Supplementary Material for
The free heme concentration in healthy human erythrocytes

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Materials and methods

Reagents and solutions

We purchased apo horse radish peroxidase (apo-HRP) from BBI Enzymes, Gwent, UK and used it without further purification. We used two batches of apo-HRP, 109 and 118; as demonstrated below, they showed identical results. We purchased hematin (Fe3+ protoporphyrin IX OH), hemin (Fe3+ protoporphyrin IX Cl), and all other reagents from Sigma-Aldrich. 96 well plates (half area flat, white) were purchased from Greiner. We used a multi-channel pipette (Mettler Toledo) for solution deposition into the wells. The consistency of the volumes disposed by the individual channels was found to be within 3%. We used an Infinite 200 PRO microplate reader (Tecan) to monitor the luminescence intensity.

Deionized (DI) water was prepared by Millipore reverse osmosis – ion exchange system RiOs-8 Proguard 2 – MilliQ Q-guard. To prepare standard solutions of hematin and hemin for the analytical procedure we dissolved the respective dry powder in DMSO at concentration ca. 1 mg mL^{-1}. We diluted these solutions by a mixture of 66.5% ethanol, 17% acetic acid, and 16.5% DI water (v/v). We spectrophotometrically determined the exact concentration of each preparation using the solvent-specific extinction coefficient 144 $mM^{-1}cm^{-1}$ at 398 nm for both reagents.[1]

We purchased luminol (3-amino-phthalhydrazide) and peroxide as two parts of the binary reagent Immobilon from Millipore. The concentration of luminol in the commercial solution is a trade secret. We spectrophotometrically determined the concentration of peroxide as 4 mM; for this we used an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm.[2] Both reagents were diluted 8-fold prior to use.

We prepared a stock solution of apo-HRP in deionized water with approximate concentration 2.5 mM. We spectrophotometrically determined the exact concentration using extinction coefficient of 20 $mM^{-1}cm^{-1}$ at 278 nm.[3] We stored the apo-HRP stock in a freezer at -80°C and thawed samples when needed.

We prepared Tris (tris(hydroxymethyl) aminomethane, $(HOCH_2)_3CNH_2$) buffer by dissolving dry powder in deionized water at concentration 100 mM and adjusting the pH to 8.4 by adding HCl. We prepared 0.15 M phosphate buffer at pH = 7.35 by dissolving K_2HPO_4 and KH_2PO_4 in water and adjusting the pH with KOH or H_3PO_4.

Source of blood

Blood from healthy adults was collected at the University of Houston Health Center (UHHC) following a protocol approved by the University of Houston Committee for Protection of Human Subjects. The blood was drawn in green cap Vacutainer tubes, which contain sodium heparin to prevent blood coagulation. The time between blood collection and the start of its analysis was approximately 30 min. During the transfer from UHHC to the laboratory, the tubes with blood were held in a Ziploc bag enclosed in a Styrofoam box that holds the temperature of the blood at ca. 23°C. In some cases, to test the effects of the time between blood collection and processing, tubes with blood were held at room temperature, ca. 23°C, for up to four hours. In a few other cases, whole blood was stored in a laboratory refrigerator for up to two days. As shown below, within these parameters of storage, the concentration of free heme in red cells was independent of the time lag between blood drawing and analysis.

Several blood samples were provided by the Gulf Coast Regional Blood Center (GCRBC) in Houston. The blood was held in grey cap tubes, which contain fluoride and oxalate to prevent blood coagulation.

All identifiers of the blood donors were removed and the blood was transferred to the University of Houston for analyses. The time between blood drawing and the start of its analysis was up to four hours. During this time, the tubes with blood were held in a Ziploc bag enclosed in a Styrofoam box that maintains a stable temperature of ca. 23°C. We found no differences in the free heme concentration between blood samples collected at UHHC and GCRBC.

