Ion Binding by Tobacco Mosaic Virus and Its Protein*

Ragaa A. Shalaby, Krishnadas Banerjee,† and Max A. Lauffer

ABSTRACT: Ion binding by tobacco mosaic virus (TMV) and TMV protein was studied, using cation- and anion-exchange membrane electrodes, for the purpose of investigating its possible role in TMV protein polymerization. Cl^-, K^+, and Ca^{2+} are not bound by TMV protein at and above the isoionic point (pH 4.3–4.6) up to pH 9. However, the binding of these ions by TMV goes from 0 at the isoionic point (pH 4.3–4.6) to 1.4 Cl^-, 2.5 K^+, and 1.8 Ca^{2+} ions bound per protein monomer (17,531) at pH 9. The binding of SCN^- to TMV and TMV protein increases with pH from isoionic point (pH 4.3–4.6) to pH 7 and changes slightly between pH 7 and 9. Below the isoionic point both the virus and the protein bind the four different ions, with the binding to protein being more than twice as much as to virus in every case.

The effect of pH is more pronounced below the isoionic point than above it. Since Cl^-, K^+, and Ca^{2+} are not bound by either polymerized protein (pH 4.3–6.5) or unpolymerized protein (pH 7–9), ion binding can play no important role in the endothermic polymerization of TMV protein.

Tobacco mosaic virus (TMV) protein undergoes reversible polymerization depending on pH, solvent, temperature, and concentration. In the direction of polymerization, the reaction is endothermic with both enthalpy and entropy increasing. Lauffer *et al.* (1958) postulated that the entropy increase stems from the release by the protein of some component of the solvent, presumably water, during the polymerization process. Stevens and Lauffer (1965) substantiated this hypothesis by directly measuring water release during polymerization. This work was undertaken to see if ion binding to protein contributes to the entropy increase during polymerization. The data in this paper, in combination with titration data (Scheele and Lauffer, 1967), enable the calculation of the charge on the molecule at different pH values. The binding to the virus was undertaken as a comparison with the protein to see the effect of the presence of RNA and the structural differences brought by it.

Materials and Methods

TMV. The common strain of TMV was isolated by differential centrifugation with a depigmentation step (Ginoza *et al.*, 1954) after the first centrifugation cycle.

TMV Protein. The protein was extracted from the virus by the acetic acid method of Fraenkel-Conrat (1957). The protein was dissolved in dilute KOH solution to a final pH between 7 and 8 and clarified by centrifugation at 40,000 rpm for 3 hr.

The protein or the virus was dialyzed in the cold against about 100 times its volume of distilled, deionized water with the water being changed every 12 hr for 3 days. Some aggregation of the protein took place during dialysis. The protein or the virus was then combined with mixed-bed ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif., AG 501-X8, 20–50 mesh). The time of deionization varied between 4 and 8 hr depending on the ratio of protein to resin. During deionization, the protein or the virus formed a heavy colloidal suspension. To get rid of the resin, the suspension was centrifuged at 5000 rpm for 5 min; the resin sedimented to the bottom of the tube and the protein was taken by a syringe. The isoionic point turned out to be the same for both the virus and the protein and varied between pH 4.3 and 4.6 from one preparation to another. Part of the difference was due to concentration difference but there was also variation at the same concentration with different samples.

Concentration Determination. Concentrations of TMV and TMV protein solutions were determined by use of a Cary spectrophotometer. In each case the ultraviolet absorption spectrum was measured from 400 to 230 mμ. Solvent and blank were 0.033 M, pH 7.5 phosphate buffer. In this solvent, any aggregated protein or virus disaggregated. The concentration of the virus was calculated from the optical density at the maximum (260 mμ) corrected for scattering, using an extinction coefficient of 27 $(g/100\ ml)^{-1}$ (Fraenkel-Conrat and Williams, 1955). Protein concentrations were similarly determined by using an extinction coefficient of 13 $(g/100\ ml)^{-1}$ at the maximum optical density at 281 mμ. The concentration used in the experiments reported here varied between 1.2 and 1.6%.

