Characterisation of cytogels using acousto-microscopy-based oscillating rod rheometry.

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1. ABSTRACT

The physical properties of cytoplasm are primarily determined by the state of cytoskeletal element, i.e. their polymerisation, crosslinking and supramolecular interactions with other molecules. These interactions are involved in signal transduction processes as well as in morphogenesis. Scanning acoustic microscopy proved to be a powerful tool to determine the mechanical properties of living cells. The interpretation of the sound propagation parameters, however, has to be based on investigation of in vitro models. Therefore polymerisation of actin and tubulin have been followed using a novel oscillating rod rheometer which allows for synchronous determination of sound velocity, sound attenuation and viscosity. Sound velocity measures the elastic propterties of cytogels, attenuation the supramolecular associations. All these parameters are evaluated with minimal strain, in the range of 1- 100 nm! In particular the interaction of actin with glycolytic enzymes not only modulated polymerisation in a specific, and substrate dependent manner, but also the stiffness of the fibrils was altered, e.g. by hexokinase in the presence of high ATP, this enzyme exhibited actin severing properties and reduced stiffness. Differences in polymerisation kinetics were observed comparing pyrene-labeled actin fluorimetry and oscillating rod viscosimetry. This comparison led to the detection of pseudocrystalline structures produced by g-actin and aldolase (in the absence of fructose-bisphophate, the substrate of aldolase). Elastic stiffness of actin filaments can be modulated by ATP/ADP and by actin binding proteins (e.g. the glycolytic enzyme hexokinase) as well. The in vitro observations support the interpretation of SAM data calculated for living cells.

Keywords: actin polymerisation, viscosity, elasticity, cytomechanics, rheometry.

2. INTRODUCTION

Subcellular mechanical properties of cells determine their shape, the direction of their locomotion and even are engaged in the control of gene expression. Cytoskeletal visco-elasticity results from the interactions of cytoskeletal elements, their polymerisation status, cross linking and their anchoring to the plasmamembrane and other organelles. The gel - like structure of cytoplasm is sensitive to deformation, i.e. it may exhibit non-Newtonian behavior when being stressed. In addition, particles percolating through such gels can cause local ruptures and thus alter mechanical properties locally. The overall mechanical properties of such gels result from the polymerisation - depolymerisation status, associations with other macromolecules including soluble enzymes and RNA, crosslinking by specific proteins (e.g. MAPS or actin associated proteins) and the stiffness of the fibrils themselves and the compressibility of the monomers. Therefore the analysis of these properties cannot be straightforward, neither in living cells nor in isolated cytogels. The measurement device has to be operated such as to apply minimum deformation to the cytogel, and it should reveal viscosity as well as elasticity to represent both these factors required to describe cytomechanics in full detail.

Acoustic microscopy is among the few methods which allow to study the mechanical properties of cells[1,2]. This might bridge the gap between the level of supramolecular structures and ultrasound signals used in medical diagnosis. The ORR simultaneously allows to calculate the volume elasticity of a fluid or gel from measurements of the velocity of longitudinal ultrasonic waves and to determine the dynamic viscosity from the oscillations (in a nanometer range) of a glass rod immersed in the viscous fluid [3]. In comparison to other rheometers (e.g. plate-plate or plate-cone rheometer), the oscillation of a glass rod acting as a sensor for viscosity causes a shear wave which is equivalent to the dimension of the rod and thus can propagate its full extent through the surrounding fluid with extremely small strain. This setup results in a more precise determination of the dynamic viscosity than that obtained by most other methods.

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Elasticity of a gel may result as well from the stiffness of fibrillar elements as from tension developed by contractile forces or crosslinking between the fibrillar elements of a gel. Actin filaments provide an excellent example to investigate these factors on the elasticity of actin gels. Therefore cross linking proteins (i.e. alpha actinin) and factors immanent to actin filaments themselves have been investigated. Among the latter, packing density and ATP or ADP binding to actin are the factors modulating elasticity of single actin filaments.

3. MATERIAL AND METHODS.

1 Preparation of actin and chemicals

G-actin was isolated from rabbit skeletal muscle[4]. α-actinin was prepared from chicken gizzard according to Feramisco and Burridge[5].

