ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM DEPARTMENT OF BIOCHEMISTRY, SCHOOL OF MEDICINE, UNIVERSITY OF VIRGINIA]

Physicochemical and Clotting Properties of *p*-Tolylazofibrinogen[1]

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RECEIVED JULY 24, 1957

Some physicochemical and clotting properties of bovine fibrinogen modified by coupling up to 50 *p*-tolylazo groups per mole fibrinogen (M = 330,000) have been examined. From the data it is estimated that only 20% of the added groups are attached to tyrosine and histidine *via* the azo linkage. The addition of these groups does not appear to affect significantly the basic structure of the fibrinogen molecule nor is the sensitivity to thrombin lost. As the number of added groups is increased, the solubility rapidly decreases. Sedimentation studies show that much of the reacted protein is in the form of soluble polymers in equilibrium with monomer. The formation of polymer is favored by increased protein concentration, increased ionic strength or decreased *p*H. The modified fibrinogen, when acted upon by thrombin, forms fibrin-like clots which become increasingly friable and granular and less compactable as the number of added groups is increased. Under most conditions examined, the rate of activation is slower for the azo fibrinogen than for the unmodified fibrinogen, but at *p*H 8.2 and μ = 0.075, the rates are the same. Polymerization, studied independently of activation, was more rapid for the modified fibrinogen at μ = 0.15 but less rapid at μ = 0.05. The results obtained are interpreted on the basis of an increased non-polarity of the fibrinogen molecule.

Introduction

Investigations of the biological and physicochemical properties of chemically modified fibrinogen have contributed greatly to an understanding of the clotting process. Iodination of fibrinogen indicates that aromatic residues are involved in clotting[3] and that the nature of the intermediate polymers formed by iodinated fibrinogen differ in size and *p*H optima from those formed by untreated fibrinogen.[4] Oxidation by tyrosinase[5] shows a progressive increase in clotting time with the extent of enzyme action and a corresponding loss in organization of the clots obtained. Formaldehyde[6] inactivates fibrinogen by a rapid intramolecular reversible reaction and a slower irreversible reaction. More recently, acetylation studies of human fibrinogen[7] have shown that clotting by thrombin is prevented when more than 35% of the free amino groups are substituted, but a peptide is nevertheless split off and soluble polymers are formed.

In this investigation, bovine fibrinogen has been coupled with *p*-tolyldiazonium chloride in order to increase the total non-polar complement of the molecule. Several lines of evidence indicate that many protein–protein interactions are at least partly stabilized by non-polar bonds.[8] It was of considerable interest, therefore, to study the effect of adding non-polar groups to fibrinogen, since this protein undergoes a series of reactions resulting in the formation of fibrils (intermediate polymers) which can ultimately cross link through secondary bonds to form a rigid structural network.

Materials and Methods

Preparation of Fibrinogen.—The fibrinogen was prepared from Armour Fraction I of bovine plasma, Lot No. 26, according to the method of Laki.[9] Solutions of fibrinogen in 0.3 M KCl were quick frozen in liquid nitrogen and stored at −20°. This product was 91–93% clottable and showed no change in clottability or physicochemical properties during the period in which it was stored. The dry weight of the protein was determined by heating 2-ml. samples to constant weight at 110°. Protein concentrations were subsequently determined spectrophotometrically at 280 mμ from the experimentally determined relation, optical density × 0.65 ± 0.01 = mg. protein/ml.

Thrombin.—Commercial dried thrombin[10] containing 22.3 N.I.H. units/mg. was used. To minimize loss by adsorption, solutions were prepared in Lusteroid tubes and used immediately.

