants are separated with a pipette.

The total amount of proteins, mercury and thiol groups are shown in Table 1, and the corresponding distributions in Table 2. EDTA was found to be of no consequence for the binding of mercury by the different fractions, corresponding to the results reported by Clarkson and Magos for binding of inorganic mercury in liver tissue homogenates.[25]

TABLE 2. PERCENTAGE DISTRIBUTION OF ENZYMIC ACTIVITY, MERCURY AND PROTEIN CONTENT, AND OF THIOL GROUPS IN THE CENTRIFUGAL FRACTIONS

	Mean ± S.D.					
	N	M	L	P	S	Recovery
Protein	16·3 ± 3·1	26·3 ± 4·4	3·8 ± 1·0	15·7 ± 2·1	30·3 ± 4·4	92·5 ± 12·4
3-Hydroxybutyrate dehydrogenase	3·7 ± 3·1	80·5 ± 11·5	0·7 ± 0·4	2·7 ± 3·0	1·7 ± 1·0	89·2 ± 12·6
Acid phosphatase	5·4 ± 0·7	45·2 ± 2·4	13·2 ± 2·9	19·5 ± 6·6	9·1 ± 4·3	92·2 ± 11·8
Urate oxydase	2·6 ± 0·3	50·4 ± 8·3	12·3 ± 5·4	10·3 ± 2·0	0·3 ± 0·3	75·8 ± 12·4
Glucose-6-phosphatase	6·2 ± 1·8	18·2 ± 3·1	10·6 ± 5·2	43·7 ± 12·6	2·6 ± 2·8	82·2 ± 17·1
Mercury	11·2 ± 2·1	26·3 ± 4·1	5·0 ± 1·3	13·3 ± 4·2	41·4 ± 6·7	97·4 ± 12·7
Thiol groups	29·9	16·8	1·3	11·3	24·4	83·7

Values are taken from the experiments in Table 1.
The fractions are, nuclear (N), mitochondrial (M), light mitochondrial (L), microsomal (P) and supernatant (S)

The recoveries are generally lower than might have been expected. However, as the recovery of mercury is satisfactory, and no major differences in the distribution of enzymic activity are found, this has been assumed to be of little consequence. To reduce errors due to different recoveries, all results were recalculated in percentage of the sum of the recorded activities in the different fractions before being used for further analysis.

A more illustrative survey of the recalculated distribution patterns from Table 2 are provided by the diagrams in Figs. 1 and 2.[14] As seen from the figures, the area of each block is proportional to the percentage of enzymic activity recovered in the corresponding fraction. The height is proportional to the degree of purification achieved over the homogenate, or an expression of the mass amount of enzyme present in the fraction. Correspondingly, the distribution of mercury and of thiol groups are expressed.

DISCUSSION

The purpose of a cell fractionation may be either analytical or preparative. To investigate the distribution of a substance or a particle in relation to known intracellular particles, it seems necessary to apply a method with an ideal combination of these purposes. To get a reasonable answer, pure cell particle populations and a good

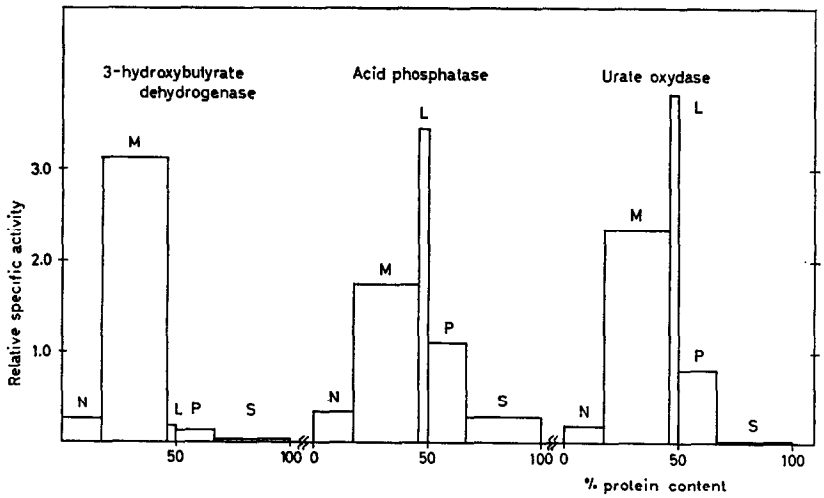

FIG. 1. Distribution pattern of the marker enzymes 3-hydroxybutyrate dehydrogenase, acid phosphatase and urate oxydase. The results in Table 2 are recalculated for variable recovery and expressed as percentage enzymic activity per percentage protein in the different fractions (relative sp. act.).