G-actin buffer: 2 mM TRIS-HCl (pH 7.4), 0.2 mM ATP, 0.5 mM DTT and 0.2 mM $CaCl_2$. Polymerization buffer: 50 mM KCl and 2 mM $MgCl_2$.

Hexokinase buffer: 2 mM TRIS-HCl (pH 7.6), 2 mM or 0.2 mM ATP (depending on the assay), 0.5 mM DTT, 0.2 mM CaCl2 and 1 mM PMSF.

Phalloidin, D-glucose, ATP-Disodium salt and GTP-Sodium salt were obtained from Sigma-Aldrich Chemie GmbH (P.O. 1120, 89552 Steinheim, Germany).

The protein concentrations were determined according to the method of Bradford6. Prior to rheological measurements, protein solutions were degassed in vacuum for 10 minutes to avoid air bubbles, which otherwise strongly influence the measurements. Sample volume was 80-120 µl.

2 Description of the oscillating rod rheometer.

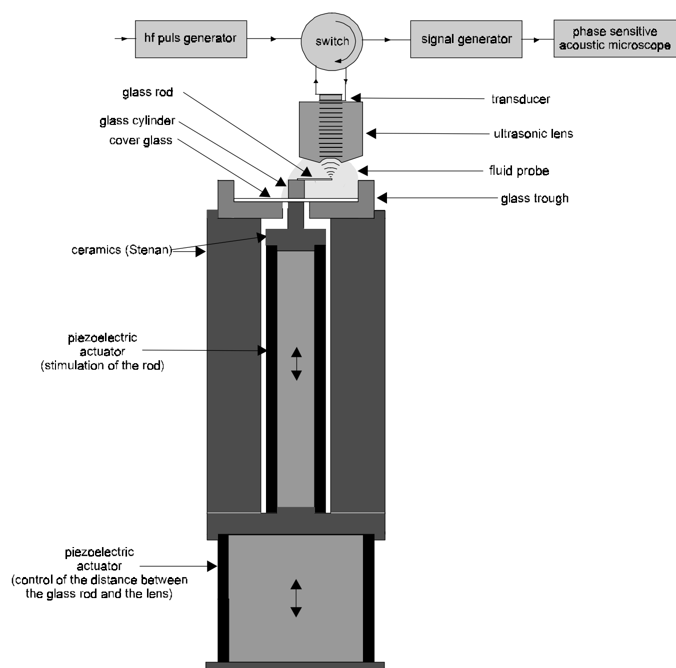

Fig. 1
Schematic overview of the oscillating rod rheometer. The motion of the piezo-electric actuators is characterized by double arrows. The broad, lower one changes the distance between the acoustic lens and the oscillating rod. This rod is induced to oscillate by the longer, thin actuator. For detailed description see text. (From[3])

A glass fiber rod (diameter ≈ 50 µm, length ≈ 2 mm) is immersed in the fluid probe and is stimulated to oscillations (1-4 kHz) via a piezomechanical bimorph actuator (Fig. 1). The *resonance frequency* of the rod oscillation is, among others (e.g. the density of the specimen), a function of the viscosity of the surrounding fluid[7]. The extremely small displacements (>1 nm) of the rod, are monitored by ultrasound reflected from the tip of the glass rod using a phase sensitive acoustic

microscope[8,9]. A Boxcar-Integrator averages the received ultrasound pulses and filters undesirable interfering signals (e.g. lens echoes).
The oscillation frequency of the rod is held at its resonance frequency via a feed back control: The phase of the rod oscillation is permanently compared with the phase of the piezo excitation frequency and is kept to a phase shift of $\pi/2$ by frequency adjustments. This function is accomplished by using a lock-in amplifier (model SR810, Stanford Research System Inc., California 94089) which also feeds the mean of the resonance frequency during one second into a PC.
Changes of the propagation speed of the longitudinal acoustic waves in the sample shift the phase difference between the reflected ultrasound wave and the generating 1 GHz electric oscillation (e.g. the detected ultrasound signal is delayed when the sound velocity decreases). This phase shift is compensated by regulating the distance between the acoustic lens and the free tip of the glass fiber via a second piezoelectric actuator (Fig. 1). The voltage controlling this piezo actuator is a measure for the change of sound velocity, which is related to the volume elasticity of the probe (for details see Wagner et al.[3]).