Preparation of hemolysate

Figure S1 illustrates the procedure for preparation of red blood cell hemolysate. We loaded 4 mL of blood in a 50 mL tube. We added 45 mL of isotonic 0.9% NaCl solution in water and gently agitated the tube to homogenize the suspension. We centrifuged the diluted blood for 20 minutes. We decanted and discarded the supernatant containing blood plasma, white blood cells, and other blood components. We repeated the sequence of washing with NaCl solution, centrifugation, and supernatant removal two additional times for a total of three washes. After that we added from 5 to 50 mL of deionized water to the precipitated red blood cells and agitated the solution to resuspend them in the added water. The red blood cells ruptured under the influence of the osmotic pressure difference between the cells and the water. We centrifuged this suspension for 35 minutes to compress the cell membranes at the tube bottom, decanted the hemolysate, and stored it in a refrigerator at ca. 5°C in another sterile capped tube.

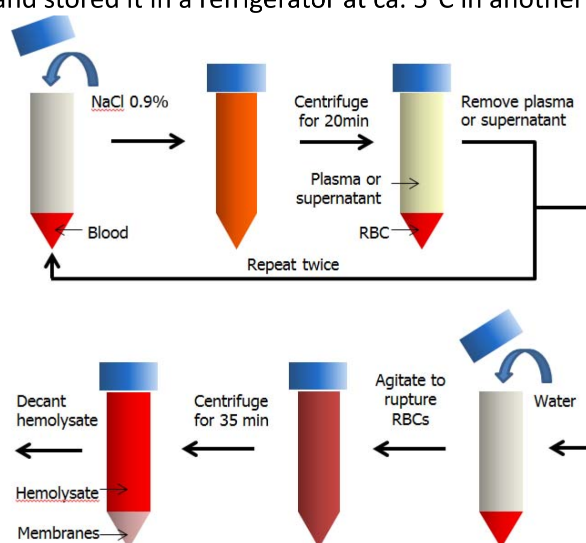

Figure S1. Schematic illustration of the hemolysate preparation.

To evaluate the dilution ratio of the hemolysate compared to the red cell cytosol, we converted hemoglobin to its cyan-met form using Drabkin's reagent. We spectrophotometrically determined the hemoglobin concentration in the hemolysate, using an extinction coefficient for cyanomethemoglobin of 1.512 mL $mg^{-1}cm^{-1}$at 540.0 nm.[4] We assumed that the hemoglobin concentration in the cytosol of healthy red cells is 350 mg mL^{-1} (corresponding to mean corpuscular hemoglobin concentration (MCHC) of 35 g dL^{-1}) and calculated the dilution ratio as the ratio of the two concentrations. Typical hemoglobin concentrations of the hemolysate were between 2 and 20 mg mL^{-1} (ca. 30 and ca. 300 μM), corresponding to dilution ratios between 175 and 17.5×, respectively.

To test the veracity of this method of evaluation of the hemolysate dilution, we weighed a sample of three-fold washed and centrifuged erythrocytes. We assumed that the measured mass is that of the cytosol only and estimated the volume using a density of 1.00 g mL^{-1}. This estimate yielded a dilution ratio lower by ca. 50% than the one based on the hemoglobin concentration. However, the estimate ignored the mass of the cellular membranes and of the saline solution trapped between the red cells. Since both

errors lead to an exaggerated erythrocyte volume and lower cytosol hemoglobin concentration, the veracity of the concentration-based estimate of the hemolysate dilution ratio is supported.

Separation of free heme from hemoglobin

We placed 3.00 mL of hemolysate into a 3 mL dialysis cassette (Slide-A-Lyser from Thermo Scientific with a 2,000 g mol^{-1} molecular weight cut-off) with a syringe. We inserted another syringe on the other side of the dialysis cassette to remove the compressed air. We suspended the cassette in a beaker containing 1.000 L (measured by a 1 L volumetric flask) of 100 mM Tris (tris(hydroxyl-methyl)aminomethane) buffer at pH = 8.4. We sealed the beaker with parafilm, placed it on a stir plate in the refrigerator at ca. 5°C, and agitated the outside solution with a 2 inch stirring bar. The duration of the dialysis was from 14 hours (overnight) to 14 days. We took samples of the dialysate and determined the concentration of free heme in them as discussed below. We calculated the concentration of free heme in the hemolysate from the ratio between the volume of the solutions in the outside beaker and the dialysis cassette. Typically, the latter ratio was 333×, leading to a dilution ratio of 334×.

Validation of the method

The tubes for blood collection.

