Characterization of Rat Heart Myosin. II. Enzymatic Properties*

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ABSTRACT: The catalytic properties of rat cardiac myosin have been studied and related to conformational changes induced by several drugs and ethylene glycol. Ca^{2+} was shown to stimulate rat cardiac myosin while Mg^{2+} completely inhibited the enzymatic activity, a result similar to that obtained with dog cardiac myosin. A value of 2.46 μmoles of P_i/sec per g of protein and 7.4×10^{-4} mole/l. are reported for V_m and K_m, respectively. Further kinetic analysis of the purified myosin gave a value of 11.35 and 14.4 kcal/mole^{-1} for $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$, respectively, and -10 cal deg^{-1} mole^{-1} for $\Delta S^{\ddagger}$. A 13.7% increase in activity was obtained in 40% ethylene glycol in the presence of Ca^{2+} and a 10% increase in 36% ethylene glycol in the absence of Ca^{2+}. No change in activity was obtained with cyclic 3′,5′-adenosine monophosphate or cortisol acetate. The helicity of the native protein was found to be 53% which did not change significantly in 30% ethylene glycol. Sodium amytal and sodium phenobarbital reduced the helicity to 51 and 45%, respectively, without affecting the enzymatic activity. However, norepinephrine and chlorpromazine lowered the helicity to 45 and 42%, respectively, and also induced a 17 and 19% increase, respectively, in the rate of adenosine triphosphate hydrolysis by myosin.

In the preceding paper (McCarl and Margossian, 1969), a detailed analysis of the physical characteristics of rat cardiac myosin was presented. The physical properties of this protein are similar to those of rabbit skeletal or dog cardiac myosin, thus suggesting conformational similarities among myosins isolated from a variety of sources.

Studies of the ATPase activity of dog cardiac myosin and skeletal myosin and their dependence upon solvent and metal ions have been reported by Brahms and Kay (1962, 1963) and a similar investigation has recently been made by Kaldor (1968). In the present paper the enzymatic properties of rat cardiac myosin are investigated and evaluated in relation to skeletal and dog cardiac myosin.

In addition, the effects of certain drugs on the helicity and ATPase activity of rat cardiac myosin are examined.

Experimental Procedures

Materials. ATP, the disodium salt, and cyclic 3′,5′-AMP were obtained from Calbiochem, Los Angeles, Calif. Samples of sodium barbital, sodium phenobarbital, sodium amytal, and chlorpromazine were gifts from Smith Kline and French Laboratories, Philadelphia, Pa. *dl*-Norepinephrine hydrochloride was purchased from K & K Laboratories, Planview, N. Y., and used without further purification. Cortisol acetate was purchased from General Biochemicals, Chagrin Falls, Ohio. The ethylene glycol used was Fisher Certified Reagent grade.

Methods. Details of the isolation of rat cardiac myosin were presented in the preceding paper (McCarl and Margossian, 1969).

Enzyme Assays. Assays were performed at 25° and the reactions were run for 5 min during which period the reaction was found to be linear. The standard reaction mixture for assays contained 0.5 M KCl, 0.01 M Tris (pH 8.5), 0.01 M $CaCl_2$, 5×10^{-3} M ATP, and enzyme solution corresponding to 0.98 mg of protein in each tube. The final pH of the reaction mixture was 8.2. Reaction conditions differing from these will be stated in the discussion of individual runs. The final volume of the reaction mixture was 2.0 ml. Reaction was stopped by the addition of 1.0 ml of 15% cold trichloroacetic acid. The protein was separated by centrifugation and an aliquot of the supernatant was taken for the determination of P_i by the method of Fiske and Subbarow (1929).

The concentration of ethylene glycol in the reaction mixture is indicated in the text. Stock solutions of 2 mg/ml each of sodium barbital, sodium phenobarbital, and sodium amytal were prepared and 10, 30, 50, 100, and 200 μl were added to the various reaction vessels. A stock solution of 0.5 and 1.00 mg per ml each of chlorpromazine and norepinephrine was prepared and 10, 25, 50, and 100 μl of each drug were included in various reaction mixtures. Cyclic 3′,5′-AMP and cortisol acetate solutions of 10^{-4} and 10^{-5} M, respectively, were prepared and the amount used in each assay is indicated in the text.