Preparation of *p*-Tolylazofibrinogens.—Solutions of the diazonium salt were prepared from crystalline *p*-toluidine[11] and immediately treated with fibrinogen. To couple 25 and 50 diazonium groups per mole fibrinogen, the reaction was carried out using final protein concentrations of 0.90 and 0.45%, respectively. To 9.0 ml. of a 1.0 or 0.50% fibrinogen solution in borate buffer, *p*H 8.3, μ = 0.15, at 22.5°, was added 1.0 ml. of 0.022 M *p*-tolyldiazonium chloride in 0.05 N hydrochloric acid. The final *p*H was 8.0. At selected times, the reaction was stopped by precipitating the protein in 9 times the volume of 8% ethanol in acetate buffer, *p*H 4.5, μ = 0.10, at 0°. Residual diazonium salt was determined by treating 1.0 ml. of the supernatant with 9.0 ml. of 0.01 M resorcinol in acetate buffer, *p*H 4.5, μ = 0.10, at room temperature for 3 hours to assure maximum color development (formation of *p*-tolylazoresorcinol). The concentration of *p*-tolylazoresorcinol was determined spectrophotometrically. The concentration of diazonium salt was equal to that of the *p*-tolylazoresorcinol since coupling was carried out in the presence of a large excess of resorcinol. The moles diazonium salt reacted per mole protein were calculated assuming a molecular weight for fibrinogen of 330,000[12,13] and that all the salt which disappeared, exclusive of slight decomposition, had reacted with the fibrinogen. Azofibrinogens are designated as ϕ_n, the subscript denoting the number of added groups.

Stock solutions of modified and unreacted fibrinogen were prepared by dissolving the precipitate obtained from the ethanol–acetate procedure in 0.1 N ammonium hydroxide. This solvent was used in order to facilitate solution of the difficultly soluble modified protein. These solutions which served as the starting material for all experiments had a *p*H ~9.5 and an added ionic strength due only to the acetate trapped in the precipitate (μ <0.01). They were either used

(1) This investigation was supported by research grants Nos. H-2347 and H-2348 from the National Heart Institute, Public Health Service. The authors are pleased to acknowledge the valuable technical assistance of Miss G. Christine Bunch and Mr. William Kingree.

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immediately or quick frozen and stored at −20°. Under these conditions, normal fibrinogen[14] and azo coupled fibrinogen are stable.

Kinetics of Activation.—The activation of fibrinogen was examined by means of the following technique developed by Waugh and Livingstone.[15] At selected times after the addition of thrombin, the conversion of fibrinogen to fibrin was stopped by the addition of formaldehyde. After compaction of the clot and centrifugation, residual supernatant fibrinogen was determined spectrophotometrically. A plot of ln $1/\theta$ *vs.* t, where θ is the fraction of unclotted fibrinogen at time t, is a straight line which extrapolates through the origin.

Kinetics of Polymerization.—In order to examine the rate of polymerization of activated fibrinogen uncomplicated by the activation phase, the fibrinogen was first activated by thrombin in 0.1 N ammonium hydroxide at an ionic strength <0.01, and then polymerized by dilution in a medium favorable to clotting.[14] Activation was carried out with a fibrinogen concentration of 5.0 mg./ml. and an initial thrombin concentration of 1 μ/ml. for 2 hours at room temperature; then, 0.5-ml. volumes of the activated material were diluted to 5.0 ml. with borate buffers which gave the same final pH and ionic strength values employed in the activation experiments. Polymerization was stopped at intervals by the addition of formaldehyde, and the fraction of fibrinogen which had clotted was determined.

Sedimentation.—Ultracentrifuge runs were made in the Model E Spinco Ultracentrifuge, using a 12 mm. 4° cell with a plastic, Kel-F, centerpiece. Rotor temperature was controlled to ±0.1° by means of the Spinco Rotor Temperature Indicator and Control unit. The sedimentation coefficients for each time interval were averaged and corrected to the corresponding value for water as solvent at 20°. The areas under the peaks were estimated using the Tiselius–Kabat[16] method by tracing an optically magnified image of the peak on ruled paper. Areas were calculated for a bar angle of 40° and extrapolated to correct for dilution as centrifugation proceeded. In relating area to protein concentration, it was assumed that the refractive index increment of modified and unmodified fibrinogen is the same.

Viscosity.—Viscosity measurements were made at 22.5° with Cannon–Fenske viscometers of standard design having flow times for water of approximately 300 seconds. Stock solutions of protein in 0.1 N ammonium hydroxide were adjusted to the desired pH and ionic strength by dialysis at 4° for 24 hours against several changes of buffer and then clarified by centrifugation.

