ROTARY DIFFUSION OF ACTOMYOSIN

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It has occasionally been pointed out by cell physiologists[1-3] that the thixotropy of the fibrous protein plays an important role in many physiological functions of protoplasma, such as protoplasmic movement, chromosomal movement in mitosis, and so on. However, the molecular mechanism of thixotropy of the fibrous protein has not yet been studied in detail.

As indicated by WEBER AND PORTZEHL[4] and others[5,6], the solution of actomyosin, the main component of muscle structure proteins, behaves as a thixotropic sol even in such a low concentration as is used for ultracentrifugal and viscosimetrical studies. Therefore, as a further step towards the understanding of the physicochemical properties of actomyosin solution, it is submitted that detailed investigation of its thixotropy merits considerable priority. Furthermore, since RIESER[7] has recently established by his oil drop method that the muscle protoplasm is a concentrated protein sol but not a gel, a study of thixotropy of actomyosin sol may be expected to aid in elucidating the mechanism of contraction of living muscle.

The dependence of the viscosity of actomyosin solution on the velocity gradient has been studied by EDSALL AND MEHL[8], the NEEDHAM group[9] and MOMMAERTS[10], but little information is available on the molecular mechanism of the thixotropy of actomyosin sol. Therefore, in the present paper the viscosity, the intensity of flow birefringence and the rotary diffusion constant of actomyosin were measured over a wide range of the velocity gradient, with a view to obtaining information about the mechanism of the thixotropic behaviour of this protein.

EXPERIMENTAL

Materials

Myosin B (natural actomyosin) was prepared from rabbit skeletal muscle by extracting for 24 h in Weber-Edsall solution and purified by repeating the dilution procedure[11] three times. The protein solution was stored at 1°C and not used for longer than a two-week period. To remove denatured aggregates of the protein the solution was passed through a layer of absorbent cotton, just before using. Adenosine triphosphate (ATP) was prepared from fresh rabbit skeletal muscle by Kerr's method modified by SZENT-GYÖRGYI[11] and used as a potassium salt.

Apparatus and procedures

The apparatus used for the study of the dependence of the viscosity (η), the intensity of flow birefringence (Δn_0), and the rotary diffusion constant (Θ) on the velocity gradient (β) was constructed according to the same principle as the one used by CONNER AND DONNELLY[12] in their study of the flow birefringence of concentrated viscose solution. Since the details of the apparatus will be reported elsewhere by the junior author (H.M.), only its principal features are presented in the following.

The sample cell was a piece of glass tubing, 38.3 cm in length and 1.20 mm in inside radius, set horizontally on the stage of a Leitz-"Panphot". The pressure head between the two ends of the glass tube was achieved by depressing pressure of a gas reservoir, 20 l in volume, and its value was

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measured by a manometer with aids of a cathetometer. A mean value ($\bar{\beta}$) of the velocity gradient was calculated from the formula of KROEPELIN[13]:

$$\bar{\beta} = \frac{8V}{3\pi r^3 t}$$

where V was the volume of the solution flowing through the sample cell of radius r in time t. Two platinum wire electrodes were fixed at an appropriate interval in a cylindrical glass vessel, 25 cm in length and 3 cm in diameter, attached to the one end of the sample cell. When the KCl solution of myosin B contacted with the tip of the electrode, a condenser began to discharge. By recording the time of the beginning of the discharge by means of an electromagnetic oscilloscope, V/t was determined. Relative viscosity (η_{rel}) was calculated from the ratio of the V/t of the KCl solution to that of the myosin B solution at a fixed pressured head.

Two Nicol prisms of the "Panphot" were crossed with each other with their axes 45° to the glass tube. Relative intensity of flow birefringence was obtained by measuring difference of intensities of light passing through the sample cell when the solution was forced to flow and when the flow was stopped. The light beam, passing through the "Panphot" and the sample cell, was received by a photomultiplier (RCA 1P21). The output current of the photomultiplier was amplified with a bridge circuit containing two power-amplifier tubes 6BQ5 (Matsushita) and was recorded on an electromagnetic oscillogram (YEW, 6 elements) with H-type vibrator. When the intensity of birefringence was low, a preamplifier employing a tube 12AX7 was also used. To make the light beam passing though the sample cell approximately parallel and to eliminate scattering from the surfaces of the lenses, the condenser lenses and several optical lenses of the "Panphot" were taken off.