In each of the above assays, appropriate controls were included and necessary corrections were performed at the end of each assay.

Optical Rotatory Dispersion Studies. Optical rotatory dispersion measurements were made with a Perkin-Elmer P-22 spectropolarimeter (calibration checked as suggested by Djerassi *et al.* (1960)).

The wavelength range employed for the optical rotatory dispersion measurements was 450–300 mμ. Blout *et al.* (1967) have presented evidence to show that the approximations made in deriving both the Moffitt–Yang and the two-term Drude equations are valid for wavelengths greater than 280 mμ. In addition, measurements on proteins of known helical content (bovine serum albumin, α-chymotrypsin, lysosyme, and ovalbumin) were in good agreement with the literature values (Urnes and Doty, 1961). Values of α were determined to

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TABLE I: Calculation of Helicity of Native Myosin and Variations in Helicity in the Presence of Ethylene Glycol and Certain Drugs.[a]

Protein	Protein Concn (g/100 ml)	Path Length (cm)	Drug and Concn	$-b_0$	HMY[a]	Std Dev	a_{193}	a_{225}	H_{193}	H_{225}	λ_c	$k \times 10^{-6}$
Rabbit skeletal myosin	0.243	1.000		−379	60	0.2	1516 (1535)[b]	−1209 (−1200)	62 (63)	58 (58)	268.4	−10.53
Rat heart myosin purified preparation 1[d]	0.080	0.500		−335	53	0.4	ND[c]	ND	ND	ND	ND	ND
	0.080	0.500	Phenobarbital (2×10^{-3} M)	−282	45	0.7	ND[c]	ND	ND	ND	ND	ND
Rat heart myosin purified preparation 2	0.140	0.199		−350	56	0.5	1350	−1151	58	55	265.5	−12.54
	0.140	0.199		−354	56	0.4	1366	−1165	58	55	266.6	−12.64
	0.140	0.199	Amytal (10^{-2} M)	−323	51	0.9	1232	−1068	54	50	264.2	−12.30
	0.140	0.199	30% ethylene glycol	−336	53	0.7	1309	−1093	56	52	267.5	−10.91
Rat heart myosin purified preparation 3	0.168	0.199		−335	53	0.7	1322	−1079	57	51	269.9	−9.72
	0.168	0.199	Norepinephrine (2×10^{-3} M)	−288	46	0.7	1108	−944	51	44	265.8	−10.21
	0.168	0.199	Chlorpromazine (1.4×10^{-4} M)	−261	42	0.9	1012	−857	48	40	273.1	−8.59

[a] HMY (Moffit–Yang helicity) = $-b_0/6.3$. [b] Literature values for rabbit skeletal myosin (Blout *et al.*, 1967). [c] ND = not determined. [d] Numbers refer to different batches of purified protein prepared from fresh heart tissue.

±0.002° and 17 or more data points were used in each determination of percentage helicity. The mean residue weight was taken as 112.0 (Kay *et al.*, 1964); λ_0 was taken to be 212.0 (Urnes and Doty, 1961). Values of refractive index were taken as those of pure water (Landolt-Bornstein, 1927). This latter assumption (Urnes and Doty, 1961; Kay *et al.*, 1964) results in errors of about 2%. In the case of ethylene glycol (30 vol %) values of refractive index were obtained from the literature (Timmermans, 1960; Kay *et al.*, 1964) and fitted to the Sellmeier dispersion equation using a least-squares method. Value of refractive index at each desired wavelength were then extracted from the graph.

In each case a fresh solution of the protein was used within 24 hr of preparation.

Cold solutions of the drug were added to an equal volume of the cold buffered protein solution to prevent high local drug concentrations.

In order to avoid errors arising from light scattering, careful monitoring of the automatic gain control unit (a means of checking the amount of light reaching the photomultiplier tube) was carried out in all experiments.

The results shown in Table I are the mean of at least two determinations agreeing within the standard deviation.

Special care was taken to ensure that the protein samples did not deteriorate. Reruns were made at the end of each determination to ensure that there had been no denaturation or aggregation.