We collect the blood in tubes containing anticoagulants, which allow easy isolation of the erythrocytes. We considered three types of tubes: with green caps, containing sodium heparin, purple caps, containing ethylenediaminetetraacetic acid (EDTA), and with grey caps, containing fluoride and oxalate. The other types of anticoagulant tubes contain the same reagents at different concentrations or reagent combinations.

To test if these anticoagulants interfere with the determination of free heme, we tested their effects on hematin and hemin dissolved in Tris buffer at pH = 8.4. For this, we filled the tubes with hematin or hemin solutions at concentrations ca. 20 μM, turned over the tubes to dissolve the anticoagulant and kept the solution in them for 30 min. We compared the spectra of the solution held in the tubes to that of identical solutions kept in the preparation containers. We observed that the spectra of hematin and hemin solutions held in green and grey cap tubes were essentially undistinguishable from those of solution kept in laboratory glassware. However, we observed that in purple cap tubes, in the presence of EDTA, the absorbance of both hematin and hemin is significantly reduced in the entire wavelength range, indicating that the concentration is reduced. A hypothetical mechanism of this reduction is through the chelation of Fe^{3+} by EDTA. These observations suggest that EDTA may interfere with the determination of free heme in the red cell cytosol; hence, we avoided using purple cap tubes and only used those with green and grey caps.

Accuracy and sensitivity of the method.

The accuracy and sensitivity of the enzymatic method of quantification of free heme can be evaluated from the reproducibility of the standard curves, such as those in Fig. 1B. We plot in Fig. S2A eight standard curves. We see that for hemin concentrations ≥ 200 pM, the relative error of the standard curves is ± (12 – 15)%. The main source of uncertainty is the inaccurate preparation of the solutions of H_2O_2, luminol, and apo-HRP, loaded in the wells: it involves several steps, in which we pipette small volumes. To minimize this uncertainty, we determine a standard curve for every analyzed sample. In this way, the standard and the tested solutions are prepared with identical reagent concentrations. With this, the error of the determination of the concentrations of free heme in the dialysate is estimated as 5 %.

The sensitivity of the method, i.e., the lowest amount of free heme that can be accurately detected and measured, is determined by the standard value of the intensity decay rate constant k at C_{heme} = 0 pM. As Fig. S2A demonstrates, this value varies from zero to the point, at which the standard data at non-zero heme concentrations intercept the k axis. Values higher than the intercept point were never

recorded. If the value of the standard curve at C_{heme} = 0 pM is lower than the intercept point, the lowest value that can be determined by this method is that of the lowest standard concentration that belongs to the linear $k(C_{heme})$ dependence. If $k(C_{heme} = 0)$ is a part of the linear dependence, the sensitivity is determined by the combination of the uncertainties of the determinations k and C_{heme}. In all cases, in which we measured low hematin concentrations, we used standard curves of the latter type. With this, the sensitivity of the method varies from 20 to 50 pM.

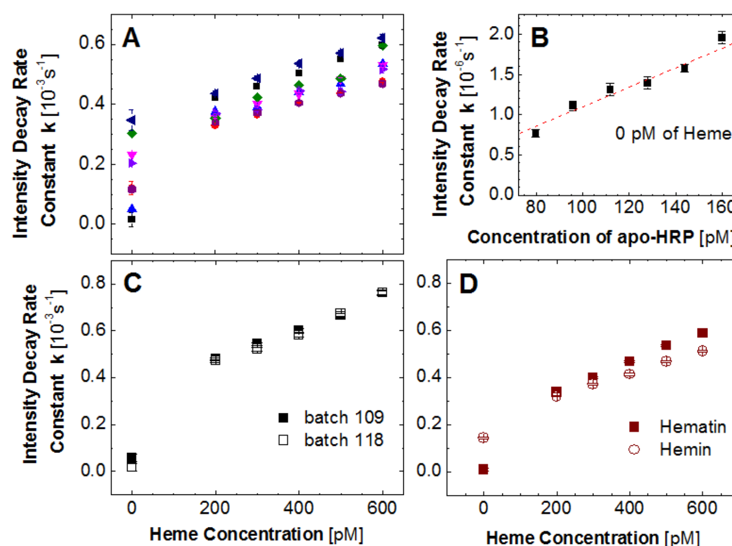

Figure S2. Validation of the method. **A.** Reproducibility of the standard curves; eight curves are shown. **B.** The luminescence intensity decay rate constant in the absence of heme. **C.** The activity of the two batches of apo-HRP used. **D.** Comparison of the activities of hemin and hematin.