Titration of the Virus and Protein. The pH measurements were made with a Radiometer pH meter Model 4 or a Beckman Research pH meter using a combination electrode. Standard buffer solutions were used for standardization of the meter. Most of the acid–base

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† Present address: Department of Biophysics, Saha Institute of Nuclear Physics, Calcutta 37, India.

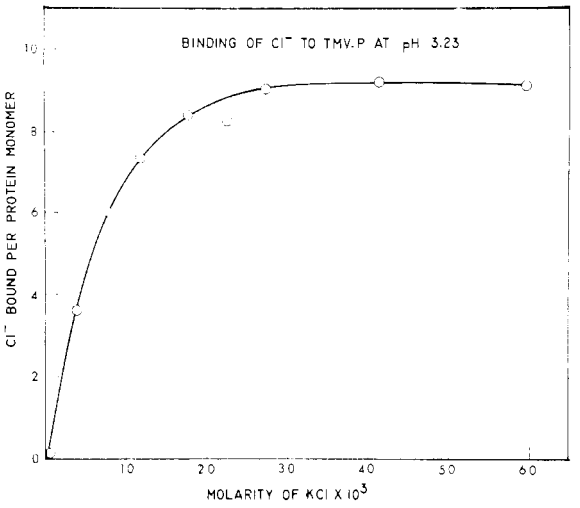

FIGURE 1: Relation between increasing molality of KCl and the number of moles of chloride bound per mole of protein monomer at pH 3.23.

titrations were carried out at room temperature except at low pH where the sample was titrated at 4° and at room temperature. The protein and the virus were brought to the desired pH by adding aliquots of the alkali or acid of the ion under investigation whenever possible; for example, when measuring calcium binding at pH levels alkaline to the isoionic point, $Ca(OH)_2$ was used for the titration and when measuring thiocyanate binding below the isoionic point, thiocyanic acid was used to adjust the pH. Otherwise, KOH and HCl were used.

Chemicals. All chemicals were analytical reagent grade and were used without further purification. KOH solutions were prepared from Acculute Standard volumetric solution in preboiled, distilled water. $Ca(OH)_2$ was prepared by weight in preboiled, distilled water and kept with minimum exposure to air. HSCN was prepared by equilibrating the H^+ form of Dowex cation-exchange resin with NaSCN. The acids and alkali were standardized before use.

Experimental Procedure

Ion binding was measured potentiometrically. The cell for emf measurements using ion-exchange membrane electrodes was modeled after Scatchard *et al.* (1959). Small pieces of ion-exchange membrane (anions A-20, Permutet Co.; A'-64; cation AMF C-60-46, AMF C-103-EE, American Machine and Foundry Co.) were first conditioned in the solvent (KCl, $CaCl_2$, or NaSCN) at low temperature for more than 24 hr with repeated change of solution. Usually in the beginning, the membrane was soaked in 0.1 *m* salt solution and, toward the end, 0.01 *m* solutions were used. The lucite cell was assembled using the minimum effective amount of Apezion grease for the three-way glass stoppers. Before introducing the membrane between the two blocks of lucite, the edge of the membrane was touched with a moderately hot soldering iron. This way a water-tight seal was made between the membrane and the lucite blocks. Four screws were tightened enough to make the junction between the two blocks water tight but without deforming the membrane too much. Each compartment of the cell makes connection through a capillary with a Beckman standard calomel electrode which was immersed in 4.5 *m* KCl to minimize the effect of small temperature fluctuations. The cell was kept in a Faraday cage in a thermally regulated room where the temperature is kept at 22 ± 1°. Potentials were measured with a vibrating reed electrometer, a Leeds and Northrop Type K_3 Universal potentiometer and a Honeywell recorder.

