Centrifugal Method of Determining Red Cell Deformability

By William D. Corry and Herbert J. Meiselman

A recently developed technique for deforming red blood cells (RBC) in which they are centrifuged through buffer and into a glutaraldehyde solution was evaluated as a method of assessing cellular deformability (i.e., the ability of the entire RBC to form a new configuration). To accomplish this, RBC populations of differing cellular deformability were tested, using three generally accepted techniques to obtain these differences: partial fixation with low concentrations of glutaraldehyde, density fractionation, and suspension of RBC in nonisotonic media. Our results indicate that at a constant deforming force the mean deformed length of the RBC decreased under conditions where cellular deformability is known to decrease, thus suggesting the usefulness of this centrifugal method for the estimation of this cellular property.

Erythrocyte deformability plays a critical role in determining the rheologic properties of blood, and it has been implicated in a number of other physiologic phenomena as well.

Although the concept of deformability is intuitively grasped as the resistance a cell offers to a change in its shape, a precise definition of cellular deformability is most difficult to formulate. In that cellular deformability is a mechanical property, physical methods have provided some of the most penetrating insights into some of the factors contributing to the mechanical properties of red blood cells (RBC). If the RBC membrane is slowly stretched over small distances it acts like a viscoelastic material with an elastic modulus of $1 \times 10^{-2}$ dyne/cm and a two-dimensional viscosity of $1 \times 10^{-3}$ poise cm. When the membrane experiences a very large deformation, it exhibits plastic flow and can be characterized by a yield stress and a plastic viscosity of $1 \times 10^{-2}$ poise cm. These measurements were performed under conditions where the cells were stretched slowly enough that cytoplasmic viscous energy losses were negligible. These studies lead to suggestions that the viscoelastic properties of the red cell were attributable to a protein matrix beneath the lipid bilayer of the cell membrane. This speculation agrees with what is known of the red cell membrane ultrastructure.

Unfortunately, techniques for measuring the intrinsic membrane properties of RBC have not evolved to the point where extrinsic cellular properties, such as deformability, can be predicted. Furthermore, it is unlikely that cellular...
deformability can be predicted solely from intrinsic membrane properties, inasmuch as cellular deformability is a function of the cellular surface to volume ratio and the hemoglobin viscosity as well as the mechanical properties of the membrane. This fact was demonstrated by Heusinkveld, who showed that the intrinsic membrane properties of fresh and metabolically depleted cells are not significantly different. However, the cellular deformability of metabolically depleted cells is much less than fresh cells. These findings suggest that studies of extrinsic cellular deformability will have to continue to rely on techniques that measure this cellular property specifically.

A variety of techniques for investigating red cell resistance to deformation are currently available. The majority of these techniques are not designed to examine the deformability of individual cells and are influenced to an unknown degree by interactions of the cells with solid surfaces. Recent work on the deformation of red cells by centrifugal forces has suggested a new method for measuring erythrocyte resistance to rapid deformation. This technique provides a means of rapidly deforming RBC with a known force without requiring the cells to be in contact with solid surfaces. In this process RBC are transformed from discocytes to cells with a roughly spherical head approximately 5 μm in diameter and with a triangular tail. The technique also offers a means of examining differences in the deformation of individual cells in a given cell population.

This study was designed to determine whether or not this centrifugal method of deforming red cells could be used as a method of assessing cellular deformability. To do this, it was necessary to demonstrate that when cells of decreased deformability were stressed by a standard centrifugal field, the strain the cells showed would be proportional to their decrease in deformability. We used generally accepted techniques for obtaining red cell populations of differing deformability (partial fixation of RBC with glutaraldehyde, density fractionation of RBC, and suspension of RBC in nonisotonic buffers), and our results established that the mean deformed length of the RBC decreased in circumstances where cellular deformability was decreased.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS) for red cell suspensions contained 0.2% salt-poor human serum albumin (American Red Cross) and 30 mM phosphate. The osmolarity of the solution was adjusted to the desired level by the addition of NaCl, and the pH was adjusted to 7.42 ± 0.02. All other reagents and materials have been previously described.