The apo-HRP preparation.

The standard curves, such as those in Fig. 1B, do not extrapolate to $k = 0$ at $C_{heme} = 0$. Similar dependencies that do not extrapolate to the coordinate origin were reported in other paper on the enzymatic method of heme determination.[5] Two feasible explanations are: (i) the apo-HRP preparation that we use contains residual amounts of holo-HRP that catalyze the decomposition of H_2O_2 even in the absence of added heme, and (ii) H_2O_2 decomposes even in the absence of HRP and the rate of this decay is reflected in the intercept.

To test the combined effect of both processes, we estimated the catalytic activity of apo-HRP in the absence of free heme. We intentionally chose relatively low apo-HRP concentrations, from 80 to 160 pM, in which we determined the rate of decay of H_2O_2. The results in Fig. S2B indicate that apo-HRP has a detectable catalytic activity. To evaluate this activity at the concentration used in the heme determination runs, we extrapolate the value of k from Fig. S2B to 600 pM and obtain $k \approx 7.5\times10^{-6}$. This value of k is (50 – 100)× lower than the value for 600 pM of hemin Fig. S2A. This ratio suggests that the apo-HRP preparation that we use contains about (1 – 2)% of residual holo-enzyme.

Figure S2B demonstrates that the $k(C_{apo\text{-}HRP})$ dependence extrapolates to $k = 0$ at $C_{apo\text{-}HRP} = 0$. This extrapolation suggests that the spontaneous decay of H_2O_2 in the absence of enzyme is an insignificant contributor to the kinetics that we monitor.

To test if the quality of apo-HRP is consistent, we compare in Fig. S2C the decay rate constant for the luminescence intensity measured in solutions with known hemin concentrations using apo-HRP from two different production batches, 109 and 118, from the same manufacturer. The two standard curves are very close, indicating that the activities of the apo-HRP preparations from the two batches are similar.

The results in Figs. S2B and S2C suggest that the standard curves do not extrapolate to zero at low hemin concentrations not because of spontaneous decomposition of H_2O_2 and that the contribution of holo-HRP, present in the apo-HRP preparation is less than (1 – 2)%. Comparing the value of *k* extrapolated form the low concentration data in Fig. 4B to $C_{apo\text{-}HRP}$ = 600 pM to the values at zero heme concentration in the standard curves, in Figs. 1B and S2A (which are at the same $C_{apo\text{-}HRP}$ = 600 pM) we see that in some cases they are close, while in other cases they differ by orders of magnitude. The inconsistency of the peroxide decay rate recorded the absence of heme suggests a non-linear kinetic response, leading to irreproducible data. However, in the presence of any amount of added hemin or hematin, this response is maximized and consistent and it does not affect the signal from the added heme.

Standard curves with hematin and hemin.

In Fig. S2D we compare standard curves determined with hematin and hemin. We see that the difference between the two curves is lower than the variability of the standard curves for hemin.

Does heme unbound to apo-HRP affect the determination?

It is possible that a fraction of the free heme in the tested solutions may remain unbound to apo-HRP and independently contribute to the decomposition of H_2O_2 and the luminescence intensity.[6] If the distribution of the heme between the bound and unbound states is inconsistent, equal total heme concentrations might elicit different intensities. Thus, the distribution of free heme between bound and unbound states may lead to an error in the determination of the total hematin concentration by this method. To evaluate the magnitude of this error, we determined the catalytic activity of hematin in H_2O_2 decomposition in the absence of apo-HRP, using the method discussed above.

Figure S3A demonstrates that: (i) the luminescence intensity is approximately two orders of magnitude lower than in the presence of apo-HRP, compare to Fig. 1A; (ii) it does not decrease, as one would expect if the concentration of H_2O_2 decreased as a result of its decay; and (iii) this intensity changes randomly in response to the increasing concentration of hematin, e.g., in Fig. S3A, the highest intensity is recorded at C_{hematin} = 450 pM and the lowest—at C_{hematin} = 300 pM. We conclude that the data in Fig. S3A do not correspond to hematin-catalyzed decay of H_2O_2, but rather to a fluctuating reading within the error range of the intensity measurement that may be due to electronic or other noise. The possible reason for the lack of activity of hematin in these solutions is the relatively low pH = 8.4 in our determinations—hematin activity has been demonstrated at more basic pH's.[6, 7] We conclude that the error in the determination of the hematin concentration by the enzymatic method that may be introduced by its incomplete binding to apo-HRP is < 1%.