Of the drugs used, chlorpromazine, barbital, amytal, and phenobarbital are not optically active and the norepinephrine used was the racemate. In order to check that there was no asymmetric binding of the drugs to the protein, giving rise to anomalous dispersion, the dispersion curves were measured to short wavelengths (230 mμ). A careful examination of these curves (particularly in the regions of ultraviolet absorption of the drugs employed) failed to reveal any evidence of asymmetric binding (*e.g.*, no extrinsic Cotton effects were observed; *cf.* Blout *et al.*, 1967).

The equations due to Moffitt and Yang (1956) and Blout *et al.* (1967) were used in the determination of helicity.

To facilitate the processing of data a least-squares regression analysis program (provided by Mr. R. C. Haddon, Department of Chemistry) was used. The programs were run on an IBM 360–67 computer. Standard deviations and correlation coefficients were also calculated for each set of data (Natrella, 1963). Only data with correlation coefficients greater than 0.99 are included.

Results

Metal Ion Effects. An important property of skeletal myosin is its response to certain divalent cations, especially Ca^{2+} and Mg^{2+}, in the presence of 0.5 M KCl. The response of rat cardiac myosin to these cations is shown in Figure 1. Ca^{2+} at a concentration of 5×10^{-4} M induces a maximal activation. As the concentration is increased the specific activity gradually decreases, and at a concentration of 0.5 M the original level of activity is restored. Thus, there is no activation by Ca^{2+} at high concentration as reported in the case of dog cardiac myosin (Brahms and Kay, 1963). Mg^{2+}, on the other hand, at a concentration of 5×10^{-5} M induces a drastic decrease in rat heart myosin activity and at 0.5 M completely inhibits the enzymatic activity as found for dog cardiac myosin

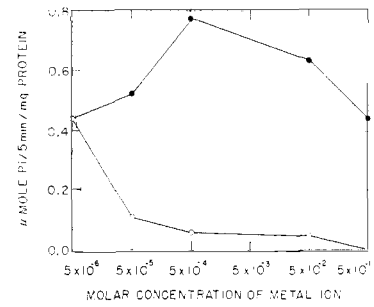

FIGURE 1: Effect of the addition of Ca^{2+} (-●-●-) and Mg^{2+} (-○-○-) on rat cardiac myosin ATPase activity. The reaction mixture contained: 0.5 M KCl, 0.01 M Tris buffer (pH 8.5), 5×10^{-3} M ATP, and metal ion as indicated on abscissa. Temperature was 25° and the final pH of the reaction mixture was 8.2.

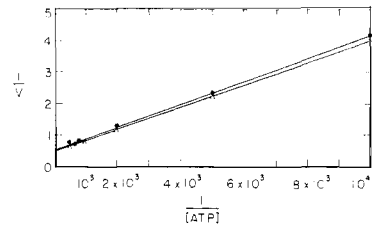

FIGURE 2: Lineweaver–Burk plot of the reciprocal of the rate of ATP hydrolysis as a function of the reciprocal of ATP concentration at 25.° (-○-○-) In 0.5 M KCl, 0.01 M Tris buffer (pH 8.5), and 0.01 M $CaCl_2$. Final pH of the reaction was 8.2. (-Δ-Δ-) 40% ethylene glycol. Remaining reagents were same as above.

(Brahms and Kay, 1963).

Evaluation of Kinetic Parameters. Figure 2 shows that rat cardiac myosin follows Michaelis–Menten kinetics. From this plot a value of 2.46 μmoles of P_i/sec g of protein was obtained for V_m. This is lower than the value reported for dog cardiac myosin (Brahms and Kay, 1963). From the same plot, the calculated value of K_m is 7.4×10^{-4} mole/l.

Kinetic studies were performed at four different temperatures. Making use of the Eyring (1935) equation

$$\ln (k_2/T) = \ln (k/h) + \Delta S^{\ddagger}/R - H^{\ddagger}/RT$$

the thermodynamic quantities $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ were estimated. The small change (0.1) in pH over the temperature range was neglected since Kaldor (1968) has shown that the reaction rates for dog cardiac myosin in the region of pH 7.0–8.5 are not significantly different. The Eyring plot of $\ln k_2/T$ *vs.* $1/T$ is shown in Figure 3. The slope of the plot gives $\Delta H^{\ddagger}$ directly and from the intercept $\Delta S^{\ddagger}$ is calculated. The enthalpy and entropy of activation of rat cardiac myosin are 11.5 kcal $mole^{-1}$ and −10.2 cal deg^{-1} $mole^{-1}$, respectively.

