Interestingly, after 3 h of reexposure to room temperature, GS activity decreased to a value almost equal to the initial room-temperature activity.

That GS activity was increased or induced by low temperature suggests that this enzyme may play a key role in the biosynthesis of RFO in response to low temperature. The increase or induction may be due to gene activation or a posttranslational process. If the former is true, the GS gene would be an interesting model for studying gene activation by low temperature in plants. Knowledge of molecular mechanisms of gene activation by low temperature in plants would be of potential importance in designing genetic engineering strategies for frost resistance or cold acclimation in plants. We are purifying GS from kidney bean in preparation to cloning its gene.

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A Stable Form of Vitamin C: L-Ascorbate 2-Triphosphate. Synthesis, Isolation, and Properties

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L-Ascorbate 2-triphosphate (AsTP) was synthesized by phosphorylating L-ascorbate (AsA) with sodium trimetaphosphate (STMP). The regioselectivity of the reaction was optimum (95% of 2-phosphorylation) when 1.62 M AsA was reacted with 1.3 equiv of STMP at pH 10.4–10.7 and 35 °C for 24 h or 55 °C for 5 h. The reaction yielded 4% unreacted AsA, 86% AsTP, 3% L-ascorbate 2-diphosphate (AsDP), 3% of a 4,5-elimination compound (ENE), and 1% L-ascorbate 2-monophosphate (AsMP). Those derivatives of AsA were isolated by anion-exchange chromatography and identified by UV and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. UV absorbance indicated that the L-ascorbyl moiety in AsTP was 1, 2, and 3 orders of magnitude more stable than AsA toward O_2 oxidation at 28–45 °C and pH 3, 6, and 8, respectively. AsMP, AsDP, and AsTP were readily hydrolyzed by acid phosphatase to give stoichiometric release of AsA. AsTP appeared to be equivalent to AsA as a source of vitamin C in guinea pigs.

The principal mechanism causing loss of vitamin C in foods and feeds is initiated by O_2 oxidation of L-ascorbic acid (AsA) catalyzed by Fe(III) and Cu(II) ions. The product, dehydroascorbic acid, retains vitamin potency but is rapidly decomposed to many compounds void of activity (Liao and Seib, 1988). In foods and feeds with high

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water activity, various strategies are used to reduce vitamin C loss. These include removing oxygen, avoiding and chelating Fe(III) and Cu(II) ions, and encapsulating AsA. In recent years, derivatives of AsA with increased O_2 stability are receiving serious consideration, especially 2-phosphorylated esters.

Cutolo and Larizza (1961) were the first to investigate phosphorylation of AsA. Since that time, a number of phosphate and phosphinate esters of AsA have been synthesized, most of which were reviewed (Seib, 1985). Those included the 2-monophosphate (AsMP), 2-diphosphate (AsDP; Nomura et al., 1969), 6-phosphate (Liao et al., 1988), 2- and 3-phenyl phosphates, 2-(diphenylphosphinate), and 3-(dimorpholinophosphinate). In addition, the 2,2′-bis(L-ascorbyl) phosphate has been reported.

AsMP has been shown to be 10–20 times more stable to O_2 oxidation than AsA (Lee, 1976). Moreover, its vitamin activity is equivalent to that of AsA in rhesus monkeys, guinea pigs, and fish (Machlin et al., 1979; Brandt et al., 1985). The 2-monophosphate ester is currently used in cosmetics for skin care (Takashima et al., 1971; Ito and Ogata, 1987) but has not been approved for foods.

To obtain high yields of AsMP by phosphorylation of AsA with $POCl_3$, the reaction must be done at high pH (12–13) in the presence of a high concentration of a water-soluble tertiary amine, such as pyridine. Isolation of a food- or pharmaceutical-grade AsMP requires removal of pyridine. AsDP is currently unavailable, since it occurs as a byproduct at only 5% yield in the synthesis of AsMP. To circumvent those problems, we sought a new method to phosphorylate AsA at its 2-position. A preliminary report of this work has appeared (Seib and Liao, 1987).

