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Incorporation of L-*O*-Ethylthreonine into Chick Muscle Protein*

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ABSTRACT: While investigating the physiological disposition of the new drug L-*O*-ethyl-^{14}C-threonine in chicks, a high level of radioactivity was observed in muscle and moderate levels in other tissues. The nature of this radioactivity in muscle was further explored by fractionating the muscle tissue into RNA, DNA, lipid, glycogen, and proteins. Most of the radioactivity was found to be associated with proteins. The protein fraction was subjected to intensive investigation to ascertain the nature of the association of the radioactivity with proteins. Results obtained indicated that L-*O*-ethylthreonine was incorporated intact into tissue proteins. This incorporation was demonstrated by studying the reaction products of dansyl chloride with tissue proteins and with protein hydrolysates, and also by fingerprinting the amino acids and polypeptides produced by hydrolytic and enzymatic degradation of muscle protein.

L-*O*-Methylthreonine, an isoleucine antagonist, has been shown to interfere with the incorporation of L-isoleucine into the proteins of Ehrlich ascites carcinoma (Rabinovitz *et al.*, 1955) and into rabbit hemoglobin (Hori and Rabinovitz, 1968), and to inhibit the rate of incorporation of isoleucine into transfer RNA (Smulson and Rabinovitz, 1968). Chick growth studies in our laboratory with the higher homolog L-*O*-ethylthreonine (B. G. Christensen *et al.*, in preparation) indicated that a similar inhibition (G. Olson and W. H. Ott, in preparation) may be occurring between L-*O*-ethylthreonine and isoleucine, *i.e.*, *O*-EtThr appeared to act as an antagonist of isoleucine.

A tracer study of the physiological disposition of L-*O*-ethyl-1-^{14}C-threonine administered orally to chicks revealed that a high level of radioactivity was retained by muscle, with moderate levels residing in other tissues. The results of this study, reported herein, demonstrated that L-*O*-EtThr was incorporated intact into chick muscle proteins. This was done as follows: (1) by studying the distribution of radioactivity retained by muscle tissue among biochemical components such as glycogen, lipid, RNA, DNA, and protein; (2) associating the protein radioactivity with intact *O*-EtThr by electrophoresis and chromatography (fingerprinting) of amino acids liberated after acid hydrolysis; (3) by fingerprinting the radioactivity distribution among polypeptides produced by enzymatic degradation; and (4) by treating the muscle protein with the end-group reagent, dansyl chloride, to demonstrate the absence of a free NH_2 group in the incorporated *O*-EtThr.

Experimental Section

Eight chicks approximately 3 weeks of age, individually housed in electrically heated, metal battery brooders with wire floors, were used for these experiments. Beacon starter broiler mash and water were supplied *ad libitum*. After a 3-day adjustment period, each chick was given a single oral dose of 7.5 mg of L-*O*-ethyl-1-^{14}C-threonine,[1] specific activity $\simeq$0.15 μCi/mg, in a gelatin capsule. The chicks were bled by cardiac puncture and then sacrificed with CO_2 gas at intervals ranging from 6 hr to 6 days. Total excreta collections were made during the experimental period. Immediately after sacrificing, the tissues were harvested, homogenized, and frozen on solid CO_2 until assayed.

Radioactivity Assay. Radioactivity in chick plasma, tissues, and excreta was determined by liquid scintillation counting, using a Packard Tri-Carb scintillation spectrometer Model 3003. Plasma was determined by direct addition of 0.5 ml of plasma to 20 ml of 70:30 scintillator solution. Tissues and excreta were homogenized, aliquots were dried and combusted by the oxygen flask method, and the CO_2-^{14}C produced was dissolved in hyamine for counting in a standard DPO—POPOP scintillator solution in 70:30 toluene–ethanol solvent.

The radioactivity of eluates obtained from papers used for fingerprinting was determined by evaporating them in 3-in. stainless steel planchets, and measuring the activity of the residues in a Sharp Low Beta counter.

Muscle Fractionation. The chick muscle was fractionated into glycogen, RNA, DNA, lipid, and protein, by means of the perchloric acid method developed by Shibko *et al.* (1967). A 20-ml sample of muscle homogenate containing 4.0 g of wet tissue was subjected to the Shibko method without modification. Protein and glycogen samples were assayed radiometrically by the oxygen flask combustion method and liquid scintillation counting. All other samples, including the combined supernatants from glycogen precipitation, were assayed by direct liquid scintillation counting.