The enthalpies of activation for skeletal myosin and dog myosin are 12.4 (Ouellet *et al.*, 1952) and 12.0 kcal $mole^{-1}$ (Brahms and Kay, 1963), respectively, while the entropy of activation for skeletal myosin is −8.0 cal deg^{-1} $mole^{-1}$ (Ouellet *et al.*, 1952). The free energy of activation for rat cardiac myosin is obtained from $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, and is calculated to be 14.5 kcal $mole^{-1}$, which compares favorably with

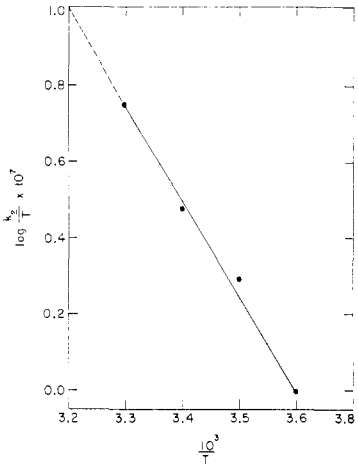

FIGURE 3: Eyring plot of ln k_2/T *vs.* $1/T$ for temperature dependence of the rate of ATP hydrolysis.

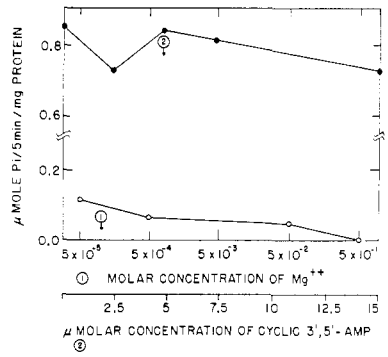

FIGURE 4: Effect of 10^{-4} M cyclic 3′,5′-AMP (curve 2) on the rate of ATP hydrolysis by myosin ATPase and also on the Mg^{2+} inhibition of myosin ATPase activity. The reaction mixture contained: 0.5 M KCl, 0.01 M $CaCl_2$, 10^{-3} M ATP, and 0.01 M Tris buffer (pH 8.5). Final pH of reaction mixture was 8.2 and temperature was 25°.

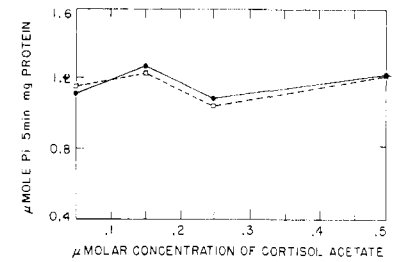

FIGURE 5: Effect of cortisol acetate on the rate of hydrolysis of ATP. Dashed line represents the result of a control with 95% ethanol only. Concentration of reagents and condition for reaction are the same as in Figure 4.

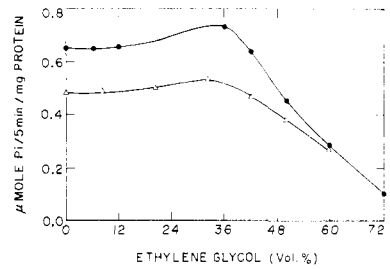

FIGURE 6: Effect of ethylene glycol in the presence (-●-●-) and absence (-Δ-Δ-) of Ca^{2+} in the reaction mixture on the ATP hydrolysis by rat cardiac myosin ATPase. Concentration of reagents and condition for reaction are the same as in Figure 4.

the value of 14.8 kcal $mole^{-1}$ for skeletal myosin (Ouellet *et al.*, 1952).