Absorption Spectra.—Absorption measurements were made with a Beckman spectrophotometer, Model DU, using 1 cm. cells. Molecular extinction coefficients, ϵ, were calculated from the equation

$$\log_{10} \frac{I_0}{I} = \epsilon c d$$

where I_0/I is the ratio of the intensity of the light emerging from the pure solvent to that of the solution, c is the molar concentration of absorbing species and d is the thickness of the cell in centimeters.

Results

Absorption Spectra.—The absorption spectra of a ϕ_{20} sample before and after dialysis *vs.* 0.1 N ammonium hydroxide are shown in Fig. 1. During dialysis, some absorbing material leaves the protein solution. If this loss is ascribed to reversibly bound dye, it would correspond to the release of 4 moles salt per mole protein.

The absorption maximum at 340 mμ corresponds to the absorption region of tyrosine monoazo derivatives.[17] The extinction coefficient, ϵ_{340}, per azo group after dialysis is 3500–3700 for all modifications up to ϕ_{46}. Since this value is much lower than those reported for azobenzene derivatives and for diazonium salts coupled to serum albumin, 19,000–26,000,[18] it appears that only 2–3 moles diazonium salt per mole protein are coupled to tyrosine *via* the azo link.

Prior to dialysis only a slight shoulder is observed at 380 mμ, the absorption region for histidine monoazo derivatives.[17] After dialysis, however, a broad plateau between 370–430 mμ appears together with increased absorption above 420 mμ. Although the plateau may have been partially obscured by the absorption of reversibly bound dye, the increased absorption above 420 mμ suggests that some of the released salt later reacts with histidine residues.

Tyrosine and histidine residues containing two tolylazo groups are expected to absorb at 480–490 mμ.[17] Since no absorption peaks have been observed in this region, it is concluded that such bis compounds are not present.

Solubility.—The solubility markedly decreases as the number of added groups is increased. At pH 7.0 and μ = 0.5 at 25°, the solubility of ϕ_{50} is less than 0.01 g./100 ml. compared to more than 4 g./100 ml. for normal fibrinogen under these conditions. Solutions of modified and unmodified fibrinogen in 0.1 N ammonium hydroxide were dialyzed for 24 hours against buffer of appropriate pH and ionic strength at 4°. At constant pH, increase in ionic strength from 0.01 to 0.30 results in a progressive increase in solubility of both ϕ_{24} and ϕ_{45}, Fig. 2. An increase in pH at constant ionic strength (μ = 0.05) increases the solubility of ϕ_0 and ϕ_{24} over the narrow range examined (pH 6.0–7.5). At the latter pH value ϕ_0 is more than three times as soluble as the modified protein.

Sedimentation.—The $S_{20,w}$ for the normal fibrinogen is 7.8 ± 0.1 which is near the values previously reported.[13,14] At concentrations above 0.2% a shoulder on the fast side is observed which represents about 5–10% of the total area of the peak and is presumed to account for the non-clottable protein known to be present.

The p-tolylazofibrinogen shows a main symmetrical peak (S = 8.0) which is similar in sedimentation and spreading to fibrinogen. A large proportion of the protein rapidly sediments without establishing observable boundaries and is completely sedimented in about 15 minutes after full speed is reached. This material has an estimated sedimentation coefficient of $S > 30$ and is recovered as a gel at the conclusion of the run. At high concentrations of reacted protein, two small subsidiary peaks preceding the main peak can be seen; but since these together represent less than 10% of the total protein, they were not sufficiently well defined to study quantitatively.

The effect of pH, ionic strength and concentration on the distribution of ϕ_{50} between monomer, $S \sim 8$, and polymer forms, $S > 30$, was studied by ultracentrifugation. The results are summarized in Table I. The fraction of protein represented by the main peak was measured directly and the

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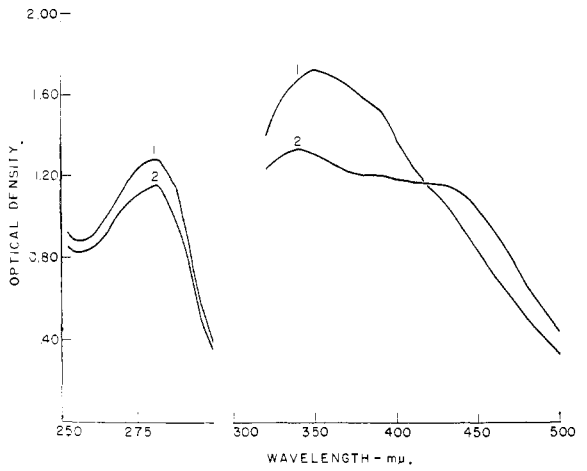

Fig. 1.—Absorption spectra of *p*-tolylazofibrinogen, ϕ_{20}, in 0.1 *N* ammonium hydroxide: curve 1 before dialysis; curve 2 after dialysis.