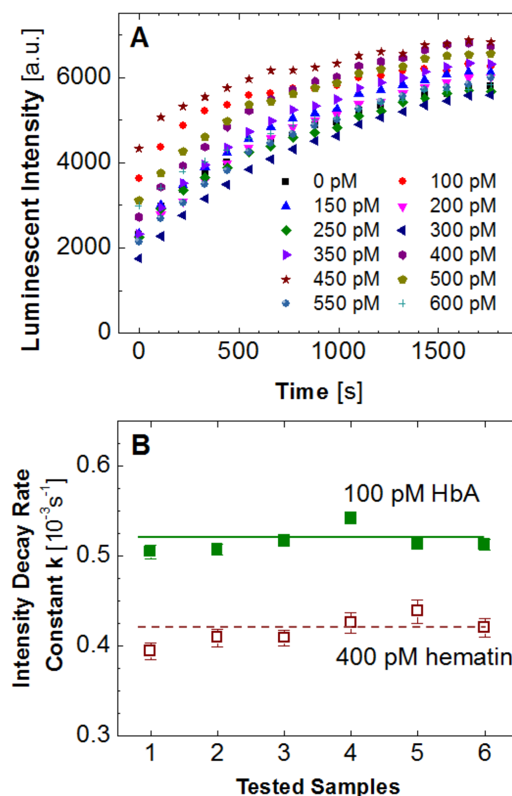

Figure S3. **A**, Evolution of the luminescence intensity emitted from solutions with hematin concentrations as indicated in the plot in the absence of apo HRP. **B**, Comparison of the activity of normal hemoglobin and hematin at the concentrations indicted in the plot.

Does hemoglobin leak into the dialysate?

A disadvantage of the luminol reaction, used for quantification of the heme concentration, is that it has very low selectivity for iron containing compounds.[6] Thus, if hemoglobin leaks through the dialysis membrane due to a defect, it may contribute to the total luminescence intensity and in this way bias the quantification of free heme.

To evaluate the possible error due to leaking of hemoglobin from the dialysis bag, we determined the catalytic activity of healthy hemolysate in which the hemoglobin concentration was 100 pM and compared it to the catalytic activity of 400 pM hematin, in which the total heme concentration is equal to that in the hemoglobin solution. We characterized the activity in terms of the rate constant for decay of the luminescence intensity. Since our goal was to simulate the leak of hemoglobin into the dialysate, we determined the activity of hemoglobin in the presence of apo-HRP.

Fig. S3B demonstrates that the activity of hemoglobin is ca. 25% higher than the activity of an equivalent concentration of hematin. Thus, if hemoglobin leaked it would introduce a significant component to the intensity of the luminescence light and to the rate of peroxide decomposition. On the other hand, it is highly unlikely that such leaks in independent dialysis runs would be identical. Hemoglobin leakage would lead to inconsistent values of *k*. Since the data presented above reveal consistent *k* values, we conclude that hemoglobin leakage did not significantly contribute to the values of *k* used for the quantification of free heme concentration.

Comparison with a previous determination of the free heme concentration

We are aware of a single previous determination of the concentration of free heme in sickle and healthy erythrocytes,[8] which found concentrations of, respectively, 0.75 and 0.15 μM. The

concentration found in healthy adult erythrocytes is more than two orders of magnitude lower than the one in Fig. 3. Liu *et al*. separated free heme from hemoglobin by charge, using ion exchange liquid chromatography,[8] in contrast to the separation by size using dialyses, employed by us. To isolate the hematin, Liu *et al*. added 2 M NaCl to the hemolysates. At this electrolyte concentration, the hematin was retained on the column. The captured hematin was then eluted by a solution of sodium dodecyl sulfate (SDS). To validate the method, Liu *et al*. added hematin to the hemolysate and retrieved 75% of the added amount after the SDS elution.