Fingerprinting of Muscle Protein Hydrolysate. Approximately 4.0 g of chick muscle processed by the above procedure yielded 894 mg of dried protein. A portion of this protein was subjected to hydrolysis in 6 N HCl at 110°, and the hydrolysate

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[1] Synthesized by our colleague Dr. H. E. Mertel.

TABLE I: Material Balance of *O*-EtThr-^{14}C in Tissue and Excreta (Total Equivalent mg in Tissues and Organs).

Bird No.	4901	4902	4403	4402	4903	635	636	4904
Time Lapse (hr)	6	24	24	36	48	72	72	6 days
Final Weight (g)	362	400	262	270	408	294	296	555
Fat			0.02	0.02		0.01	0.01	0.01
Intestine	0.11	0.13	(0.1)	(0.1)	0.07			0.02
Kidney	(0.1)	(0.1)	0.11	0.14	(0.1)	0.11	0.13	0.07
Liver	(0.2)	(0.2)	0.22	0.24	(0.2)	0.18	0.23	0.09
Muscle	4.84	4.76	2.67	3.81	4.17	7.19	5.62	4.50
Plasma	0.50	0.28	(0.4)	(0.4)	0.18	0.44	0.55	0.04
Skin	(0.2)	(0.2)	0.13	0.25	(0.2)	0.08	0.05	0.06
Excreta	(0.50)	(1.37)	1.15	2.05	(2.02)	2.23	2.41	(2.6)
Intest. Content	0.04	0.03			0.01			0.0
Total mg	6.49	7.07	4.80	7.01	6.95	10.24	9.00	7.39

was investigated using the fingerprinting technique of Ingram (1958). Prior to performing this experiment, tests were made of the stability of the ethoxy-1-^{14}C group in *O*-EtThr-^{14}C and in the radioactive protein. This was done by heating 1 mg of *O*-EtThr-^{14}C in a sealed tube with 1.2 ml of 6 N HCl for 1, 2, and 4 days at 110°. In a similar fashion, 25 mg of dried protein was heated in 4 ml of 6 N HCl. At the end of the specified periods, the tubes were opened, the contents were distilled at reduced pressure (room temperature), and the distillates were collected in liquid nitrogen. A 0.5-ml aliquot of each distillate was measured by liquid scintillation counting to determine the amount of ethanol-1-^{14}C liberated by the hydrolytic treatment.

The protein was hydrolyzed for 24 hr, and small aliquots (10–40 μl) of the hydrolysate were spotted on 15 × 18 in. Whatman No. 3MM paper and subjected to high-voltage electrophoresis in a Savant high-voltage electrophoresis instrument for 1.5 hr in a pH 2.2 buffer composed of pyridine and formic acid. After electrophoresis, the paper was dried in air, and descending chromatography was applied for 18 hr using a solvent system butanol–butyl acetate–HOAc–H_2O (19:1:5:25, v/v). The paper was then dried and sprayed with ninhydrin reagent (0.10% in alcohol containing 5% collidine) to visualize the component amino acids, and the identity of ninhydrin-positive components was established by comparison with the fingerprint of a mixture of known amino acids (Richmond and Hartley, 1959).

In order to measure the radioactivity of the ninhydrin-positive components, colored areas were cut out of the paper and eluted with slightly acidified water, and the eluates were concentrated under vacuum to a small volume. These concentrates were transferred to 3 in. stainless steel planchets and evaporated to dryness for measurement in the Low Beta counter.

A separate experiment was performed to make certain that *O*-EtThr was unquestionably separable from isoleucine (Ile) and leucine (Leu). To test this separation a mixture of Ile, Leu, and *O*-EtThr-^{14}C was subjected to successive high-voltage electrophoresis and chromatography.