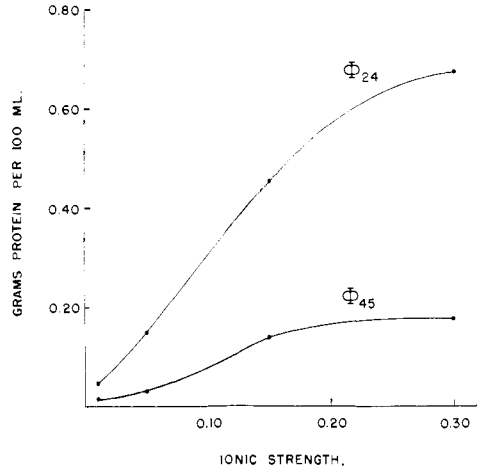

Fig. 2.—Solubility of *p*-tolylazofibrinogen at *p*H 6.6, phosphate buffer.

fraction in the form $S > 30$ was obtained by difference. The reversible formation of polymer is favored by higher ionic strength, increased protein concentration or decrease in *p*H. When the polymer, recovered as a gel, is redissolved, the solutions contain a monomer–polymer distribution in the proportion predicted from Table I.

TABLE I

SEDIMENTATION DISTRIBUTION OF SOLUBLE COMPONENTS OF ϕ_{50}

*p*H	μ	Concn., g. %	Distribution, % $S \sim 8$	$S > 30$
9.7	0.15	0.99	15	80
		.48	23	67
		.32	31	60
9.7	.01	.57	52	38
		.28	60	30
		.14	72	18
8.5	.05	.91	18	76
		.53	21	70
		.26	26	63
8.5	.01	.88	24	66
		.44	25	64
		.22	25	64

Viscosity.—Viscosity studies were carried out at *p*H 8.2 and 8.8 and ionic strengths of 0.05 and 0.15 (borate buffers) with reacted (ϕ_{24}) and unreacted (ϕ_0) fibrinogen. Typical results are presented in Fig. 3. Both ϕ_0 and ϕ_{24} have an extrapolated intrinsic viscosity of 0.25 ± 0.01 which is in good agreement with values reported elsewhere.[13] In all cases examined, however, the slope of ϕ_{24} is greater than for ϕ_0.

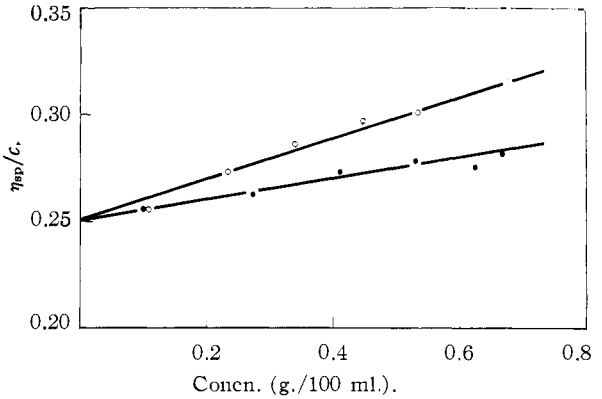

Fig. 3.—Specific viscosity per unit concentration *vs.* concentration: ●, fibrinogen; ○, *p*-tolylazofibrinogen, ϕ_{24}. *p*H 8.2, μ = 0.15, borate buffer.

Clotting of Modified Fibrinogen.—Modified fibrinogen "clots" upon the addition of thrombin. However, as the number of *p*-tolylazo groups is increased, the clots become more friable and granular and less compactable. Nevertheless, the clotting goes to completion and is thrombin dependent.