To understand the effect of NaCl to the state of hematin in solution, we added 50 mM or 2 M NaCl to a 20 μM solution of hematin in 0.15 M phosphate buffer. To test for hematin aggregation or crystallization, we filtered these solutions through a 0.22 μm filter and compared the UV-Vis spectrum of filtered and unfiltered solutions. Since hematin dissolution in aqueous solvents is slow,[9] we expect the presence of undissolved aggregates in the initial solution. The spectra in Fig. S4A confirm this aggregation: the solution is turbid, judging from the absorbance at wavelengths greater than 700 nm, and filtration leads to reduction in absorbance. The spectrum of the filtered initial hematin solution corresponds to those in the literature.[10]

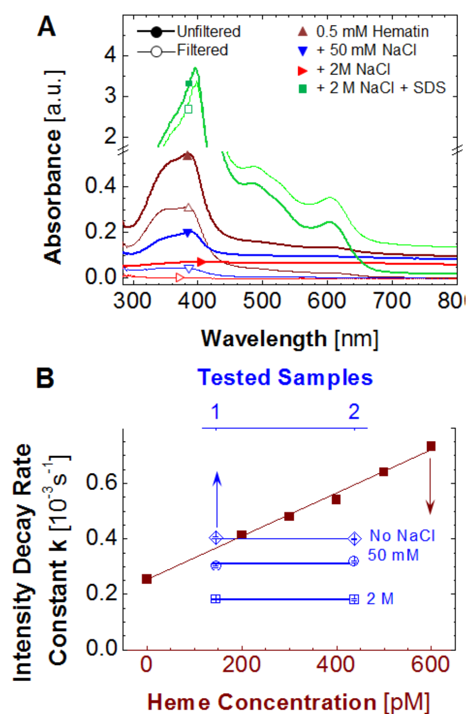

Figure S4. The effects of NaCl addition on the free heme in solution. **A.** Spectra of a 0.5 mM hematin solution in 0.15 M phosphate buffer at pH = 7.34 and after the addition of 50 mM NaCl, 2 M NaCl, and 2 M NaCl followed by the addition of 20 mM sodium dodecyl sulfate, SDS. **B.** Determination of the concentration of free heme in the dialysate after 20 hours of dialysis of healthy hemolysate in the presence in the dialysis

The addition of 50 mM or 2 M NaCl to an unfiltered hematin solution lowers the absorbance at all wavelengths, Fig. S4A. The turbidity in the respective spectra is removed by filtration, indicating that it is due to hematin aggregation. Microscopic observation of the unfiltered solutions revealed the presence of brownish clusters that are, likely, precipitated hematin. Comparing the spectra of filtered solution before and after the additions 50 mM and 2 M NaCl reveals that of the additions remove, respectively, ca. 80 % and nearly 100 % of the dissolved hematin.

The addition of SDS to unfiltered solutions holding 2 M NaCl fully solubilizes the precipitate (note the disappearance of turbidity) and likely some undissolved hematin and the optical absorbance increases to levels above those of the initial solution. The dissolved hematin is likely held in SDS micelles. Filtering the SDS containing solution increases its turbidity, likely due to micelle flocculation. These observations suggest that the hematin added for calibration by Liu *et al*.[8] was precipitated, retained as a solid at the top of the column, and subsequently dissolved and eluted by SDS for nearly complete recovery. This conclusion casts doubt on the ability of the column employed by Liu *et al*.[8] to retain free heme and in this way separate it from hemoglobin and quantify it.

To test if the presence of NaCl may affect the release of heme from hemoglobin, we added 50 mM or 2M NaCl to hemolysate samples. In this way, we reproduced the conditions of the determination of Liu et al.[8] We determined the concentration of free heme in these solutions; to preserve the NaCl concentrations during the isolation of free heme from hemoglobin by dialysis, NaCl was added to the dialysis buffer at the same respective concentrations. Fig. S4B demonstrates that the addition of NaCl arrests the release of heme from the hemoglobin. Microscopic observation of the hemolysate extracted from the dialysis cassette revealed the absence of solid residue. Thus, the hematin in stoichiometric ratio to apoglobin was not precipitated by NaCl, but likely driven back to the apoglobin due to its increased chemical potential. The reattached heme cannot be separated from hemoglobin, leading to the low amounts of free heme found by Liu *et al*. [8].

Supporting References

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