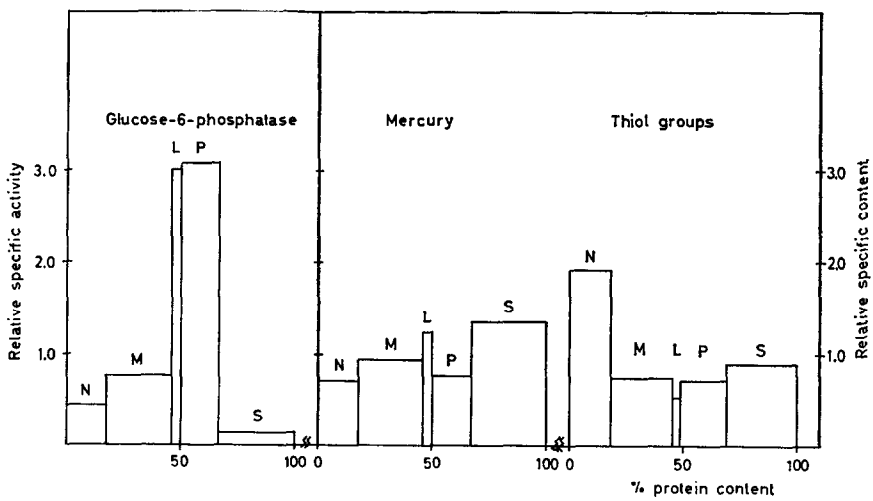

FIG. 2. Distribution pattern of the marker enzyme glucose-6-phosphate, and of mercury and thiol groups expressed as for the other marker enzymes in Fig. 1.

recovery are fundamental. These problems have been thoroughly reviewed by de Duve,[12] and it may be concluded that a pure analytical view is necessary for a handling of such a problem. The use of marker enzymes to characterize the different cell particle populations, or cell components, is one approach to this analytical problem.

When the distribution of enzymic activity is taken to represent the distribution of cell components, it rests on a postulate of biochemical homogeneity.[12] This postulate, which has thus far proved useful, rests upon the principle that "granules of a given population are enzymically homogeneous, or at least cannot be separated by centrifuging into subgroups differing significantly in relative enzymic content".[12] It is thus assumed that the cytoplasmic particles are biochemically sufficiently homogeneous to allow meaningful extrapolation from enzymes to host particles. The marker enzymes used in this study are 3–hydroxybutyrate dehydrogenase for mitochondria, acid phosphotase for lysosomes,[14] glucose–6–phosphatase for membrane fragments or microsomes, and urate oxydase for peroximes.[26]

Some pseudoanalytical methods have formerly been used for the centrifugal investigation of mercury distribution in tissue homogenates. Mercury cannot be related to cell components by such methods, and previous attempts to do so have been, to a certain degree, misleading.[10, 11] Minor variations in centrifuges, and in handling of the tissues and homogenates may change the composition of the fractions in centrifugal cell fractionation.[4] Marker enzyme distribution is thus necessary to know for standardization and comparative studies. These principles are of profound value when distribution of mercury under different conditions are investigated. A more detailed distribution analysis in relation to cell components will, however, be dependent upon the value of the principle of biochemical homogeneity for binding of mercury to cell components. Because there are no reasons for restricting this principle to enzyme proteins and not comprising other proteins and small molecules, it seems that the homogeneity principle is also valuable for the analysis of mercury distribution in the cells.

As shown in Figs. 1 and 2, no obvious resemblance between mercury distribution and the distribution of any of the marker enzymes is found. The distribution pattern indicates some accumulation of mercury in the lysosomes or in the peroximes, but no conclusions can be given. The interpreting of the amount of mercury in the nuclear fraction and in the superntant may be misleading. The homogenate contains small, but unknown amounts of blood with unknown content and distribution of mercury.