Different ion-exchange cells were then calibrated by using KCl, $CaCl_2$, NaSCN, and KCl over the concentration range 0.001–0.100 *m*. At the lower concentrations, measured electromotive force values were within 1% of the theoretically calculated values. The agreement between theoretical and experimental electromotive force decreases with increasing concentration until at 0.1 *m* there was about 4% difference. Equation 1 was used to calculate the electromotive force

$$\Delta E = \frac{RT}{nF} \ln \frac{m_1\gamma_{1\pm}}{m_2\gamma_{2\pm}} \qquad (1)$$

where the subscripts 1 and 2 refer to the two half-cells. ΔE is the electromotive force across the ion-exchange membrane, m is the molality of the salt, R is the gas constant, T is the absolute temperature, n is the valency of the ion, and F is the Faraday constant.

The mean activity coefficient $\gamma\pm$ for KCl was taken from Harned and Owen (1943). For $CaCl_2$, $\gamma\pm$ was calculated according to Coleman (1953) and for SCN, $\gamma\pm$ was calculated according to Yap (1964). Molal solutions were made for standardization except at low salt concentrations where dilutions were made volumetrically. Before making any electromotive force measurements, the potentiometer and the recorder were calibrated against a standard unsaturated cadmium cell.

To measure the binding of a particular ion to protein, protein solutions were prepared in different concentrations of the ion. On one side of the membrane was placed the protein solution in a known concentration of the ion under investigation, on the other side, a solution of the same composition except for the absence of protein. The electromotive force across the calomel electrodes was then measured and using eq 1, m_2, the molality of the unbound ions in the protein side, was calculated. The number of moles of ions bound ($\bar{\nu}$) per mole of protein monomer (17,531) was then obtained by eq 2.

$$\bar{\nu} = (m_1 - m_2)/m_p \qquad (2)$$

where m_1 is the added molality of the ion and m_p is the molarity of the protein.

This procedure is repeated at different concentrations of the ion and $\bar{\nu}$ calculated. Plotting $\bar{\nu}$ *vs.* salt concentration produces a plot typified in Figure 1, where binding increases with increasing ionic strength of the solution until a certain concentration is reached, after which no

further increase in binding takes place. The value of $\bar{\nu}$ at the plateau, then, represents the maximum number of ions that could be bound under these conditions and will be called n. The maximum number of ions is also obtained by plotting the data in two ways. First is the reciprocal plot where $1/m$ is plotted as abscissa against $1/\bar{\nu}$ as ordinate. The second way is the Scatchard plot, where $\bar{\nu}$ is plotted on the abscissa *vs.* $\bar{\nu}/m$ as ordinate. If there are n equivalent and independent sites on the protein molecule we should get a straight line in both cases according to the equation $\bar{\nu} = n_2km/(1 + km)$, $\bar{\nu}$ is the average number of ions bound per protein molecule at ionic molality (m), n_2 is the maximum number of $\bar{\nu}$, k is the association constant, and m is the ionic molality.

In the reciprocal plot, the intercept on the ordinate axis gives $1/n_2$, the slope is $1/kn_2$. In the Scatchard plot, the intercept on the abscissa gives n_2, the maximum number of groups; the intercept on the ordinate axis gives kn_2. The Scatchard method of analyzing the data gives more even relative weight to the different points on the curve.

The accuracy of the electromotive force measurements is about 0.2 mV and electromotive force values less than 2 mV were discarded. By applying eq 1, it is assumed that the presence of protein does not affect the ionic species activity coefficient $\gamma+$ or $\gamma-$ and that the ionic species activity coefficient is equal to the mean activity coefficient $\gamma\pm$. It is also assumed that the salt is completely dissociated, *i.e.*, the ionic strength of the solution is the same as the solution molality.

Results

Cl^- Binding. In this case an anion-exchange membrane conditioned in KCl solutions acts as a reversible chloride electrode. The binding of Cl^- by TMV and TMV protein was studied at pH values from 2.7 to 9. At each pH, a plot similar to Figure 1 is obtained. The plateau value of $\bar{\nu}$, representing the maximum number of ions that bind under these particular conditions (n_1), is plotted *vs.* pH in Figure 2a. Table I gives n_1, n_2, k (the association constant), and the saturation concentration after which no increase in binding occurs at each pH.