Dansylation Experiment. The possibility that *O*-EtThr-^{14}C was bound to protein by a noncovalent bond instead of being incorporated in the polypeptide chain was tested by reacting chick muscle protein with dimethylaminonaphthalenesulfonyl chloride (dansyl reagent) before and after hydrolysis, and then performing two-dimensional thin layer chromatography. Hydrolyzed protein was also dansylated. The reaction product would be a highly fluorescent sulfonamide. Dansylation was accomplished by mixing reagent and substrate in $NaHCO_3$ solution for 1 hr at room temperature. This reagent reacts with amino groups of amino acids to form sulfonamides. For the two-dimensional chromatography, the following successive solvent systems were employed. (1) Methyl acetate–2-propanol–ammonium hydroxide (45:35:20, v/v) and (2) *tert*-butyl alcohol–HOAc–hexane (15:15:75, v/v). Appropriate areas on thin-layer chromatography plates were scraped into liquid scintillation counting vials containing 70:30 phosphor solution for radiometric assay.

Digestion of Muscle Protein with Trypsin and Chymotrypsin. Hydrolytic degradation converted muscle protein into individual amino acids, among which intact *O*-EtThr-^{14}C was identified. Another approach adopted to prove that *O*-EtThr-^{14}C was actually incorporated into protein was to degrade chick muscle protein enzymatically to polypeptides, many of which should contain *O*-EtThr-^{14}C, hence show radioactivity. Such a result would not be explainable in terms of noncovalent bonds. Accordingly, a mixture of 90 mg of muscle protein, 2 mg (in 0.2 ml) of trypsin (Worthington), and 50 μg of chymotrypsin (Schwarz Bioresearch) in 100 ml of pH 7.8 phosphate buffer was incubated overnight at 37° and then lyophilized. The dried polypeptide mixture was dissolved in 10–15 ml of 0.1 M acetic acid, and then purified (*i.e.*, desalted and fractionated) by passage through a Sephadex G-25 column. Fractions (10 ml) were collected on a fraction collector and monitored by measurement of absorption at 280 mμ in a Cary 14 spectrophotometer *vs.* 0.1 M acetic acid as a blank.

One fraction (No. 1) was investigated further by the fingerprinting technique based on (1) high-voltage electrophoresis for 1.5 hr at 2000 V using a Savant high-voltage electrophoresis instrument, followed by (2) descending chromatography for 18 hr using 1-butanol–butyl acetate–acetic acid–H_2O (19:1:5:25, v/v). A pyridine–acetate buffer of pH 6.4 was used for the electrophoresis step. Paper was sprayed with ninhydrin reagent to visualize polypeptide locations.

Results

Retention and Elimination. Excretion of *O*-EtThr-^{14}C was slow and incomplete, amounting to approximately 35% of the dose in 6 days. Very small amounts (0.15% of dose) of CO_2-^{14}C were observed in exhaled carbon dioxide. Highest

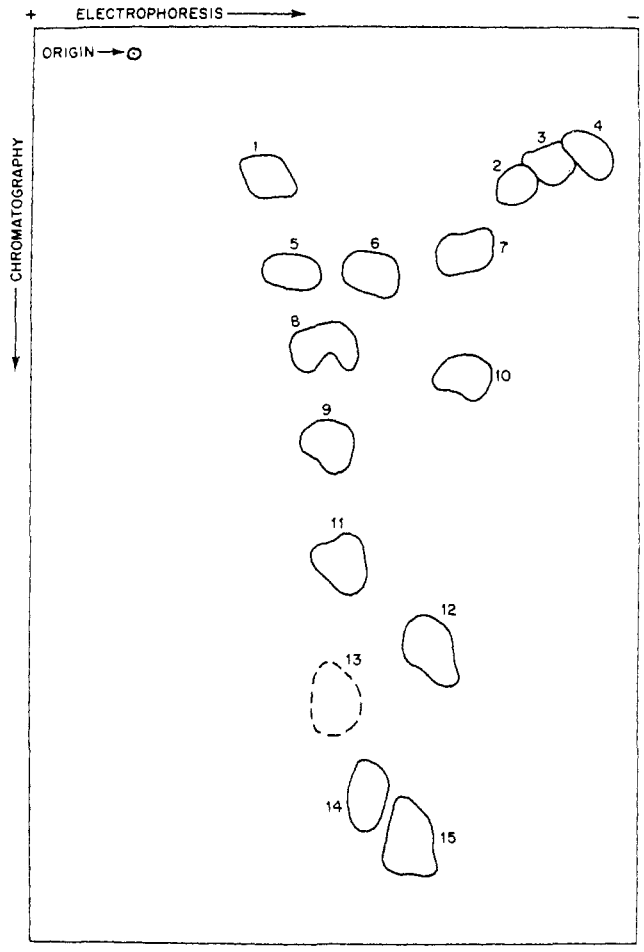

FIGURE 1: Fingerprint of chick 635 muscle protein hydrolysate (500 μg).