Effect of Cyclic 3′,5′-AMP and Cortisol Acetate. It was shown that cyclic 3′,5′-AMP (Sutherland *et al.*, 1965) and cortisol acetate (McCarl *et al.*, 1965) both have positive inotropic effects. The effect of these compounds on the ATPase activity of rat heart myosin is shown in Figures 4 and 5, respectively. It can be seen that neither the cyclic nucleotide nor the steroid hormone have any significant effect. At higher concentrations both have a slight inhibitory effect. Since cortisol acetate was dissolved in 95% ethanol, a control was run at the same time and the same concentrations of ethanol. In order to check if cyclic 3′,5′-AMP prevents Mg^{2+} inhibition, a reaction was performed in which Mg^{2+} and cyclic 3′,5′-AMP were included. No such protective effect was observed (Figure 4). Mg^{2+} inhibition was complete in the presence or absence of the cyclic nucleotide.

Effect of Ethylene Glycol. Brahms and Kay (1962, 1963) reported that ethylene glycol (45 vol %) increased the activity of dog cardiac myosin ATPase activity some two- to threefold in the presence of 0.5 M KCl and 0.01 M $CaCl_2$. This enhanced activation of enzyme activity was accompanied by a decrease in helicity. However, Kaldor (1968) has recently reported that the enhanced activation of myosin ATPase activity in the presence of ethylene glycol was dependent upon the concentration of certain metals. He reported that the maximum activation occurred in ethylene glycol (40 vol %) when the KCl concentration was 0.5 M. He also found that this activation was 75–80% inhibited by 5–10 mM $CaCl_2$. Furthermore he was unable to detect any change in helicity upon activation by ethylene glycol.

Figure 6 shows that the specific activity of rat heart myosin ATPase is lower in the absence of $CaCl_2$ than in its presence, and, further, a certain threshold level of calcium is required for activation even though high concentrations are inhibitory. These data support Kaldor's results.

The observation that no significant change in the helicity of rat cardiac myosin is obtained in 30 vol % ethylene glycol is also in agreement with Kaldor's results.

Effects of Drugs. Several drugs which are known to affect the central nervous system and which also inhibit certain enzyme systems were tested *in vitro* for their effects on rat cardiac myosin.

Figure 7 shows the results obtained from a study of the effects of sodium barbital, sodium amytal, and sodium phenobarbital on the ATPase activity of rat cardiac myosin. It can be seen that none of these drugs shows any significant increase on the catalytic effect of the protein. In fact a slight inhibitory effect occurs at lower concentrations. Slightly higher concentrations of phenobarbital (2×10^{-4} M) and sodium amytal (8×10^{-4} M) reduced the helicity to 45 and 51%, respectively (Table I).

The results obtained with norepinephrine and chlorpro-

TABLE II: Effect of Chlorpromazine (0.5 mg/l) and Norepinephrine (1.00 mg/ml) on ATP Hydrolysis.

Control and μg of Drugs Added	Chlorpromazine[b]		Norepinephrine[b]	
	Prep I	Prep II	Prep I	Prep II
Control	0.680	0.796	0.680	0.796
Control + 10 μg	0.680	0.881	0.783	0.796
Control + 25 μg	0.840 (23.6%)	0.881 (10.7%)	0.830 (22.2%)	0.909 (16.8%)
Control + 50 μg	0.753	0.756	0.830	0.930
Control + 100 μg	0.783	0.849	0.835	0.856
Average increase in activity (%)	17.2		19.5	

[a] Protein concentration in preparation I is 0.92 mg and in preparation II, 0.69 mg. [b] In μmoles of P_i/5 min per mg of protein.

mazine differ from those observed with the barbiturates. Table II summarizes the effects of these two drugs on the ATPase activity of rat cardiac myosin. Both drugs caused a definite increase in the ATPase activity.

The change in helicity induced by norepinephrine and chlorpromazine are shown in Table I. Norepinephrine at a concentration of 2.4×10^{-3} M reduced the helicity to 46%. Chlorpromazine at much lower concentrations (1.4×10^{-4} M) (comparable with those involved in the kinetic investigations) reduced the helicity to 42%. High concentrations of chlorpromazine (10^{-2} M) cause visual denaturation of the protein.

Discussion

No basic differences were observed between rat cardiac myosin and that isolated from either dog cardiac muscle or rabbit skeletal muscle in physical properties, and the enzymatic characteristics appear to differ only slightly.

The complete inhibition of the enzymatic activity by Mg^{2+} parallels that of myosin isolated from other sources. However, the activating effect of Ca^{2+} seems to be pronounced at lower concentrations since at high concentrations no activation was observed (Figure 1), in contrast to dog cardiac myosin.