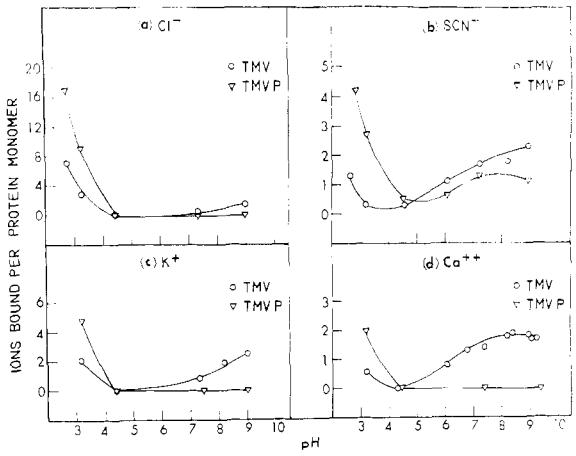

FIGURE 2: Effect of pH on the maximum number of moles of ions bound per mole of protein monomer for both TMV and TMV protein. (a) Chloride binding, (b) thiocyanate binding, (c) potassium binding, and (d) calcium binding.

On addition of KCl to the protein, slight increase in turbidity takes place with increasing concentration of KCl. This effect is also observed with the virus but to a lesser extent. This is a reflection of increased aggregation with increasing ionic strength. When titrating isoionic protein with KCl, the pH of the solution increased with increasing KCl concentration.

Thiocyanate Binding. An anion-exchange membrane electrode is modified to the thiocyanate form. Binding by TMV and TMV protein is then measured from pH 2.7 to 9. In Figure 2b is plotted n_1 *vs.* pH. Table II gives n_1, n_2, k, and the saturation concentration at each pH.

Potassium Binding. A cation-exchange membrane is used as a potassium reversible electrode by condition-

TABLE I: Binding of Chloride by TMV and TMV Protein.

pH	n_1	n_2	k	Satn Concn
		TMV		
2.68	7.0	8.2	0.58×10^2	0.04
3.23	2.8	3.4	2.2×10^2	0.015
4.4	0.0			
7.3	0.5			0.002
8.96	1.4	2.0	3.2×10^2	0.007
		TMV Protein		
2.68	17.0	18.0	0.8×10^2	0.04
3.23	9.1	9.6	2.4×10^2	0.03
4.35	0.0			
7.3	0.0			
8.94	0.0			

TABLE II: Binding of Thiocyanate by TMV and TMV Protein.

pH	n_1	n_2	k	Satn Concn
		TMV		
2.66	1.3	1.5	2.8×10^2	0.008
3.2	0.3			0.005
4.55	0.3	0.4	1.3×10^3	0.002
6.08	1.1	1.3	1.6×10^3	0.003
7.2	1.7	1.9	1.6×10^3	0.002
8.18	1.8	1.9	1.1×10^3	0.004
8.95	2.3	2.6	5.1×10^2	0.008
		TMV Protein		
2.82	4.2	4.6	2.4×10^2	0.012
3.2	2.7	3.1	4.0×10^2	0.01
4.52	0.5	0.6	1.1×10^3	0.003
6.07	0.6			0.0015
7.2	1.3	1.6	5.9×10^2	0.004
8.94	1.1	1.5	9.5×10^2	0.0025

TABLE III: Potassium Binding by TMV and TMV Protein.

pH	n_1	n_2	k	Satn Concn
			TMV	
3.2	2.1	2.5	4.1×10^2	0.01
4.4	0.0			
7.3	0.8			<0.0033
8.2	1.9			<0.0044
9.0	2.5			<0.0048
			TMV Protein	
3.2	4.8	5.7	1.9×10^2	0.015
4.43	0.0			
7.45	0.0			
9.0	0.0			

ing in KCl solution. Measurements were taken between pH 3.2 and 9. The results of these experiments are shown in Figure 2c and Table III.