4. RESULTS AND DISCUSSION

3 The ORR as a non-destructive measuring device

Viscous fluids or gels prepared from cytoplasmic polymers are very sensitive to deformation. For instance the entangelments of actin fibrils might be destroyed by a falling ball in a ball viscosimeter, or even the filaments themselves may be broken by the interaction with the measuring probe. Therefore several techniques have been developed to use an interacting probe deforming the polymers as little as possible. The lower limit of deformations is given by the sensitvity of the measuring device, and this is limited finally by the signal to noise ratio. Sensitivity of phase measurements in general exceeds that of intensity measurements by orders of magnitude. Combination of an oscillating signal with a lock-in amplifier adds to the reduction of noise because it allows to extract the signal according to the phase of the stimulating oscillation, then this signal is avaraged a few thousand time by a box-car further reducing noise relative to the signal. A further reduction of noise influence is brought about by determining frequency only (the important parameter for the measurement is the resonance frequency of the rod). All these procedures in combination with an extremely stable mechanical setup allow viscosity determinations by deformations of a few nanometers only. Thus the deformations applied by the tip of the oscillating rod can be smaller than the thickness of an actin filament, and still reliable data on viscosity and elasticity (Fig.2).

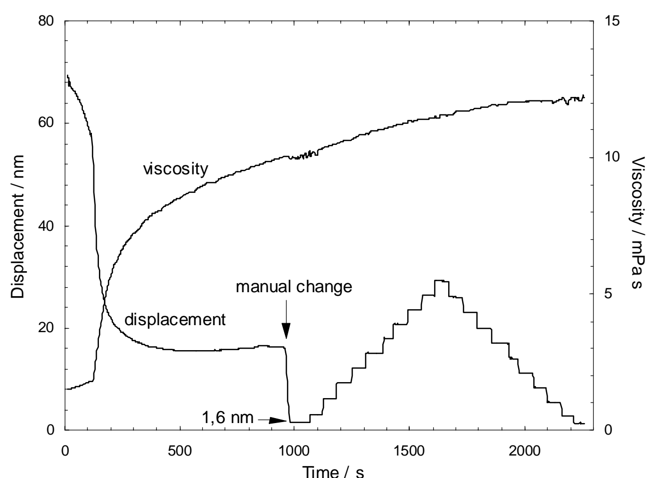

Fig. 2
Dynamic viscosity of 100 µM actin during polymerisation and amplitude of the rod oscillation. The amplitude of the rod oscillation was varied systematically starting at the point marked by the arrows: First the oscillation amplitude was brought to 1.6 nm and then increased/decreased in 3 nm steps. Obviously in the range up to 30 nm, these amplitudes of rod oscillation do not influence the result of the viscosity measurement.
(From [3])

This extreme sensitivity may raise the question whether the data still can be interpreted assuming continuum mechanics rather than considering the interaction with single fibrils. The simple approach assuming a continuum interaction between the measuring device and the filament containing solute is justified by the relatively large size of the rod, the mass distribution along its total length contributes to its resonance frequency.

4 Compressibility of polymerising actin

Sound velocity (c), in a body is a function of the appropriate elasticity modulus. In fluids this is primarily the commpression modulus (K):

$$c = \sqrt{\frac{K + \frac{4}{3} \cdot G}{\rho}} \qquad (1)$$

where G is the shear modulus and ρ • is the density of the liquid in g/cm^3.

Commonly G << K, therefore Eq. 1 is approximated to:

$$c = \sqrt{\frac{K}{\rho}} \qquad (2)$$

The relation between the elastic modulus, E and the compression modulus, K is:

$$K = \frac{E}{3 \cdot (1 - 2\mu)} \qquad (3)$$

where μ is the Poisson ratio (e.g. for actin filaments about 0.4. The elastic modulus, E, is then 0.6 times the compression modulus, K). K is often expressed as the compressibility κ:• K = 1/ κ• .

Neither mass nor density of a protein solution change during polymerisation. Thus each change in sound velocity monitors elasticity properties related to polymerisation as may be stiffness of the forming filament or alterations of the hydration shells of the protein molecules. However, polymerisation is initiated by the addition of ions which cause an increase of sound velocity of 2-3 m/s. In polymerising actin solutions with protein concentrations exceeding 10 µM, a biphasic response in sound velocity can be observed (fig. 3). After the increase due to the addion of saline a further small increase of sound velocity of 0.5 m/s accompanies polymerisation. After polymerisation comes to an equilibrium, i.e. viscosity does not increase anymore, sound velocity is reduced to levels lower than those of g-actin (fig. 3).