1. Activation.—A comparison of the rates at which thrombin "activates" modified and unmodified fibrinogen was necessarily examined over a narrow *p*H range since below *p*H 8 the modified fibrinogen is relatively insoluble and above *p*H 9 at low ionic strength fibrinogen is not readily clottable. First order kinetics were, however, observed at *p*H 8.2 and 8.8. Figure 4 illustrates the kinetic curves obtained. The values of the slopes are directly proportional to the rate of activation. The pertinent data are summarized in Table II.

TABLE II

ACTIVATION KINETICS OF UNMODIFIED AND *p*-TOLYLAZOFIBRINOGEN[a]

Slope × 100

*p*H	8.2		8.8		8.6
μ	0.15	0.05	0.15	0.05	0.075
ϕ_0	25.4	7.2	23.8	9.7	10.6
ϕ_{24}	16.4	4.1	13.4	4.5	10.6
ϕ_{50}					10.6

[a] Initial fibrinogen concentration is 0.55 mg./ml.

Under most conditions the modified fibrinogen is activated at a slower rate than the unmodified fibrinogen. However, at *p*H 8.6 and μ = 0.075 the activation rates of ϕ_0, ϕ_{26} and ϕ_{50} are the same. The initial portions of the curves in Fig. 4 deviate from linearity and are due to the accumulation of the unclotted ("uncompactable") fibrin.[15] Examination of the data reveals no significant difference

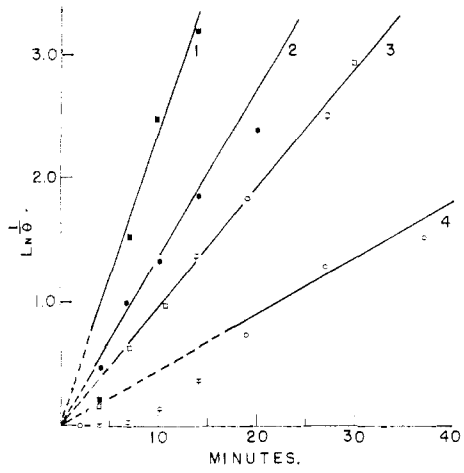

Fig. 4.—Typical activation curves: *p*H 8.8, borate buffer; initial thrombin concentration, 0.1 μ/ml.; initial fibrinogen concentration, 0.55 ± 0.07 mg./ml. Unmodified brinogen: curve 1, μ = 0.15; curve 3, μ = 0.05. Modified brinogen (ϕ_{24}): curve 2, μ = 0.15; curve 4, μ = 0.05. The dashed lines represent linear extrapolations of the straight line portions of the curves.

between the modified and unmodified fibrinogen in this region of the curves.

2. **Polymerization.**—The polymerization rates of ϕ_0 and ϕ_{24} at μ = 0.15 and μ = 0.05 at *p*H 8.7 are given in Fig. 5. At the lower ionic strength, the onset of polymerization of the unmodified fibrinogen is immediate, but at the higher ionic strength, it is delayed 9 minutes. The modified fibrinogen is less sensitive to change in ionic strength but polymerizes somewhat more rapidly at higher ionic strength. At *p*H 8.2, the only difference noted is an earlier and more rapid polymerization of both proteins.

Discussion

The coupling of diazonium salts with proteins may not be primarily to tyrosine and histidine residues *via* the azo linkage,[18] as had been generally accepted.[19] Calculations of ϵ per azo group, based on the assumption of complete uptake of *p*-tolyldiazonium chloride by fibrinogen, indicate that less than 20% of the salt which initially reacts with fibrinogen is coupled to tyrosine and histidine *via* the azo linkage. Some diazonium salt is reversibly bound and some reacts with other amino acids to give products which are less colored. These results are similar to those of Gelewitz, *et al.*,[18] who have shown that serum albumin reversibly binds a variety of diazonium salts and that of the remaining irreversibly reacted groups only about a third are attached to the protein by an azo linkage. Similar results have been noted by Koltun[20] for insulin. A colorless addition compound of benzenediazonium chloride and glycine has been isolated[21] suggesting that *p*-tolyldiazonium chloride may react with ϵ-amino or terminal amino groups of fibrinogen.