Ca^{2+} Binding. Binding of Ca^{2+} by TMV and TMV protein was studied at different pH values from 3.2 to 9. In each case, the value of n_1 was obtained from the plot between calcium concentration and $\bar{\nu}$. Figure 2d and Table IV show the effect of pH on calcium binding.

The protein seems to aggregate considerably on addition of $CaCl_2$, especially at lower pH values. The same effect is observed with the virus but to a lesser extent.

Discussion

If binding of the different ions follows electrostatic charge, then an increase in cation binding and a decrease in anion binding would be expected on increasing the pH of the virus or the protein solution above the isoionic point. Experimentally, no binding of Cl^-, K^+, or Ca^{2+} takes place to the protein while the binding of these ions by the virus increases with increasing pH up to pH 9. Both the virus and the protein bind thiocyanate with increasing affinity on increasing pH. If the ions bind to the same sites on both the virus and the protein, then we would expect more binding to the protein on increasing pH, since we start with a completely polymerized, highly aggregated sample at the isoionic point, and we end with a dissociated protein (a trimer or a hexamer) at pH values between 7 and 9. With the virus, however, we also start with an aggregated sample which becomes less aggregated with increasing pH but the protein subunits are still held in the higher structural arrangement of the virus rod, so more sites are exposed in the protein than in the virus at the higher pH values. The fact that the protein does not bind any Cl^-, K^+, or Ca^{2+} and that it binds about half as much thiocyanate as the virus points out that RNA might be playing a role in the binding of these ions. The increased binding with increasing pH above pH 7 might be due to slight structural change of the virus particle which makes RNA more accessible to the solvent. In the titration data of Scheele and Lauffer (1967), the same phenomenon was observed, that is, the virus binds more hydroxyl ions than the protein in the pH range 7–9.

Below the isoionic point, both the protein and the virus bind the four different ions. In this region, however, the protein binds at least twice as much as the virus. Thus, the effect of pH on binding is the same for both anions or cations, indicating that the major part of the interaction taking place is not electrostatic. The isoionic point seems to separate two distinctly different patterns of binding between the virus and the protein. Above it, the protein binds little thiocyanate and no Cl^-, K^+, or Ca^{2+}, while binding of these ions by the virus increases with increasing pH. Below it, both the protein and the virus bind all the ions studied, but the trend is reversed, that is, the protein binds significantly more than the virus.

The possibility of protein denaturation at these low pH values presented itself as a possible cause for the observed high binding. Scheele and Lauffer (1967) have found that TMV protein titrates reversibly down to at least pH 2.7. Their titration, however, was carried out at 4° and in 0.1 *m* KCl. For ion binding, on the other hand, the protein was titrated at 22–23°, starting with deionized protein where the ionic strength is very close to zero. Titration of the protein under the same conditions as for ion binding was performed and found to be very close to being reversible. (This could not be very accurately determined due to the change of ionic strength during titration.) In another set of experiments, the protein was titrated at 4° and kept at this temperature except when measuring ion binding which takes about 2–3 min for a sample. No difference in the number of ions bound by the protein was observed. These two experiments indicate that the protein is native at low ionic strength and low pH.

Binding of cations K^+ and Ca^{2+} at low pH values

TABLE IV: Calcium Binding by TMV and TMV Protein.

pH	n_1	n_2	k	Satn Concn
			TMV	
3.2	0.58	0.7	1.5×10^3	0.003
4.3	0.0			
6.04	0.8			<0.002
6.8	1.3			<0.0025
7.41	1.4			<0.0015
8.2	1.8			<0.0028
8.4	1.9			<0.0028
9.0	1.8			<0.0029
9.1	1.7			<0.0019
9.2	1.7			<0.003
			TMV Protein	
3.2	2.0	2.3	1.0×10^3	0.06
4.4	0.0			
7.4	0.0			
9.4	0.0			

where a positive charge is dominant on the protein is difficult to understand especially when the protein is in such high state of aggregation (pH 3.2). Many instances of this nature have been reported in the literature and lack an explanation (Carr and Topol, 1950; Carr, 1953a,b; Carr and Wood, 1955). It could be that binding of a particular ion (the chloride ions either from HCl used in titrating or from the KCl or $CaCl_2$ used for binding measurements) makes binding of its counter ion more feasible. It is also likely that an explanation might lie in the structural relationships between the various active groups as they occur in the protein, or RNA, or in the interaction between the protein and the RNA.