Preparation of Cells

Venous blood was taken from healthy donors into a heparinized syringe (0.05 mg heparin/ml blood). The blood was centrifuged, the plasma and buffy coat removed, and the packed cells washed twice in PBS. The tonicity of the PBS was the same as that in which the cells would ultimately be deformed. All centrifugations for washing the cells were at 20°-24°C for 10 min at 2000 g. After the second wash, the cells were suspended in PBS to a concentration of <2 x 10^7 cells/ml.

Deformation of Cells

RBC were deformed via a recently developed high-speed centrifugal technique, the technical details of which have been presented elsewhere. The deformation system consisted of a high-
speed microfuge (Beckman Model 152) mounted in a horizontal position, standard round-bottom, 0.55-ml-capacity disposable microfuge tubes, and a teflon insert designed to fit snugly inside the microfuge tube and to extend within 1.0 cm of the bottom of the tube. The teflon insert served to minimize mixing of the PBS with the glutaraldehyde fixative solution (see below) and to keep the RBC near the axis of the microfuge tube during centrifugation.

Prior to centrifugation, three fluids were placed in the microfuge tube: (1) 75 μl of 2% glutaraldehyde in albumin-free PBS was first placed on the bottom of the tube; (2) 100 μl albumin-free PBS was then gently layered on top of this fixative solution; (3) lastly, following gentle insertion of the above-mentioned teflon insert, 3.4 μl dilute RBC suspension in buffer (≤ 2 × 10⁷ cells/ml) was placed in the top portion of the insert with the aid of a 26-gauge needle. Immediately following this loading procedure, the complete microfuge tube assembly (Fig. 1) was placed in the microfuge and centrifuged at 16,000 rpm (15,000 g average field strength in the glutaraldehyde region) for at least 5 sec. Thus as the cells traversed the length of the tube toward the centrifugal end they passed from the buffer in which they were suspended into the same buffer less albumin and finally into the same buffer containing 2% glutaraldehyde. By the time the cells reached the bottom of the microfuge tube they were fixed in their deformed state.¹⁶

Normal RBC deformed by this process have a nearly spherical head (approximately 5 μm in diameter) and a triangular tail (Fig. 2). The overall length of the deformed cell was determined via light microscopy, using a 100×, NA = 1.30 oil immersion objective and a 12.5× eyepiece equipped with a 140-division eyepiece reticle. RBC were measured for length only when one end of their greatest dimension lay within 2 μm of the focal plane in which the other end lay. Employing these deformation and measurement methods, RBC from several normal donors were found to have an average overall deformed length 3 of 11.0 ± 1.2 μm (mean ± 1 SD) at a centrifugal field strength of 15,000 g.¹⁶

### Determination of Red Cell Density

Red cell densities were determined by the phthalate oil method of Danon and Marikovsky¹⁶ using an IEC Model MB microphototocrit centrifuge. Heat-induced changes in the density distributions were minimized by keeping the centrifuge lid open during the 10-min centrifugation.

### Partial Fixation of Red Cells

Partial fixation of red cells was achieved by suspending red cells to a 2% hematocrit in 0°C, albumin-free PBS containing various concentrations of glutaraldehyde for 80 min as outlined by

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**Fig. 1.** Microfuge tube with teflon insert. Head of teflon insert 0.08 cm from the center of rotation (⊙). Regions to the left of microfuge tube indicate locations of the various fluids immediately prior to centrifugation: A, RBC in albumin-containing PBS in head of teflon insert; B, albumin-free PBS; C, 2% glutaraldehyde in albumin-free PBS. Fluid extends continuously from the bottom of the microfuge up through the bore of the insert to the top of the insert. (With permission, from Corry and Meiselman.)¹⁶
Effect of Intravascular Complement Activation on Granulocyte Adhesiveness and Distribution