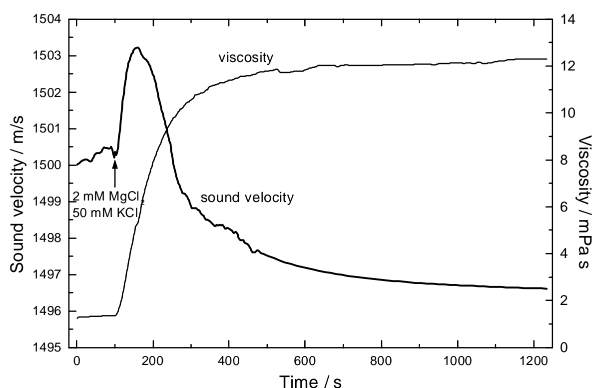

Fig. 3
Dynamic viscosity and sound velocity changes during the polymerization of 100 µM actin. The increase of the sound velocity (about 0.5 m/s) at the very beginning is due to the heating of the cold suspension. An increase of 3 m/s occurs during the early phase of polymerisation as a consequence of the addition of Mg and KCl for the initiation of polymerisation of actin, followed by a a slow and small increase of 0.5 m/s due to actin polymerisatoin and a larger decrease of the sound velocity (6.5 m/s) which lasts.

This biphasic elasticity changes could be due to two factors counteracting each other: An small increase in sound velocity by the formation of stiff actin filaments and a decrease in the hydration shells reducing sound velocity. The subsequent decrease in compression modulus is missing at actin concentrations ≤ 10 µM actin (Fig. 4). The relation between sound velocity decrease and actin concentration is sigmoidal, it approaches a lower limit which we have interpreted to represent a nematic phase of the f-actin [3] according to the observations of nematic actin structures reported by Käs et al.[10]. The fact that polymerisation of actin reduces the compression modulus can be alloted to a decrease of the hydration shells of the actin molecules by water release during polymerisation11.

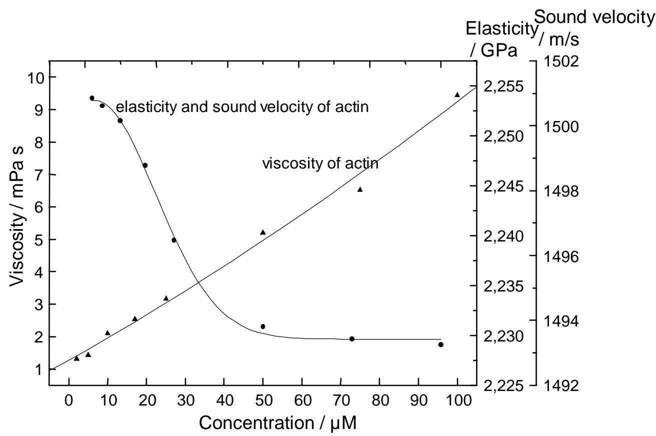

Fig. 4
Concentration dependence of viscoelasticity of skeletal actin after having completed polymerisation. Viscosity = • , Elasticity and sound velocity = ▲. Sound velocities of f-actin (monitored at 25°C). One symbol represents the mean of 2-5 different measurements.

5 Modulation of actin filament stiffness

Despite the dependence of compressibility of f-actin solutions on protein concentration the stiffness of the single filaments itself has to be considered as a siginificant mechanical parameter. This should better be revealed at low protein concentrations which maintain a higher elastic modulus in the polymerised state than that one of g-actin. Such stiffness modulations might be brought about by the interaction with actin binding proteins and by the turnover of ATP bound to G-actin which in the f-actin state becomes dephosphorylated. Previous studies on single actin filaments revealed the influence of ATP on filament elasticity [12,13]. In the following we concentrate on the role of ATP for f-actin stiffness.