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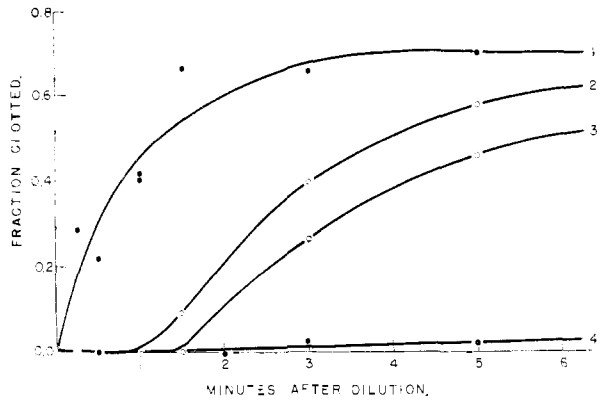

Fig. 5.—Polymerization of activated fibrinogen: ●, unmodified fibrinogen: curve 1, μ = 0.05; curve 4, μ = 0.15; ○, modified fibrinogen, ϕ_{24}: curve 2, μ = 0.15; curve 3, μ = 0.05.

The sedimentation and viscosity studies demonstrate that the addition of *p*-tolylazo groups produces no great change in the size and shape of the unit fibrinogen molecule. Fine structure also appears to be preserved at least to the extent that the potential for activation by thrombin is not destroyed. The changes noted in solubility and clotting properties can be attributed to an increase in the non-polar complement of the molecule resulting from the addition of the *p*-tolylazo groups.

Several proteins exhibit reversible monomer-polymer reactions in aqueous solution.[8] In the case of insulin, equilibrium is attained when a constant attractive force, presumably due to lipophilic groups, is in balance with a variable electrostatic repulsive force.[22] This equilibrium has been shown to shift markedly toward association when the lipophilic character of the insulin molecule is increased.[8,20] Normal fibrinogen does not form soluble polymers under any of the conditions studied here. However, after the addition of *p*-tolylazo groups, soluble polymers are formed and these dissociate when the *p*H and ionic strength are adjusted to increase the electrostatic repulsive force.

The physical characteristics of the clots obtained from *p*-tolylazofibrinogen suggest that a non-specific aggregation occurs in addition to the normal oriented polymerization during the clotting process. Such aggregation would give rise to the packing irregularities in the final clot structure and result in the granular, non-compactable "clots" observed. In fact, when the number of added groups is large, the "clots" grossly resemble a disordered precipitate more than an ordered fibrous structure. The addition of large chemical groups might also cause a steric interference with the formation of an oriented structure or occlude a specific interaction site leading to a similar end result.

The activation of *p*-tolylazofibrinogen proceeds according to first-order kinetics but generally at a slower rate than is observed for normal fibrinogen. This suggests that the reaction equations[15] for the two proteins differ in the constants involved, and, thus, either active or inactive complex formation or

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both may be involved. Since thrombin adsorbs readily to fibrinogen, additional non-polar groups on the fibrinogen molecule may be expected to increase inactive bonding and thus reduce the free thrombin available for active complex formation. However, at $\mu = 0.075$ and pH 8.6, inactive binding of thrombin by modified fibrinogen appears to be compensated since the activation rates are the same under these conditions.

Ferry[23] has suggested that the structure of fibrin is built up by a lateral association of oriented molecules and that the orientation may be due to long-range electrostatic forces between charged groups on the activated fibrinogen molecule. If this is the case, once activation is accomplished, a low ionic strength medium may be expected to promote orientation and consequently polymerization, provided that the ionic strength is not so low that the molecules are prevented from approaching one another at all. This last condition is encountered during activation at ionic strength $\mu < 0.01$.

With normal activated fibrinogen, the expected results are observed; *i.e.*, the onset of polymerization at $\mu = 0.05$ is immediate, but at $\mu = 0.15$ it is delayed. The behavior of p-tolylazofibrinogen, however, is distinctly different since non-oriented bonding which is insensitive to ionic strength here plays an important role in the aggregation process.