A sophisticated treatment of ion binding data should take into account the electrostatic interaction on the protein molecule. The calculation of the electrostatic factor in this polymerizing system of widely different particle sizes is impossible. Neglecting electrostatic interactions and analyzing the data according to both the reciprocal plot and the Scatchard plot, a straight line was obtained but there was much scattering of the points, especially at lower binding values. But since in these cases there is one or less than one ion bound per protein molecule and since the fit is better when higher binding is obtained, the assumption that there are n_2 equivalent and independent sites seems to be justified. The values of the maximum number of groups (n_2) and the association constant for binding (k) calculated from the Scatchard plot are shown in Tables I–IV. The values of these parameters calculated from the reciprocal plot are in close agreement.

In studying binding of potassium and calcium above the isoionic point, it was not possible to use as low ionic concentration as desirable since the protein or the virus was titrated with the hydroxide of the ion under investigation. In the case of the protein, no binding was observed on increasing the concentration of these two ions. Binding of these two ions by TMV was found to take place. However, it seemed that the concentration of potassium or calcium ions required to saturate TMV under a particular condition was lower than the concentration of these ions required to adjust the pH. Consequently, on increasing ionic concentration, $\bar{\nu}$ remained constant within experimental error. As a result, these data could not be analyzed for n_2 and k.

No inferences could be made about the binding sites since, as has been discussed, the binding does not correspond with the electrostatic charge on the molecule. Hulett (1964) measured proton release from TMV on addition of divalent metal ions. He found that four protons could be released per protein subunit. He concluded that two divalent metal ions react per protein subunit and that the site of reaction is two carboxyl groups held together by hydrogen bonding. The average pK of the protein-releasing group is about pH 8. He speculated that each subunit participates in four such binding sites and that this binding is a major factor in holding RNA-free protein rods together. Since our results indicated that protein does not bind any Ca^{2+} between pH 4.4 and 9, while the virus binds 1.8 Ca^{2+}/protein monomer at pH 8–9, then the proton-releasing groups should be involved in binding between the protein and RNA or else groups on the RNA.

One final point to make is that it is clear that ion binding does not contribute to the entropy increase observed in the reversible endothermic polymerization of TMV protein between pH 6 and 7 since no binding of Cl^-, K^+, or Ca^{2+} by the protein is observed at these pH values.

Combining the charge due to H^+ (Scheele and Lauffer, 1967), K^+, and Cl^- binding and calculating a net charge on the virus and the protein at different pH values from pH 5.0 to 3.2, we get the following. For the virus, the point of zero net charge lies at about pH 4.6, which stands in contradiction with the electrophoresis results in which the point of zero mobility is at pH 3.23 in 0.1 m KCl (R. B. Scheele, unpublished data). For the protein, however, there is a range of zero net charge between pH 3.2 and 4.7, but it is hard to draw conclusions since electrophoretic mobility measurements for the protein in KCl are not available. The discrepancy between the isoionic point and the isoelectric point for the virus can be explained by the same reasoning given by Ansevin *et al.* (1964) and Scheele and Lauffer (1967) which goes as follows. TMV has a deeply grooved surface (Franklin and Klug, 1956); it is proposed that ions that go into the inside of the groove would have little influence on the electrophoretic mobility while it would contribute to the charge calculation. This could also explain the lack of Donnan pressure for TMV, which, if calculated from the charge from base binding, would be very significant (Banerjee and Lauffer, 1966).