When the flow of myosin B solution was suddenly stopped by a spring-loaded knife valve, the birefringence, and consequently the current from the phototube, decreased in a manner determined by the BENOIT equation[14]:

$$\Delta n = \Delta n_0 \, e^{-6\Theta t}$$

where Δn is the intensity of flow birefringence at time t after the stop of the flow and Θ is the rotary diffusion constant. The lower limit of the time constant of our apparatus was about 0.5 msec. By putting a glass bottle, in which myosin B solution was reserved, into a thermostat, the temperature of the sample was kept at 20° ± 0.1°C.

Sedimentation studies were performed with a Spinco model E ultracentrifuge at 16°C at 59,780 r.p.m. Concentration of KCl of myosin B solution was determined by measuring electrical conductivity of the solution. pH was measured by a Beckman G-type pH meter. The content of protein was calculated by multiplying by a factor of 6 the nitrogen content determined by the micro-Kjeldahl method.

RESULTS

Ultracentrifugal study

The 24 h myosin B behaved polydispersedly, and two major peaks and one minor peak were revealed in its sedimentation pattern. Sedimentation constants at 16°C in 0.5 M KCl and 0.1 M phosphates (pH 7.0) solution of 2 mg protein/ml were about 4.8 and 29 for the two main peaks and about 34 Svedberg units for the minor peak, respectively. The sedimentation constant 4.8 might be that of pure myosin.

Rotary diffusion at higher protein concentration

In Fig. 1 are drawn the dependence on $\bar{\beta}$ of η_{rel}, Δn_0 and Θ of 0.55 M KCl solution of myosin B at the protein concentration of 5.94 mg/ml. η_{rel} decreased remarkably with increase of $\bar{\beta}$, as observed already by EDSALL AND MEHL[8] and others[9,10].

At low $\bar{\beta}$, the relation log $\Delta n - t$ was not given as a linear one (Fig. 2); that is, myosin B behaved polydispersedly with respect to rotary diffusion, showing good correspondence with the results of ultracentrifugation. For example, at $\bar{\beta}$ 17.45 and 335 sec^{-1}, Θ distributed over wide ranges of 0.1–1 and 1–10 sec^{-1}, respectively. In spite of the polydispersity, the greatest part of the curve was approximated by a single value of Θ. Accordingly, in the range of polydispersity, the best single value of Θ was plotted in Fig. 1.

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At the higher velocity gradients, the polydispersity of the rotation decreased and a single value of Θ prevailed. Where $\bar{\beta}$ was higher than 1,000 sec^{-1}, the logarithmic decrease of flow birefringence was observed rather completely (Fig. 3). The rotary diffusion constant increased with increase of $\bar{\beta}$, particularly in the range of 100–1,000 sec^{-1}, and when $\bar{\beta}$ was higher than 1,500 sec^{-1} it approached to a constant value (about 20 sec^{-1}). As given in Table I, the preparation-to-preparation variability of Θ at sufficiently high $\bar{\beta}$ was not evident.

It is remarkable to note that when $\bar{\beta}$ was higher than 1,500 sec^{-1}, Δn_0 rose conspicuously with the increase of $\bar{\beta}$ (for example, Δn_0 at $\bar{\beta}$ 12,500 sec^{-1} was 4 times as high as at $\bar{\beta}$ 335 sec^{-1}), while in the range of low $\bar{\beta}$, it was almost independent of $\bar{\beta}$.

Effect of repeating flow

When myosin B solution was once subjected to a high shear stress, its rheological properties were altered considerably. Within 1 min after the subjection to high $\bar{\beta}$ (about 10,000 sec^{-1}), the relation $\Delta n - t$ at low $\bar{\beta}$ appeared to be more logarithmic, though imperfect, than that of the original solution. However, the discrepancy of the relation $\Delta n - t$ from a logarithmic curve and the decrease of Θ became apparent with time, that is, Θ at $\bar{\beta}$ 230 sec^{-1} decreased from 1.2 to 0.6 sec^{-1} during 15 min after the subjection to the high shearing stress. It is to be noted that Δn_0 of the solution once forced to flow through the capillary with high shearing stress was considerably greater than the original one, *i.e.*, its Δn_0 value at $\bar{\beta}$ 230 sec^{-1} was found to be 40% greater than the original one.