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[CONTRIBUTION FROM THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTE OF MENTAL HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Some Physical and Chemical Properties of the Ribonucleic Acid Contaminant of Rabbit Muscle Myosin Preparations

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RECEIVED JUNE 20, 1957

Myosin B isolated from rabbit muscle by standard methods contains up to 1% ribonucleic acid (RNA) by weight as an impurity. This paper describes procedures for separating this RNA from the protein as well as some of its physical and chemical properties. Methods involving selective denaturation of the myosin yield a product with a protein/RNA ratio of 1.3–1.4, similar in RNA content to fish nucleo-tropomyosin. The protein is not myosin B but rather a heat stable protein with a low aromatic amino acid content, similar to tropomyosin. The RNA is not tightly bound to this protein, however, as they can be further separated by electrophoresis or by chromatography on ECTEOLA columns under mild conditions, yielding a product with a protein/RNA ratio of less than 0.1. The mean nucleotide composition of the RNA is 18.3% adenylic acid, 31.9% guanylic acid, 30.9% cytidylic acid and 18.9% uridylic acid. All samples analyzed were of this same composition despite wide variations in the extent and methods of fractionation of the RNA. The RNAs were electrophoretically homogeneous but highly heterogeneous in the ultracentrifuge, having a concentration independent, mean sedimentation coefficient of 6.6 S. The preparations showed a typical RNA ultraviolet absorption spectrum with a mean $\epsilon_{(P)}$ value of 7528 at 260 mμ. This $\epsilon_{(P)}$ value increased to 10,782 upon base hydrolysis to the nucleotides, and the spectrum of the hydrolyzate coincided with the sum of the absorptions of the component nucleotides as calculated from the measured nucleotide composition. The spectrum of the hypochromic effect is shown to have two maxima, which nearly coincide with those of guanylic and cytidylic acids.

Introduction

In a previous paper[1] it was shown that myosin prepared from rabbit skeletal muscle contains ribonucleic acid (RNA). In the present study we have isolated this RNA by a variety of methods and have made physical and chemical studies on the isolated material. The physicochemical measurements have been used to compare the extent to which the various isolation procedures tend to degrade, denature and fractionate RNA, and therefrom to estimate the properties of the "native" RNA. Because the isolated RNA possessed both a high sedimentation coefficient and a low $\epsilon_{(P)}$ value and therefore appeared to be one of the better RNA preparations, studies were made of the changes in its physicochemical properties under controlled conditions. Certain conclusions were drawn as to the structure and spectrum of this RNA.

Experimental

Myosin Preparation.—Myosin B was isolated from rabbit muscle as described in detail in the previous paper.[1] The back and hind leg muscles were minced, suspended in twice their weight of 0.9 M KCl, and extracted in the cold (0–4°) for 6 to 10 hours. The suspension was diluted with two liters of ice-cold deionized water and strained through cotton gauze. The filtrate was diluted with 12 liters of ice-cold deionized water to precipitate the myosin and allowed to settle overnight at about 4°. After decanting the supernate, the myosin precipitate was packed by centrifugation and dissolved by adding enough 3 M KCl to bring the final concentration to 0.6 M KCl. Insoluble impurities were removed by centrifugation for 10 minutes at 3000 × g. The myosin was reprecipitated by diluting the supernatant with 4 liters of ice-cold deionized water (to a final ionic strength of 0.05). It was then separated from the solution by centrifugation and redissolved in sufficient 3 M KCl to give a final concentration of 0.6 M KCl. Myosin solutions prepared in this way contained approximately 0.15 mg. of nucleotide material/ml. and about 150 times as much protein.

Deproteinization.—The RNA was routinely separated from the bulk of the myosin B by coagulating the protein in hot 10% NaCl solution.[2] The myosin solution was slowly mixed with half its volume of 30% NaCl and heated in a steam-bath for 8 minutes after coagulation began (at 45°), for a total of about 10 minutes (final temperature about 97°). The suspension was blended for 20 seconds in a Waring Blendor, stirred for 30 minutes at 0°, and then centrifuged at 3000 × g for 10 minutes. The slightly turbid supernate was either filtered through a medium porosity sintered glass filter or centrifuged at 40,000 × g for 20 minutes to remove suspended, denatured protein which otherwise strongly adsorbed RNA in the following ethanol precipitation step.

The 10% NaCl extract was treated with two volumes of ice-cold ethanol and allowed to stand overnight at 4°. The

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