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Tropomyosin from Smooth Muscle of the Uterus*

Mary E. Carsten†

ABSTRACT: Tropomyosin was isolated from smooth muscle of human and pregnant sheep uterus. The tropomyosin preparations were shown to be essentially free of other proteins. The sedimentation constant and the intrinsic viscosity of human uterus tropomyosin were similar to those of rabbit skeletal tropomyosin but those of sheep uterus tropomyosin were higher. Other methods revealed differences between the uterus tropomyosins and rabbit and sheep skeletal tropomyosins, but no differences between human and sheep uterus tropomyosins. In starch gel electrophoresis at pH 7.6 the smooth muscle tropomyosin migrated faster toward the positive pole than the skeletal tropomyosin. Quantitative amino acid analyses showed significant differences in their amino acid composition. Peptide maps of tryptic digests of human and sheep uterus tropomyosins, though resembling those of skeletal tropomyosin, consisted of fewer peptide spots.

Physiological differences between uterine smooth muscle and skeletal muscle may be a reflection of chemical differences in the contractile proteins of the myofibril. There are three major proteins recognized in the myofibril: (1) actin, (2) myosin, and (3) tropomyosin. While it has been established that actin and myosin interact in muscle contraction, the function of the third protein of the myofibril is still not completely understood; it has been suggested that tropomyosin is implicated in sensitizing actomyosin to EGTA or calcium control when combined with the cofactor troponin (Ebashi and Kodama, 1966).

Tropomyosin found in skeletal and heart muscle (Bailey, 1948) appeared essentially identical (Katz and Converse, 1964). Whether the proteins of smooth muscle are identical with those of skeletal and heart muscle is still largely unknown because of the difficulty in preparing the smooth muscle proteins in adequate yield and purity for extensive characterization. Actin from uterus was found highly similar if not identical with skeletal muscle actin (Carsten, 1965). Myosin from uterus muscle has not been prepared in pure form. Differences between tropomyosin from smooth and skeletal muscle have been suggested (Kominz *et al.*, 1957a,b) but no extensive investigation has been made. In the present communication tropomyosin was obtained from human and from pregnant sheep uterus muscle. It was compared with rabbit skeletal tropomyosin because this is best characterized in the literature. We are referring to tropomyosin or tropomyosin B of vertebrate smooth muscle in contrast to tropomyosin A or paramyosin of invertebrate smooth muscle. Some analyses of sheep skeletal tropomyosin are included in order to be certain that any differences encountered are due to organ specificity and not to the class of animal.

Experimental Procedures

The human uteri were surgical specimens removed because of uterine prolapse and showed no signs of pathology. The sheep uteri were from pregnant sheep near term. They were obtained after removal of the fetus but with the placenta still attached to the walls. The placental cotyledons and major blood vessels were resected and the uterine muscle strips were frozen. The sheep skeletal muscle was from a fetus at term.

The procedure of Bailey (1948) for tropomyosin preparation was used with some modification (Katz and Converse, 1964). After the third precipitation in 70% $(NH_4)_2SO_4$, the protein was dissolved in H_2O and dialyzed against three changes of 0.09 M KCl–0.01 M HCl for 72 hr. Protein concentration was determined by the biuret method, standardized with a freeze-dried sample of tropomyosin corrected for moisture.

To minimize aggregation the same solvent (Tsao *et al.*, 1951) was used for sedimentation and viscosity studies. The former were carried out in a Spinco Model E ultracentrifuge equipped with RTIC temperature control and the latter in an Ostwald-type viscometer at

* From the Departments of Obstetrics and Gynecology and of Physiology, Mary E. Carsten Research Laboratory, University of California at Los Angeles School of Medicine, Los Angeles, California. *Received July 25, 1967.* This investigation was supported by a U. S. Public Health Service grant (HD-00010) from the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md., and by cancer research funds of the University of California. Presented in part at the 51st Annual Meeting of the Federation of American Societies for Experimental Biology.

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