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The effects of manipulations that activate complement (C) by the alternate pathway upon the concentration of circulating granulocytes and their adhesiveness were studied in rabbits and humans under several conditions. Whether C is preactivated in vitro and then infused intravenously, activated in vivo by administration of cobra venom factor, or activated during extracorporeal circulation in human hemodialysis, a close correlation between changing granulocyte adhesiveness and granulocyte levels is noted. Shortly after C activation, circulating granulocytes disappear, while their adhesiveness (measured by nylon fiber filtration) increases strikingly. Thereafter, when circulating granulocytes return and actually rebound to above-baseline levels, their adhesiveness declines in parallel. The results suggest that activated C components induce stickiness of granulocytes, which engenders their sequestration. When C-activated plasma is infused intravenously, granulocytes are sequestered selectively in the pulmonary circulation. When C is activated throughout the entire body by the intravenous infusion of cobra venom factor, granulocytes sequester nonselectively. In the former instance, animals develop hypoxemia; in the latter instance, hypoxemia is not observed. Thus granulocytes made hyperadhesive sequester in the first capillary network traversed, and selective organ dysfunction may occur. The results help to explain the recently reported pulmonary leukostasis and dysfunction accompanying hemodialysis.

Interaction of Granulocytes with Various Activated Complement Components

In vitro, the interaction of granulocytes with various activated complement components provokes several alterations in their function and metabolism. Thus such components may cause granulocytes to demonstrate chemotaxis, attach to and phagocytose particulate matter, and release lysosomal constituents. However, the importance in vivo of these complement-related effects is uncertain, although it seems likely that complement-granulocyte interactions are critical to host defense and inflammatory phenomena. For instance, animals depleted of complement capable of being activated are inefficient in localizing granulocytes to diverse inflammatory sites. Patients who genetically lack certain complement components may suffer severe and chronic bacterial infection.

To study the interaction of complement with granulocytes in vivo, other investigators infused animals with activated plasma, purified components of activated plasma, or various complement activators such as cobra venom factor. In rabbits, each technique has been shown to induce profound, albeit transient, granulocytopenia. Our laboratory confirmed and extended these results by showing that the granulocytopenia following intravenous infusion of plasma

**Density Separation of Cells**

Cells were separated on the basis of their density by a modification of Murphy's method consisting of centrifuging the cells in their plasma rather than in their serum.

**Measurement of Cell Volume**

Mean cell volumes were calculated from measurements of the hematocrit and concentration of cells in a given suspension. Hematocrits were measured using the microhematocrit technique, and cell concentrations were determined on an Electorzone-celloscope, an orifice-type electronic cell counter and sizing apparatus (Particle Data Systems, Elmhurst, Ill.). Mean cell volumes were determined on nondeformed cells.

![Fig. 2. Scanning electron micrographs of typical RBC deformed in relative centrifugal field of 15,000 g. (A) RBC with flat tail; (B) deformed RBC with three lobed tails. Both tail shapes are commonly observed.](image)

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**Fig. 3.** Mean deformed length of red cells after they have been partially fixed in various concentrations of glutaraldehyde. Each point represents the mean and standard deviation of at least 90 cells. All RBC were obtained from the same donor.

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activated with inulin, zymosan, polycellulose, or nylon fibers resulted from sequestration of granulocytes, particularly in pulmonary capillaries. Of pathophysiologic interest is our finding that the polycellulose membrane utilized in hemodialysis coils is capable of potently activating complement predominantly through the alternate pathway (much as do other polysaccharides such as zymosan, endotoxin, and inulin). From these data we suggested that complement-induced granulocytopenia with its accompanying pulmonary leukostasis causes the acute and chronic pulmonary dysfunction that can occur during hemodialysis of uremie patients. We also suggested that dialysis-coil cellophane might be utilized as a convenient, sterilizable, and easily removable complement activator.

In the present studies we explored the mechanism whereby activated complement components produce neutropenia. A previous, serendipitous observation that exposure of granulocytes to complement-activated plasma rendered them excessively adherent to plastic Petri dishes suggested that activated complement components in the circulation might increase granulocyte adherence to endothelium, i.e., might promote their "margination." Hence the potential for localized organ dysfunction from capillary plugging by granulocytes would seem real.

Indeed, utilizing a newly described assay of granulocyte adhesiveness to nylon fibers, we demonstrated in the present experiments that granulocyte adhesiveness, measured in vitro, is altered rapidly and inversely to circulating granulocyte counts during complement activation. Adhesiveness strikingly increased as granulocytes disappeared from the circulation shortly after complement activation. Conversely, when circulating granulocytes reappeared and actually rebounded to supernormal levels, adhesiveness in vitro decreased markedly. That the correlate in vivo of this complement-induced excessive adhesiveness (i.e., vessel plugging) can cause organ dysfunction—particularly of the lungs—is also shown.