Rotary diffusion at lower protein concentration

When the protein concentration was 1.5 mg/ml, Θ rose with increase of $\bar{\beta}$, though not so remarkably as in the case of the higher protein concentration, and at higher gradients it approached to a constant value which is almost equal to that of the higher protein concentration (13–18 sec^{-1}). As in the case of the high protein concentration, over a higher range of $\bar{\beta}$, Δn_0 increased with increment of $\bar{\beta}$* (Fig. 4).

Influence of ionic strength

When KCl concentration increased to 2.34 M, the relaxation time of birefringence was observed to be shorter than 18 msec ($\Theta \geqq 10$ sec^{-1}) even in low range of $\bar{\beta}$ ($< 1{,}000$ sec^{-1}).

Influence of adenosine triphosphate

In one experiment, 1.1 mM ATP and 1.5 mM Mg^{++}** were added to myosin B solution (1.5 mg/ml), and the relaxation of flow birefringence was observed at high shearing stress, but it was too fast to be followed by the present apparatus. This means that in the presence of ATP Θ should be larger than 100 sec^{-1}.

* In the case of high protein concentration the turbulence of the flow could be neglected over all $\bar{\beta}$ employed, whereas in the case of low concentration it could not be neglected when $\bar{\beta}$ became higher than 4,000 sec^{-1}. Therefore, in the case of low protein concentration and high $\bar{\beta}$, the relation between Δn_0 and $\bar{\beta}$ could not be accurately measured.

** The addition of 1.5 mM Mg^{++} to 0.6 M KCl solution of "actomyosin" has no effect on the rheological properties of the solution[9].

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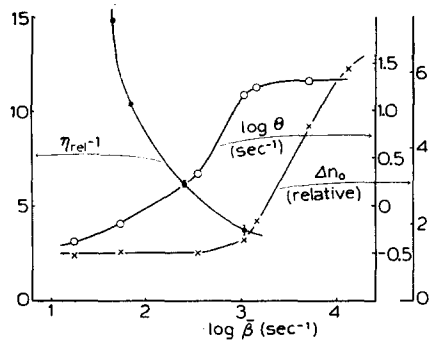

Fig. 1. Dependence on gradient ($\bar{\beta}$) of specific viscosity ($\eta_{rel} - 1$), flow birefringence (Δn_0) and rotary diffusion constant (Θ) at high protein concentration; 0.55 *M* KCl, pH 6.3, 20°C, myosin B 5.94 mg/ml.

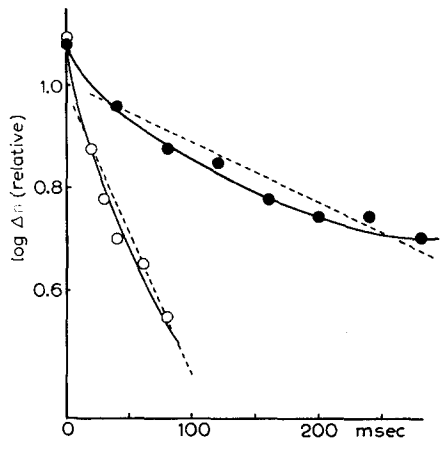

Fig. 2. Relaxation of flow birefringence at low gradient, ●, velocity gradient 17.45 sec^{-1}; ○, velocity gradient 335 sec^{-1}. Experimental data as in Fig. 1.