levels of radioactivity were found in muscle tissue at all sacrifice intervals. Thus the average equivalent concentration of drug in chick muscle 3 days after administration was $\simeq$45 μg/g as compared with $\simeq$27 μg/g of plasma, $\simeq$15 μg/g of kidney, $\simeq$14 μg/g of liver, $\simeq$5.6 μg/g of skin, and $\simeq$1 μg/g of fat. After 6 days, the respective concentrations were 16.9 μg/g of muscle, 1.01 μg/g of plasma, 5.0 μg/g of kidney, 3.2 μg/g of liver, 2.5 μg/g of skin, and 0.56 μg/g of fat.

TABLE II: Distribution of Radioactivity among Various Tissue Components of Chick Muscle.

Tissue Component	Equivalent μg of *O*-EtThr-^{14}C	% of Total[a] Radioactivity
Supernatant (after precipitation of glycogen)	6.24	2.94
RNA	0.15	0.07
DNA	1.00	0.47
Lipid	1.61	0.76
Protein	187.9	88.63
Glycogen	0.28	0.13

[a] Based on initial value of 212 μg/4 g of tissue.

TABLE III: Stability of the Ethyl-^{14}C Group in *O*-EtThr and Radioactive Muscle Protein (Hydrolysis at 110° in Sealed Tubes Containing 6 N HCl).

Sample	Duration (Days)	% Release
O-EtThr-^{14}C	1	13.7
	2	19.6
	4	36.0
Protein-^{14}C	1	12.4
	2	21.0
	4	19.8

All retention data have been converted from micrograms per gram to total drug content by multiplying these concentrations by total weights of the respective tissues. These results are reported in Table I which shows the total drug content of chick organs and excreta expressed as total milligrams equivalent to radioactivity resident in these specimens. Total organ weights per bird needed for these calculations were estimated by multiplying the total body weights shown in Table I by the following percentages: 3% fat, 2% intestines, 2.6% kidneys, 5% liver, 48% muscle, 6.3% plasma, and 4.2% skin. Excreta values in parentheses are average values of all birds. Tissue values in parentheses are also estimated average values. Concentration in individual tissues can be derived from these totals by dividing by respective total tissue weights.

Muscle Fractionation. Table II illustrates the distribution of radioactivity among the various natural components derived from whole muscle of chick No. 635. Approximately 89% of the radioactivity was found to be associated with protein. Traces of radioactivity associated with other natural components accounted for $\simeq$4.5%; the remaining 6% may have been lost during procedural manipulations.

Acid Hydrolysis. The stability of the ethyl-1-^{14}C group in *O*-EtThr, as well as in the radioactive protein, under hydrolytic conditions was verified prior to undertaking an extensive degradation study. Results presented in Table III indicate that the per cent release of C_2H_5OH-^{14}C during prolonged autoclaving in 6 N HCl for the first 48 hr is similar for both *O*-EtThr-^{14}C (19.6%) and protein-^{14}C (21.0%). No additional carbon-14 was released by protein during a second 48-hr period. These stability results suggest that acid hydrolysis for 24 hr will leave the bulk of the radioactivity attached to the *O*-EtThr-^{14}C molecule. This is in agreement with the inconsequential production of CO_2-^{14}C which is a major ethanol metabolite.

The identification of intact L-*O*-ethylthreonine was established by application of the fingerprinting technique to protein hydrolysate from chick 635. Figure 1, representing the fingerprint of the radioactive muscle protein hydrolysate, showed 14 ninhydrin-positive spots, none of which was radioactive. Most of the radioactivity was concentrated at the *O*-EtThr position 13, which bore no ninhydrin color because of the low *O*-EtThr concentration ($\lesssim$0.1 μg). The fingerprint of the protein hydrolysate plus $\simeq$10 μg of *O*-EtThr-^{14}C contained 15 ninhydrin-colored spots, of which only spot 13 was radioactive. Locations 13 in both fingerprints were superimposable, which identified the radioactive component in the protein hydrolysate as *O*-EtThr-^{14}C. Only 14 ninhydrin spots appeared in a fingerprint of control muscle protein hydrolysate, with

TABLE IV: Radioactivity Distribution in Muscle Protein Hydrolysates (Chick 635).