MATERIALS AND METHODS

Infusion of Preactivated Complement Into Rabbits

Under local anesthesia (1% procaine) a femoral artery and vein were cannulated in each of a number of albino white rabbits weighing 2-4 kg. Arterial blood was immediately placed in ice and centrifuged at 10,000 g for 10 mm at 0°C. The derived plasma, with heparin (1 U/ml) added, was placed in plastic containers, and its complement was activated as previously by exposure to either polycellulose dialysis membrane (Gambro, Newport News, Va.) (20 sq cm/cc) or inulin (0.2 mg/cc) for 30 mm with gentle agitation at 37°C. Thereafter, plasma was freed of dialysis membrane manually or of inulin by recentrifugation (at 10,000 g for 10 mm). Documentation of predominant alternate pathway activation of C by these two methods was provided previously by us and by others.

The activated plasma was rapidly reinfused into the venous catheter of the donor animal. At various times thereafter, samples from the arterial catheter were assayed for granulocyte counts and adhesiveness and compared to samples obtained 30 sec prior to infusion.

Activation in Vivo of Complement in Rabbits

Cobra venom factor (CVF), prepared and purified by the technique of Cochrane et al., was stored at -10°C until rewarmed and infused (40 U/kg) into the venous catheter of rabbits prepared as above. To measure pulmonary clearance of granulocytes after infusion of activated plasma or after injection of CVF, the arterial and venous catheters were advanced into the thorax and granulocyte counts were performed on samples obtained simultaneously from both catheters.

RESULTS

Deformation of Partially Hardened Cells

Partial fixation of red cells decreased cell deformation by the centrifugal technique. This effect is seen in Fig. 3, where the mean length of the deformed cells \( \bar{x} \), is shown to decrease as the concentration of glutaraldehyde used to treat the cells increases. With the exception of the cells fixed in 0.0189\% glutaraldehyde, the mean deformed length of each cell population is significantly \( (p < 0.001) \) less than the length of the cells treated with a lower concentration of glutaraldehyde. Determinations of cell density showed that the concentrations of glutaraldehyde used to partially fix the cells did not change their density.

The effect of glutaraldehyde and the capacity of the technique to detect differences in cell deformability are also shown in Fig. 4, a histogram of the frequency of the deformed cell lengths from a cell suspension made by mixing equal numbers of unfixed cells and cells partially fixed with 0.0125\% glutaraldehyde. The unfixed and glutaraldehyde-fixed cells were mixed after half the cells had been exposed to glutaraldehyde but before they were deformed. The two populations are clearly resolved in the histogram.

Deformation of Density-separated Cells

Figure 5 shows the mean deformed length of density fractionated cells as a function of the mean density of the various cell fractions for three individuals. In all cases \( \bar{x} \) decreased with increasing cell density.

Effect of Tonicity

Figure 6 demonstrates the manner in which the relative deformed length of the cell changes as a function of relative cell volume. The relative volume of the cell refers to the ratio of the nondeformed cell’s volume in a buffer of given tonicity to its volume under isotonic conditions. The relative deformed length of the cell is the ratio of \( \bar{x} \) in a given buffer to \( \bar{x} \) in isotonic conditions, \( x_0 \). Cells deformed in isotonic PBS have by definition a relative cell volume and a relative deformed length of 1.0. When cells were placed in increasingly hypotonic
media, the mean volume of the cells increased while their relative deformed length decreased. In hypertonic media the relative deformed length of the cells initially increased as their volume decreased; however, with further increases in the tonicity of the suspending buffer the relative deformed length of the cells dropped precipitously.

Experimental measurements of cell volumes and density in each suspending medium showed that the mean buoyant weight of the cells did not change with the tonicity of the suspending medium. The mean buoyant weight of the cells was calculated as the product of mean cell volume and the mean buoyant density of the cells.