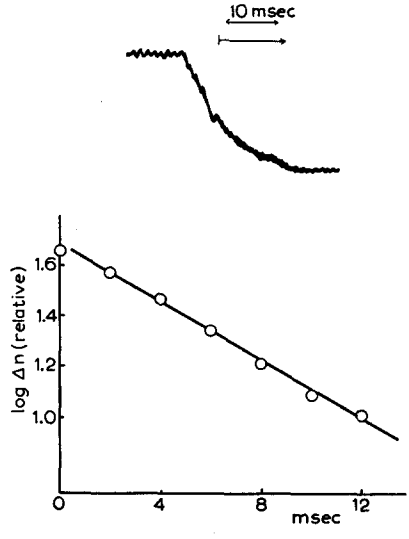

Fig. 3. Relaxation of flow birefringence at high gradient, calculated from the upper oscillogram. Velocity gradient 5,230 sec^{-1}. Experimental data as in Fig. 1.

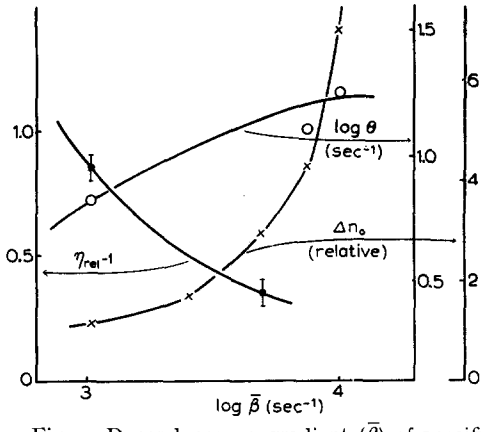

Fig. 4. Dependence on gradient ($\bar{\beta}$) of specific viscosity ($\eta_{rel} - 1$), flow birefringence (Δn_0) and rotary diffusion constant (Θ) at low protein concentration. 0.6 *M* KCl, pH 6.7, 20°C, myosin B 1.5 mg/ml. When $\bar{\beta}$ was higher than 4,000 sec^{-1}, its value was rather inaccurate because of turbulence of flow.

TABLE I

ROTARY DIFFUSION CONSTANT AT HIGH VELOCITY GRADIENT

Preparation	*Concentration (mg/ml)*	*Condition*			$\bar{\beta}$ *(sec^{-1})*	Θ *(sec^{-1})*
		pH	*KCl (M)*	*Temp.*		
No. 1	6	6.3	0.6	20	(6,000)	29.0
No. 2	5.94	6.3	0.55	20	1,508	17.6
					5,230	20.8
No. 3	1.5	6.7	0.6	20	(7,480)	13
					(11,800)	18

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DISCUSSION

One of the principal points of interest in the present study is that even in low concentration the rotary diffusion constant of myosin B increases with increment of gradient. JOLY[15] has already deduced the increase of Θ of gelatine sol at higher shear stresses, indirectly from his flow birefringence method. Furthermore, it was observed that Θ of the solution of higher protein concentration depends on $\bar{\beta}$ more pronouncedly than that of the solution of lower concentration and that Θ at sufficiently high $\bar{\beta}$ shows a constant value independent of $\bar{\beta}$ and protein concentration. Thus, we may conclude that a kind of network is built up in actomyosin solution by the interferences between elementary particles of a constant length (see below) and this network is easily broken by velocity gradient. This conclusion may be supported also by the observation[16] that the higher the protein concentration, the smaller the sedimentation constant obtained. The interferences may be of electrostatic nature, since they are broken, as stated above, by increasing ionic strength of the solution.

As described above, the rheological properties of myosin B solution changed gradually during several minutes after subjection to high shear stress. Therefore it appears probable that the re-formation of the network once broken by gradient is not accomplished instantly but proceeds gradually. The observation made by JAISLE[17], that the viscosity of concentrated actomyosin solution decreases to a definite value by repeating the passage through a capillary, would lend support to this deduction.

The length of the constituent elementary particles of myosin B can be estimated from the value of Θ at sufficiently high $\bar{\beta}$. Using the PERRIN equation[18]

$$\Theta = \frac{3kT}{16\eta_0 a^3}\left(-1 + 2\ln\frac{2a}{b}\right)$$

and taking the axial ratio a/b as 100[19] and the viscosity of solvent η_0 as 9.9 millipoises[20], we obtain for the molecular length 8,600–10,100 A for rigid elongated ellipsoid*. In magnitude these agree satisfactorily with results from light scattering[6, 21], which show a distribution of lengths of the order of 5,000 to 6,500 A.