Mercury might be expected to distribute as the proteins in the fractions. This was found by Yoshino *et al.*[11] A statistical evaluation of the relative specific mercury content in fractions shown in Fig. 2, indicates, however, differences for some fractions (Table 3). As the protein content in the different cell components are unknown, this comparison gives no answers with regard to the mercury content in relation to protein content within the cellular components.

Mercury distribution might be expected to resemble that of thiol groups in the homogenate. The single experiment demonstrating the thiol distribution allows for no conclusions, but there seem to be greater differences between thiol and mercury distribution than between the distribution of mercury and proteins. As for protein-mercury, no conclusions concerning distribution among cell components can be deduced.

Some remarks are necessary concerning the distribution of mercury in the homogenate compared to the distribution in the cell *in situ*. There is no reason for assuming a redistribution of mercury during the homogenization or fractionation. The cell components are mostly intact, as shown by the constituent distribution figures, and probably only small amounts of previous masked organic ligands appear during

these procedures. The microsomes, however, most likely consisting of broken intracellular membranes, are a possible source of some new ligands with a possible redistribution.

TABLE 3. MERCURY CONTENT IN RELATION TO PROTEIN CONTENT OF FRACTIONS

Fraction	Mercury content Mean ± S.E.M.	P*
Nuclear	0·72 ± 0·04	<0·0025
Mitochondrial	0·94 ± 0·05	0·2960
Light mitochondrial	1·25 ± 0·12	0·1086
Microsomal	0·78 ± 0·04	0·0054
Supernatant	1·36 ± 0·05	<0·0025

Mercury content is given as relative specific values.
*P is computed from the Students *t*-test as the probability of being different from 1 (relative specific protein content).

Statistical distribution analysis

A more detailed analysis of the distribution patterns of mercury in relation to the marker enzymes allows some conclusions to be drawn about the mercury distribution among cell components which are impossible without marker enzymes. The same principle has been used by de Duve *et al.*[14] analysing the binodal distribution of cytocrome reductases, but the multinodal mercury distribution is more complicated. Because no marker enzymes are used for the nuclear fraction and for the supernatant, this analysis is restricted to the microsomal fraction and the two mitochondrial fractions. It must further be assumed that the relative specific enzymic activities really express the relative mass amount of the cell components, and that no mercury is bound to cell components other than those characterized by the four marker enzymes used.

If we assume that the amount of mercury per mg protein in a fraction is the sum of the mercury content in each component of the fraction, and that the mass amount of each component is given by the relative specific enzymic activity of the corresponding marker enzyme, the amount of mercury in a certain fraction is expressed in the following equation

$$\mathrm{Hg} = ax + by + cz + dv. \tag{1}$$

Hg is the relative specific amount of mercury, a, b, c and d are the relative specific activities of the marker enzymes, and x, y, z and v are unknown coefficients.

A reasonable method for estimating these coefficients is by calculating the mean relative specific enzymic activities and mean relative specific mercury content for the five experiments reported, and work out the system of three equations representing the three different fractions as indicated in equation (1). As there are four unknown values, however, it is necessary to add together two of the mean enzymic activities to get a system of equations giving reasonable answers. Peroximes and lysosomes distribute almost identically in this centrifugal system as indicated by the marker enzyme distribution, these enzymic activities are therefore added for the calculations.

The final system of equations is thus

$$\overline{Hg}_M = \alpha_1\overline{X}_{1M} + \alpha_2(\overline{X}_{2M} + \overline{X}_{3M}) + \alpha_3\overline{X}_{4M}$$

$$\overline{Hg}_L = \alpha_1\overline{X}_{1L} + \alpha_2(\overline{X}_{2L} + \overline{X}_{3L}) + \alpha_3\overline{X}_{4L}$$

$$\overline{Hg}_P = \alpha_1\overline{X}_{1P} + \alpha_2(\overline{X}_{2P} + \overline{X}_{3P}) + \alpha_3\overline{X}_{4P}. \quad (2)$$

Hg represents the mean relative specific amount of mercury, $\overline{X}_1$, $\overline{X}_2$, $\overline{X}_3$ and $\overline{X}_4$ represent the mean relative specific activities of 3–hydroxybutyrate dehydrogenase, acid phosphatase, urate oxydase and glucose–6–phosphatase respectively, and α_1, α_2 and α_3 represent the corresponding unknown coefficients. The following probability model which gives identical estimates of the unknown values, indicates that under certain assumptions the above intuitive method will give reasonable and unbiased estimates.