DISCUSSION

New techniques for measuring a given property are usually validated by testing the technique on systems whose properties are known. A recently developed method of centrifugally deforming red cells was evaluated as a means of assessing red cell deformability by determining if the strain exhibited by cells, which had been stressed by a standard centrifugal force of known duration, was proportional to the deformability of the cells. Erythrocytes with varying degrees of deformability were obtained by partially fixing the cells with...
glutaraldehyde, using erythrocytes of various ages and suspending cells in media of differing tonicity.

The most direct method of demonstrating that the mean deformed length of a cell, $\bar{x}$, decreased with decreasing cell deformability was to measure $\bar{x}$ for cells exposed to low concentrations of glutaraldehyde. Cells exposed to glutaraldehyde or various other lower aldehydes show different properties than unfixed cells owing to the ability of the aldehydes to act as protein crosslinking agents.\(^{20}\)

Whereas complete fixation of red cells with various aldehydes has been shown virtually to eliminate cellular deformability,\(^{12}\) incremental decreases in erythrocyte deformability can be achieved by exposing the cells to very low concentrations of glutaraldehyde.\(^{10}\) As shown in Fig. 2, the mean deformed length of the cells was inversely proportional to the concentration of glutaraldehyde used to treat them. Since the process of glutaraldehyde fixation did not alter the density of the cells, all cells experienced the same deforming forces. Thus decreases in cellular deformability were reflected by a decrease in $\bar{x}$.

A second test of the technique involved a determination of $\bar{x}$ for cells separated on the basis of their density. The rationale behind this test lay in the observations that cell density increased with cell age\(^{21}\) and that cell deformability decreased with cell age.\(^{12}\) Figure 5 shows that $\bar{x}$ decreased with the increasing average density of the density fractionated cells of the three individuals tested. The mean deformed length of the older cells was always less than that of the younger cells. The differences between adjacent points in the figure were statistically significant ($p < 0.05$) even though the actual differences in the deformability of the various density fractions were underestimated. In this study all cells were deformed by equal centrifugal fields (15,000 g). Since the older cells were more dense but presumably of the same mass as the younger cells, they should have experienced a greater deforming force than the younger cells. Thus if the deforming forces on the various cells were equalized, the differences in $\bar{x}$ between young and old cells would have been even greater.

The last test of the relationship between $\bar{x}$ and cellular deformability was to examine $\bar{x}$ after the deformability of a red cell population had been changed by manipulating the surface area to volume ratio and the hemoglobin viscosity of the cells. The rationale for this test was derived from a number of studies showing that hypotonic\(^{22,23}\) and hypertonic\(^{12}\) solutions altered the deformability of red cells. Figure 6 corroborates previous findings on the effect of viscosity on red cell deformability. Cells suspended in hypotonic solutions showed increases in mean relative cell volumes and decreases in $\bar{x}/\bar{a}$ that paralleled the decrease in the viscosity of the suspending medium.

Decreased cell deformability in hypotonic solutions is usually attributed\(^{1}\) to a decrease in the cellular surface area to volume ratio. In the present system, increases in the volume of the cellular contents should have decreased the total deformed length of the cell by increasing the amount of cell membrane required to encompass the cellular contents. Since the total red cell membrane area was constant, less membrane was available for tail formation.

When RBC were suspended in hypertonic media, their volume decreased while their density and surface area to volume ratio increased. Cells deformed in mildly hypertonic suspensions were slightly longer than those deformed in isotonic PBS (Fig. 6). This finding was presumably due to simultaneous in-
creases in their density and surface to volume ratio. These changes should have increased the fluid drag forces on the cell and the amount of cell membrane available for tail formation. However, when the tonicity of the suspending medium decreased cell volumes to less than 80% of their original size (Fig. 6), precipitous decreases in $(\bar{x}/\bar{x}_0)$ were observed. Although tonicity increases raised the cellular surface to volume ratio, they also produced large increases in the viscosity of the intracellular hemoglobin solution. The data of Cokelet and Meiselman suggest that the viscosity of a hemoglobin solution should increase approximately 600% when the solution concentration of hemoglobin is raised from the normal intracellular level (33 g/dl) to a concentration of 43 g/dl. This increase in hemoglobin concentration should be produced by a 30% reduction in cell volume. Several authors suggested that when intracellular hemoglobin is concentrated to this extent, a liquid-gel or liquid-crystal phase change in the hemoglobin should occur. Such a transformation could easily cause a stiffening of the cell. Thus dehydration of erythrocytes in hypertonic solution probably decreases cell deformability by causing increases in the viscosity of the intracellular hemoglobin that more than offset the effect of an increased cellular surface area to volume ratio.