The non-Newtonian character of viscosity is generally attributed to orientation of the particles and breaking of the interparticular entanglements. As shown in Table II, over the range of gradients employed, the ratio $\bar{\beta}/\Theta$ was extremely large and the orientation factor (f) of myosin B particles was almost independent of gradients. Accordingly the decrease of viscosity of myosin B with increase of $\bar{\beta}$ should be attributed to the GOODEVE mechanism[23], *i.e.*, the breaking of the network.

VON MURALT AND EDSALL[24] have found that the increase in Δn_0 of "actomyosin" continues in that range of β where the angle of isocline is already constant. In the present study Δn_0 was measured over much higher range of gradients and it was found that Δn_0 of myosin B solution of both low and high concentrations exhibits an increase of 4–5 fold when $\bar{\beta}$ increases from 1,000 to 10,000 sec^{-1}. One possible mechanism of the increase of Δn_0 is the polydispersity of the system. If the system is polydisperse, the

* As is well known, myosin B preparation contains pure myosin and disaggregated actomyosin as well. Therefore η_0 must be higher than that of the KCl solution. The myosin B particle is, as will be described below, somewhat flexible, and its shape may be somewhat complicated. Consequently, the true length is possibly shorter than the one given above. If the solute were made up of elongated ellipsoids 8,600–10,100 A in length, in 0.6 % solution the particles could not rotate freely, and Θ at sufficiently high $\bar{\beta}$ would be strongly dependent on the concentration.

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TABLE II

ORIENTATION FACTOR AND INTENSITY OF FLOW BIREFRINGENCE

0.55 M KCl, pH 6.2–6.3, 20°C, 5.94 mg/ml

$\bar{\beta}$ (sec^{-1})	Θ (sec^{-1})	$\bar{\beta}/\Theta$	f*	Δn_0
17.45	0.43	41	0.56	1.2
53.3	0.65	83	0.65	1.3
335	2.15	156	0.68	1.25
1,083	14.5	75	0.63	1.6
1,508	17.6	90	0.65	2.1
5,230	20.8	251	0.72	4.6
12,500	—	—	—	6.1

* Orientation factors were estimated from the table of SCHERAGA, EDSALL AND GADD[22] for extremely elongated ellipsoid. In the present study $\bar{\beta}$ and Θ in low gradients are given as average values. Moreover, the factors computed by them apply to a rotor-type flow birefringence apparatus where the light enters in a direction perpendicular to the velocity gradient, and this condition is not satisfied in our experiments. Therefore, the orientation factors given above must be considered as rough estimates.

longer particles will be oriented more easily at lower $\bar{\beta}$ and, as $\bar{\beta}$ increases, the shorter particles become oriented. But, since myosin B behaved monodispersedly in the range of $\bar{\beta}$ higher than 1,000 sec^{-1}, this possibility may be excluded*. As is well known[26], the intensity of flow birefringence of a monodisperse system is given by the following equation:

$$\Delta n_0 = \frac{2\pi\phi}{n_0}(g_1 - g_2)\cdot f$$

where ϕ is the volume fraction of the solute particles, n_0 is the refractive index of the solvent, and f and $g_1 - g_2$ are the orientation and the optical factors, respectively. As indicated in Table II, the orientation factors were almost constant. Consequently, it may be concluded that the optical factor is increased several times on subjection to higher shearing stresses. It would appear then that myosin B particles are somewhat flexible and the orientation of polypeptide chains in the particles are improved by shearing stress. It is also interesting to note that the increase of the optical factor by shearing stress seems to be of more or less enduring character, since, as previously mentioned, the increase of Δn_0 was preserved to some extent even several scores of minutes after removal of high gradient**.