No.	Amino Acid	Net cpm	
		Figure 1	+*O*-EtThr-^{14}C
1	CySH	0.0	1.49
2	Basic amino acid	0.96	0.0
3	Basic amino acid	0.0	0.0
4	Basic amino acid	1.2	0.0
5	Asp	0.0	1.60
6	Ser	0.0	0.85
7	Gly	0.0	0.0
8	Glu	0.0	1.77
9	Pro	0.0	3.03
10	Thr + Ala	2.35	0.0
11	Tyr + Met	0.92	9.17
12	Phe	2.55	203[a]
13	*O*-EtThr	17.3	3605
14	Ile	0.0	170[a]
15	Leu	1.56	2.82
	2σ	±0.40	±0.48

[a] Probably due to added *O*-EtThr-^{14}C.

TABLE V: Radioactivity of Polypeptides from Chick 635 Muscle Protein.

No.	Net cpm
1	8.6
2	4.1
3	11.1
4	22.5
5	13.0
6	9.0
7	23.7
8	12.8
9	23.0
10	24.4
11	9.9
12	0.0
13	0.0
2σ	2.0

no color visible at location 13. This eliminates the possible presence of an unknown amino acid derivative at location 13.

It was further verified, by fingerprinting of a mixture of *O*-EtThr, leucine, and isoleucine, that the separation of these three amino acids was unmistakable and unequivocal.

Results of radiometric analyses corresponding to individual amino acids are reported as "Net cpm" in Table IV. Radioactivity measurements were performed with a $2\sigma_{net}$ of 0.45 cpm. Radioactivity was localized chiefly at spot 13, both in muscle protein hydrolysate, and in the same plus *O*-EtThr-^{14}C.

Treatment with Enzymes. Polypeptides produced by enzymatic hydrolysis were separated by chromatography on Sephadex G-25. Measurements of the absorbance of cuts at 280 mμ indicated the elution of five fractions, only one of which (fraction 1) retained significant (64% of total) radioactivity. Fingerprinting this fraction revealed the presence of 13 ninhydrin-positive areas, all but two of which (12 and 13) proved to be radioactive as seen in Table V. This suggests that radioactivity associated with intact *O*-EtThr-^{14}C was widely distributed among polypeptides as a result of incorporation into protein.

Dansylation Experiments. The reaction mixture produced by direct dansylation of muscle protein from chick 635 was examined without prior hydrolysis, by two-dimensional thin-layer chromatography. All of the applied radioactivity (86 cpm) remained at the origin. No activity was associated with either *O*-EtThr or dansylated *O*-EtThr. Obviously no free NH_2 group attributable to *O*-EtThr was present in the protein.

Chromatography was repeated after hydrolysis of the dansylated protein, and the distribution of the radioactivity on the plate redetermined. Significant activity (173 cpm) was observed only at the *O*-EtThr location, with a trace remaining at the origin (3.3 cpm). The other three areas associated with reagent, hydrolyzed reagent (dansyl free acid), and dansylated *O*-EtThr-^{14}C were devoid of radioactivity. Obviously no dansylated *O*-EtThr-^{14}C was present; and only *O*-EtThr-^{14}C can be responsible for the observed radioactivity. By contrast, treatment of protein after hydrolysis liberated the fluorescent and radioactive dansyl derivative of *O*-EtThr-^{14}C which could be isolated by thin-layer chromatography.

Discussion

The prominent association of a high level of radioactivity from *O*-EtThr-^{14}C with chick muscle protein is quite interesting in view of its antagonistic nature *vis-à-vis* isoleucine. Such massive retention suggests the involvement of intact *O*-EtThr or a closely related metabolite. That administered *O*-EtThr-^{14}C is retained as such, and is responsible for the tissue protein radioactivity, was demonstrated by the fingerprinting technique which revealed the presence of intact *O*-EtThr in the muscle protein hydrolysate. This finding also suggests that transethylation was not very extensive if it occurred at all.