The ATPase activity is increased only slightly in the presence of ethylene glycol with 0.1 M Ca^{2+}. In addition, no significant change in the helicity of rat cardiac myosin was observed in 30 vol % ethylene glycol.

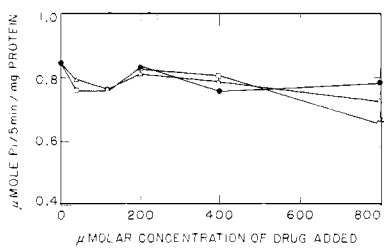

FIGURE 7: Effect of sodium phenobarbital (-□-□-), sodium amyal (-●-●-), and sodium barbital (—△—△—) on the rate of ATP hydrolysis by rat cardiac myisin ATPase. Concentration of reagents and condition for reaction are the same as in Figure 4.

The decrease in helicity associated with the introduction of norepinephrine and chlorpromazine cannot necessarily be correlated with increased enzymatic activity since phenobarbital causes a decrease in helicity with a slight decrease in the ATPase activity. In any case changes in the over-all helicity of the protein may take place in sections not associated with the active site. The helical sections disrupted by the different drugs may also be in totally different sections of the protein. Thus any attempt to correlate enzymatic activity with the over-all helicity of the macromolecule is not justified.

The fact that drugs such as chlorpromazine, phenobarbital, and norepinephrine cause changes in the structure of macromolecules at relatively low concentrations is of considerable interest. Such changes in structure and ATPase activity have recently been reported (Levine *et al.*, 1968) for the action of chlorpromazine and related phenothiazine drugs on myosin B isolated from horseshoe crab muscle (*Limulus polyphemus*). These authors reported that chlorpromazine caused changes in the structure of myosin B, together with an increase in the ATPase activity of the purified protein.

Consideration of the structure of chlorpromazine suggests that the 3-dimethylpropylamine residue may confer amphipathic qualities on the molecule which may lead to a detergent-like disruption of protein hydrophobic bonds. An analogy is found in the well-known effect of sodium dodecyl sulfate on the structure of proteins (see, for example, Jirgensons, 1966; Reynolds *et al.*, 1967).

A more recent report (Akera and Brody, 1968) suggests that chlorpromazine free radicals caused inhibition of Na^+- and K^+-activated ATPase activity. The inhibition of ATPase systems sensitive to Mg^{2+}, Na^+, and K^+ has been linked to altered membered permeability (Skou, 1965).

The action of norepinephrine as a neurotransmitting substance at the synaptic junction in the sympathetic nervous system has been known for some time (Cavallido, 1968; Belleau, 1965; Bloom and Goldman, 1966). A number of mechanisms (Cavallido, 1968; Ahlquist, 1962) have been proposed for the transmission of the impulse without a clear demonstration of the inherent mechanism. The observation that norepinephrine can influence both the helicity of myosin and its rate of hydrolysis of ATP is of particular significance with regard to the neuromuscular junction. Bloom and Goldman (1966) have sug-

gested that the adrenergic receptor machinery is built around phosphorolytic enzymes which utilize ATP as their substrate, and function at a basal rate in the absence of stimulation. The hormonal roles ascribed to the catecholamine agonists at these "receptors" involve their ability to stimulate the rates of these phosphoryl group transfer reactions. The observation that drugs such as norepinephrine and chlorpromazine effect the conformation and ATPase activity of myosin suggests that similar studies on the ATPase systems at the neuromuscular junction may help to shed light on the mechanism of nerve transmission.

It should be pointed out that direct extrapolation of these *in vitro* results to the intact organism are not necessarily valid. In particular the precise concentrations of these drugs at the synaptic junction or in surrounding areas are not available. Norepinephrine, however, is stored in synaptic vessels where it is released in "bursts." Such a release of norepinephrine may then produce local concentration within the range of those employed in the *in vitro* experiments.

As to the effects of cyclic 3′,5′-AMP and cortisol acetate, the present investigations do not suggest that either has any direct effect on the contractile protein and that the explanation for their observed positive inotropic effects should be looked for elsewhere, possibly in the mechanisms governing the mobilization of energized calcium within the myofilaments.

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