It is a well established fact[4] that viscosity, sedimentation constant, intensity of flow birefringence, and intensity of scattered light of actomyosin solution are decreased by the addition of ATP. In spite of extensive investigations of many workers, it remains to be determined whether ATP dissociates actomyosin into actin and myosin or not. As pointed out by several investigators[5,6], one of the reasons that makes this problem difficult to solve lies in the fact that even at low protein concentration actomyosin sol is thixotropic. As previously described, ATP increases remarkably the

* As previously mentioned, our myosin B samples contained pure myosin. But in our experiments the contribution of myosin to Δn_0 might be negligible, because its content was lower than the one of actomyosin and its orientation factor was lower than 0.14 (estimated from the table by SCHERAGA *et al.*[22], taking its molecular length as 1,600 A[25]).

** As, in the range of high $\bar{\beta}$, the relaxation of Δn_0 is given as a logarithmic curve and the relaxation time is almost constant regardless of whether Δn_0 is increased by shearing stress or not, it may be deduced that the relaxation time of the deformation is longer than 20 msec.

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rotary diffusion constant at high shearing stress and low protein concentration where thixotropy may be negligible, that is, the rotation of the elementary particles is accelerated by the addition of ATP. This observation is compatible with the view that ATP dissociates actomyosin into actin and myosin. This, however, does not exclude the view proposed by BLUM AND MORALES[6] that ATP elongates actomyosin without changing its molecular weight, because our results can also be explained if by addition of ATP the structure of myosin B particles becomes so loose that they are disaggregated when subjected to shearing stress.

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SUMMARY

1. The viscosity, the intensity of flow birefringence, and the rotary diffusion constant of myosin B, prepared from rabbit skeletal muscle, were investigated over a wide range of the velocity gradient (17–12,500 sec^{-1}). The following results were obtained.

2. The viscosity decreased with increase of the gradient.

3. At low gradient myosin B particles behaved polydispersedly with respect to the rotary diffusion, while at high gradient they were monodisperse. The rotary diffusion constant increased with increment of the gradient, and at sufficiently high gradient it was a constant value (*ca.* 20 sec^{-1}), independent of gradient and the protein concentration.

4. The length of the equivalent elongated ellipsoid calculated from the rotary diffusion constant at sufficiently high gradient was 8,600–10,100 A, in satisfactory agreement with results obtained from light scattering.

5. At a low range of gradient ($< 1{,}000$ sec^{-1}) the intensity of flow birefringence was almost independent of gradient, while at gradient $\sim 5{,}000$ sec^{-1} it was about 4 times as large as that at gradient $\sim 1{,}000$ sec^{-1}.

6. If the protein solution were once subjected to a high gradient, the intensity of flow birefringence observed at low gradient was considerably higher than the original value.

7. In high KCl solution, the rotary diffusion constant was observed to be higher than 10 sec^{-1} even at a low range of gradient.

8. Consequently, it was concluded that a network is built up by the electrostatic interferences of the constituent elementary particles with each other and the entanglements are broken more and more as the gradient increases, that the length of the elementary particles is almost constant, and that the orientation of polypeptide chains in these particles is improved by high shearing stress.

9. The rotary diffusion constant at high shearing stress increased remarkably on the addition of ATP. The molecular mechanism of this effect was briefly discussed.

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HORMONAL INFLUENCE ON THE PHOSPHORYLASE ACTIVITY OF THE HUMAN MYOMETRIUM*, **

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INTRODUCTION

Profound changes in the composition and structure of the human myometrium have been shown to take place under the influence of ovarian hormones[1,2]. The glycogen-content, especially, exhibits an impressive increase in connection with hormonally induced growth of this tissue. Thus, it could be demonstrated that the amount of glycogen per tissue unit*** is increased about 50 times in a pregnant uterus at term as compared with the value found in a post-menopausal uterus[2].

It may be postulated that the hormone-induced changes in the myometrial glycogen-content are mediated by a series of enzyme systems, and that the action of substances which regulate cellular metabolism may be elucidated by studying their effect on these systems. This paper reports some investigations on the phosphorylase

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** The abbreviations or contractions used in the paper include AMP, adenosine-5'-phosphate; DNAP, deoxypentose nucleic acid-phosphorus; EDTA, ethylenediaminetretaacetate.

*** The expression "tissue unit" is used in the sense of a statistical unit and implies the average amount of extracellular and intracellular tissue per nucleus. For further explanation, see BRODY[1].

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