It also appears that the *O*-EtThr-^{14}C was incorporated by covalent bonding into the peptide chains, presumably in competition with isoleucine, and not by simple physical binding or by a noncovalent bond. The ubiquitous disposition of radioactivity among peptides released by enzymatic degradation of radioactive protein also suggests chemical bonding. This was supported by the experiments with the end-group reagent, dansyl chloride, which confirmed the involvement of the α-amino group in peptide bond formation. Even though no information is available concerning the carboxyl group of *O*-EtThr, one is forced to conclude that *O*-EtThr in chick muscle is chemically incorporated as an intact molecule in the protein.

Muscle appears to be the target organ for *O*-EtThr. At 3 days, the muscle concentration was ≃1.7 times that in plasma, ≃3.0 times the kidney level, ≃3.3 times the liver level, ≃8 times the skin concentration, and ≃45 times the fat residue. The muscle concentration at 6 days was 17 times plasma, ≃3 times kidney, ≃5 times liver, ≃7 times skin, and ≃30 times fat. Except for plasma, which appears to exhaust its *O*-EtThr burden, the concentration ratios for other tissues remain approximately constant between 3 and 6 days despite a loss of ≃$^2/_3$ of total residual *O*-EtThr during this interval. The exceedingly low *O*-EtThr-^{14}C residue in fat is probably due to

its low protein content. The preference for and persistence of the drug in muscle after a single dose are not surprising in view of the growth depressing effect of *O*-EtThr in chicks. A lower metabolic activity of muscle tissue than of other tissues would account for a slower turnover of *O*-EtThr-^{14}C.

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Role of Cation and Anion in the Polymerization and Depolymerization of Tobacco Mosaic Virus Protein*

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ABSTRACT: Potassium and chloride ion binding by unpolymerized TMV protein (2–6°), by polymerized TMV protein (22–26.2°), and by TMV, all in buffers with pH values near 6.5, were measured. Potassium and chloride specific electrodes were used. No binding of either ion was detected for the virus or for the protein in both the polymerized and the unpolymerized states. The estimated error of measurement was such that the binding of one ion per protein monomer (molecular weight 17,500) should have been detected. Therefore, there is no appreciable change in the binding of potassium or chloride when TMV protein polymerizes at pH 6.5 and the large entropy increase which drives the polymerization must be associated entirely or almost entirely with the previously demonstrated release of water molecules.

The negative change in free energy necessary to drive the endothermic polymerization of tobacco mosaic virus (TMV) protein in 0.1 M phosphate buffer at pH 6.5, first observed in this laboratory, was assumed by Lauffer *et al.* (1958) to come from the release of water molecules, resulting in an increase in enthalpy and in entropy. Since then other aspects of this reversible polymerization have been studied in detail. From all these results, Lauffer (1966) proposed a model, subsequently modified (Lauffer *et al.*, 1967). But there was originally no experimental evidence to exclude the possibility of the role of ions in this reaction system. Even in the previous paper on ion binding by Shalaby *et al.* (1968), this point was not fully clarified because no experiments were done at pH 6.5 on both polymerized (23°) and unpolymerized (4°) protein. With the availability of new specific ion electrodes, the work was undertaken again to find out if ions, particularly potassium and chloride ions, participate in the polymerization reaction and contribute anything toward the thermodynamic parameters mentioned above.

Materials and Methods

TMV. The common strain of TMV was isolated by alternate high- and low-speed centrifugation with a step involving depigmentation with EDTA according to Boedtker and Simmons (1958).

TMV Protein. The protein was prepared from the virus by the acetic acid method of Fraenkel-Conrat (1957) with slight modification.

The aggregated protein obtained after removal by dialysis of the acetic acid was picked up in dilute KOH to a final pH near 7.5. It was then centrifuged at 40,000 rpm for 3 hr in the cold to remove any undegraded virus or denatured protein. The supernatant fluid was then exhaustively dialysed for 3 or 4 days against deionized water containing mixed-bed, ion-exchange resins (Bio-Rad Laboratories, Richmond, Calif., AG 501-X8 mesh). This step was necessary for obtaining native isoionic protein. It was observed that a large amount of material leaked out of the dialysis tubing and this material could not be redissolved even in 0.1 M KOH. If the ion-exchange resin was mixed directly with the protein, some of the protein denatured during stirring, and it was difficult to remove the denatured protein from the aggregated isoionic protein.

Isoionic TMV was also prepared in the same way by dialyzing against mixed-bed resin. For each experiment, freshly prepared isoionic TMV or TMV protein was used because,

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