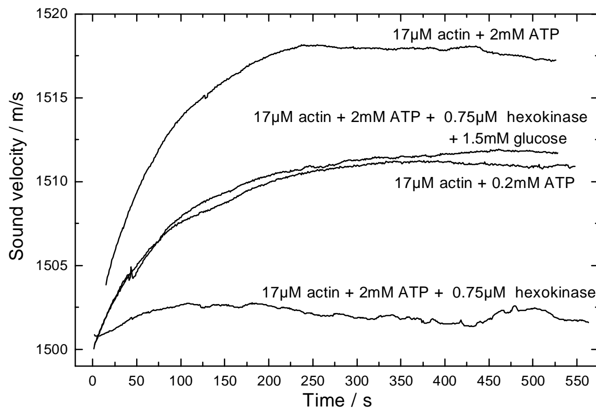

Fig. 5
Sound velocity changes during the polymerisation of 17µM rabbit skeletal muscle actin. ATP (2mM) stiffens the actin filaments, and this stiffening is abolished by hexokinase (without glucose) and filaments are less elastic than under control conditions (with 0.1mM ATP). Hexokinase together with glucose reduce filament elasticity depending on glucose concentration (see Fig. 6). The curves are the means of 3-7 independent measurements.

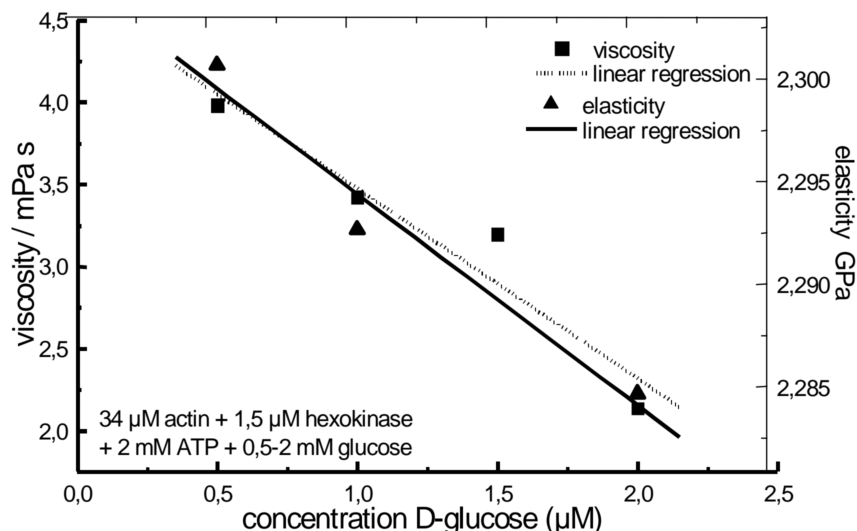

Fig. 6
Final viscosity and elasticity of f-actin (34 µM) polymerised in the presence of hexokinase (1.5 µM) and ATP (2mM) with varying concentrations of glucose to modify ATP concentration. Both, viscosity and elasticity decrease linearily with decreasing free ATP concentration.

Modulation of viscoelasticity by ATP

ATP (2 mM) increased actin polymerization when compared with 0.2 mM ATP, simultaneously, sound velocity revealed higher elasticity (Fig. 5). Depletion of ATP, on the other hand, reduced sound velocity significantly. ATP depletion was achieved by the addition of hexokinase and glucose as its substrate. The immediate effect of ATP concentration is shown by the linear relation of glucose concentration to viscosity and elasticity (Fig. 6).

In the absence of glucose this actin binding enzyme (type I from bovine heart) accelerates the polymerization rate and increases the final viscosity of actin. In electron micrographs actin filaments with hexokinase appeared shorter than without hexokinase. However, hexokinase without glucose considerably decreases f-actin elasticity in the presence of high ATP concentrations (Fig. 6). This influence may be attributed to an ATP-induced conformation of the hexokinase interacting with f-actin and thereby loosening g-actin interaction in the filament. This conclusion is supported by electron micrographs showing very short actin filaments [3]. Similar morphology and reduction of elasticity take place if actin polymerises in the presence of cytochalasin D[14].

Higher elasticity of ATP-f-actin than that of ADP-f-actin has first been reported by Janmey et al.[12]. This is consistent with the lower elasticity of actin filaments in presence of hexokinase and glucose described above (Fig. 6). The action of ATP in the buffer is supposed to facilitate ATP exchange within the filament. Janmey et al. [12] showed that such an exchange may occur, indeed. Hydrolysis of the bound ATP is supposed to strengthen the interaction between the g-actih subunits. This is not accompanied by an immediate release of Pi which destabilises the filaments by a change in conformation [16,17].