It is apparent from the above data that decreases in cell deformability are consistently paralleled and can be detected by decreases in $\bar{x}$. This contention is further substantiated by Fig. 4, which shows a histogram of $\bar{x}$ versus frequency of occurrence in a population of cells where 50% of the cells have been partially hardened with glutaraldehyde. The histogram indicates the presence of two populations and demonstrates one of the technique's most important assets: it provides an average measure of cellular deformability as well as estimates of the range and scatter of cellular deformabilities in a given sample. Thus it can be used to examine rapidly a sample of cells for subpopulations that are more or less deformable. An additional advantage is its ability to examine the deformation shown by cells deformed in the absence of contact with solid surfaces.

While the present technique for measuring cell deformability meets the traditional definition of measuring cellular strain in response to a given stress, the type, rate, and extent of cellular strain caused by centrifugal forces are different from those the cell experiences in vivo or with other techniques for assessing cellular deformability. This difference makes a comparison of the results of different techniques difficult, since they may measure different cellular properties. Furthermore, in the absence of a precise mechanical description of shape changes in vivo, the relationships between the measured quantity "cellular deformability," and cellular resistance to shape changes in vivo are unknown. However, until a molecular or quantitative definition of cellular deformability is obtained, semiquantitative techniques for studying cellular resistance to deformation must be employed. The present technique presents another method for studying this important area of cellular physiology.

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Fig. 5. Site-dependent effect of C activation upon pulmonary function and PMN clearance. (A) When cellophone-activated plasma (2 mL/kg) was reinfused into venous circulation of rabbits, pulmonary clearance of PMN rapidly increased accompanied by arterial hypoxia. (B) In contrast, in T transfusion of purified cobra venom factor, which activates C through-out the entire vascular system, did not significantly alter pulmonary PMN entrapment or arterial oxygenation. Each point represents duplicate assays in each of three animals; points significantly different (p < 0.05) from A post-infusion time compared to pre-infusion levels.

DISCUSSION

These data demonstrate that during complement activation in animals or in hemodialyzed patients a close association exists between excessive adhesiveness of granulocytes, as measured in vitro, and their sequestration from the circulation. A reasonable explanation is that granulocytes induced to be "sticky" by interaction with activated complement components attach to endothelium and/or to other granulocytes and are thereby removed from the circulating pool of cells. We acknowledge that rigorous proof of a causal relationship between these two phenomena is not yet available; other more recent observations, however, also tend to support a possible causal relationship. When infused into rabbits, highly purified Cs induces neutropenia and simultaneous changes in GA similar to those found in the present studies. Moreover, pulmonary capillary plugging by granulocytes can be demonstrated in animals infused with activated C components during the same period of particularly excessive GA (e.g., within 5 mm of C infusion). It is noteworthy that others have also suggested that the nylon fiber adherence assay may predict granulocyte stickiness to endothelium and have reported changes in GA identical to those presented here that occur during human extracorporeal hemodialysis. Moreover, these authors have demonstrated that two fluid-phase constituents, one of which is probably activated C, may augment GA. The existence of other possible GA augmenters remains to be determined, although coagulation, fibrinolytic, and kallikrein activation products seem worthy potential candidates. In addition, the possibility that bacterial endotoxins might alter GA, independent of C activation, should also be considered. Thus others have observed that neutropenia may follow endotoxin administration to C-depleted or genetically C6 deficient rabbits.

Further knowledge of possible variables in the GA assay was also gained in the present studies. We became particularly concerned that changing concentrations of the various filtered blood elements might systematically affect granulocyte entrapment by the nylon fibers. Since levels of granulocytes themselves changed so drastically during C activation (Figs. 2-4), analysis of the effect of varying leukocyte numbers on GA was required before an interpretation of our results became feasible. In fact, GA increased directly with the logarithm of granulocyte count (Fig. 1). Since in the present studies adhesiveness increased measuring the deformability of the red cell membrane. Blood 45:581-586, 1975.

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