We must assume that the expected mercury content (the 'true' value) of a certain fraction is a linear function of a given (observed) set of enzymic activities. Independence between mercury content in the different pairs of rats is assumed, and the conditional distribution of the mercury content with a given set of enzymic activities is assumed normal. The expectation of the mercury content in one fraction with a given set of enzymic activities must be the sum of the products of the unknown coefficients and the corresponding enzymic activities, and the probability distribution of the enzymic activities must be independent of the coefficients. The covarians in the different pairs of rats must be equal, that means the same sources of variation must exist from one experiment to another. These assumptions seem reasonable, and as the conditional distribution of the mean content of mercury in the fractions also follows the normal distribution curve, the coefficients may be estimated from the probability distribution of the mean mercury content in the different fractions. This will give the same results as the indicated intuitive system of equations. These results are shown in Table 4, and Table 5 shows the computed distribution of mercury among cell components.

TABLE 4. RESULTS OF THE STATISTICAL DISTRIBUTION ANALYSIS (EQUATION (2))

Enzyme	Coefficient	Upper limit of standard deviation*
3–Hydroxybutyrate dehydrogenase (α_1)	0·134	0·034
Acid phsphatase and urate oxydase (α_2)	0·090	0·024
Glucose–6–phosphatase (α_3)	0·188	0·027

* The probability model do not allow accurate calculation of the standard deviations.

A corresponding statistical computation gives a reasonable test for the assumption that mercury does not combine with other components in the three fractions used for the estimations than those characterized by the four marker enzymes. By estimating

the joint mercury content in two groups consisting of two populations of cell components, only two fractions are necessary to get a set of equations. Three fractions are then left for comparison between computed and actually found mercury content under the above assumption. As seen from Table 6, the computed value for the

TABLE 5. MERCURY DISTRIBUTION AMONG CELLULAR COMPONENTS IN CENTRIFUGAL FRACTIONS AND IN THE CELL

Cellular component	Percentage (of total) mercury content in fractions						Percentage in components*
	N	M	L	P	S	Total	
Mitochondria	0·6	12·1	0·1	0·3	0·2	13·3	26·6
Lysosomes and peroxímes	0·9	10·5	2·7	3·0	0·9	18·0	36·0
Microsomes	1·5	4·2	2·3	9·8	0·9	18·7	37·4

* Mitochondria, lysosomes, peroxímes and microsomes taken as 100 per cent.

TABLE 6. COMPUTED AND FOUND MERCURY CONTENT IN FRACTIONS

Fraction	Computed	Found
Microsomal	11·7	13·4
Nuclear	3·5	11·5
Supernatant	1·6	42·5

Values are given as percentage of total mercury content. The found values are from Table 1 corrected for a recovery of 97·3 per cent.

microsomal fraction is in accordance with the found value. The standard deviation of these estimates must be assumed to be of the same magnitude as shown in Table 4. Table 6 also shows that for the nuclear fraction and the supernatant, where mercury is known to combine with components other than those characterized by the marker enzymes, the differences between the computed and the found value are much greater.

No comments are necessary to the distribution of mercury shown in Table 5, but the possibility to adopt this model system for further evaluation of the different aspects of intracellular mercury distribution must be pointed out. Small modifications of this assay would provide more accurate values for the computation of the mercury distribution in the cell. A continuous gradient for further analysis of one of the fractions will provide a system of equations giving more accurate answers. Conditions for differentiating lysosomes and peroxímes are obtainable in certain gradients, and by injecting the animals with Triton WR–1339 to change the sedimentation of lysosomes.[26] The amount of mercury in the nucleus and supernatant may also be computed if the blood content of the tissue is washed out.

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