Whether an elasticity difference between ATP- and ADP-actin can be measured depends on the sensitivity of the measuring device. High precision 1GHz ultrasound measurements provide a very sensitive method to clarify this question as is evident from the sound velocity differences shown in Figs. 5 and 6. Further evidence should be brought forward by replacing ATP with an analogon which will not be hydrolysed as fast as ATP. AMPPNP is such an analogon. It supports actin polymerisation, although less efficiently than the same concentration of ATP (insert in Fig. 7). Elastic stiffness of f-actin (15 µM) increases during polymerisation and finally reaches a constant value in the presence of AMPPNP (0.1mM). If ATP (0.1mM) is present, the initial elasticity increase is faster, followed by a continuous but slowlier rise of sound velocity (Fig. 7). This behaviour can be explained by the higher stability of AMPPNP-f-actin[15] , thus the internal tension exerted by the f-actin-ADP-Pi complex cannot be developed, yielding less elastic filaments. A continuous addition of new ATP-g-actin units to the filaments increases continuously the relative amount of ATP-f-actin (fig. 7). At higher ATP concentration this process is much faster and therefore reaches equilibrium conditions earlier (comp. fig. 5 and Fig. 7)

In living cells local stiffness variations have been described[19] and reduction of elasticity was related to malignancy (e.g.[20]. In these cases actin - myosin interactions and binding of other proteins to f-actin are supposed to represent the main source for local mechanical properties. The elasticity modulations evoked by the phosphorylation state of ATP in f-actin obviously

are less prominent than those caused for example by alpha-actinin binding or actin-myosin based contractions but they may support these functions by providing stiffer material to construct the three-dimensional fibrillar net penetrating the cytoplasm.

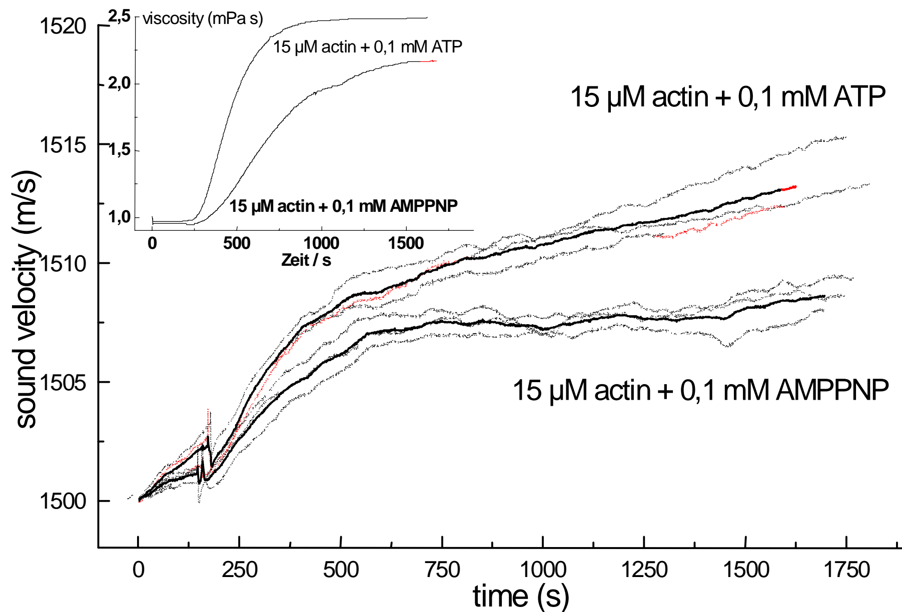

Fig. 7
Sound velocity and viscosity changes during the polymerisation of 15µM rabbit skeletal muscle actin in presence of 0,1mM ATP or 0,1mM AMPPNP, respectively. The AMPPNP-buffer still contained abut 0,015 mM ATP.
The dotted lines show single measurements. The full lines represent the mean of 3 independent measurements. Polymerisation was induced by the addition of KCl and MgCl2 (peak in sound velocity at ca. 130 s). Standard deviation for viscosity curves was less than 5%.

5. ACKNOWLEDGEMENTS

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