EXPERIMENTAL SECTION

General Procedures. Evaporations were done under diminished pressure below 40 °C. TLC was performed on flexible polyethylene terephthalate plates coated with cellulose (Eastman Kodak Co., Rochester, NY) in 30:35:15:20:0.4:5 (v/v/v/v/v/w) water–ethanol–2-methyl-1-propanol–2-propanol–concentrated ammonium hydroxide–trichloroacetic acid (Seiler, 1969). Components were detected with spray A, 1% ferric chloride in 95% ethanol (Vestling and Rebstock, 1945), or spray B, acid–molybdate (Hanes and Isherwood, 1949), followed by UV irradiation (Bandurski and Axelrod, 1951). NMR spectra were recorded on aqueous solutions with a Bruker WM-400 instrument. The pH values of solutions that had been previously exchanged with D_2O were adjusted prior to measurement. The pH reported was that read from the pH meter. Disodium ethylenediaminetetraacetate (EDTA; 0.2 mM) was added to samples before ^{31}P spectra were recorded. Signals were reported in δ values versus reference standards as follows: ^1H and ^{13}C, internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS); ^{31}P, external 50% phosphoric acid, unless otherwise stated. UV spectroscopy was done with use of a Model DMS-80 Varian spectrophotometer. Extinction coefficients were calculated from phosphorus levels (Cooper, 1977) of pure components isolated as ammonium salts by ion-exchange column chromatography. Ionization constants were determined from UV data (Albert and Serjeant, 1984).

High-performance liquid chromatography (HPLC) was carried out with a Knauer pump fitted with a loop injector (20 μL), reversed-phase column, integrating recorder, and electrochemical (EC) or UV detector. For HPLC–UV analysis, samples (20 μL) were injected and components eluted with a 83:17 (v/v) mixture of 0.1 M acetate buffer (pH 4.7) and methanol containing 1.0 mM tetrabutylammonium phosphate (TBAP) and 0.2 mM EDTA. The column was maintained at 45 °C, the flow rate was 1.0 mL/min, and the detector was set at 250 nm. The retention times for AsA, AsMP, AsDP, L-ascorbate 2-triphosphate (AsTP), and (2*Z*,4*Z*)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate (ENE) were approximately 3.4, 4.5, 5.3, 6.3, and 10.6 min, respectively. AsA was assayed with HPLC–EC, with a mobile phase of 1:19 (v/v) methanol–0.08 M acetate buffer (pH 4.0) containing 1.0 mM TBAP and 0.1 mM EDTA. The column was maintained at 35 °C, the flow rate was 0.8 mL/min, and the detector was set at +0.72 V vs a Ag/AgCl reference electrode. The retention time for AsA was approximately 6.8 min. Samples (20 μL) were injected, and AsA was quantitated by comparison of peak heights with those of known amounts of standard AsA.

2-Triphosphorylation of L-Ascorbic Acid. Reactions were run at pH 9.5–11.5, temperature 25–55 °C, time 4–72 h, concentration of AsA 0.81–1.75 M, and molar ratio of sodium trimetaphosphate (STMP) to AsA 1.1–1.5. Unreacted AsA was determined by iodometric titration (Food Chemicals Codex, 1981), 2-phosphorylation and 4,5-elimination by UV analysis, and the yields of derivatives of AsA by HPLC–UV.

A 250-mL beaker was fitted with a magnetic stirring bar, a pH electrode, an automatic pH controller, and a peristaltic pump. To the beaker, which was placed in a water bath at 35 °C, were added, in sequence, water (70 mL), AsA (20 g, 114 mmol, initially 1.62 M), and 10 M sodium hydroxide to attain pH 10.5. STMP (47.6 g, 95% purity, 148 mmol; Sigma) was added, and the pH was maintained at 10.4–10.7 by automatic addition of 10 M alkali. The reaction mixture was stirred continuously and stirring stopped after 24 h. The total volume of 10 M sodium hydroxide added during the reaction was approximately 30 mL. The reaction mixture (120 mL) was diluted to 500 mL with water, and an aliquot (5.0 mL) was titrated immediately with 0.05 N iodine. The iodine titer (1.7 mL) indicated 3.7% unreacted AsA.

A second aliquot (5.0 mL) of the diluted reaction mixture was diluted an additional 500-fold with water. Then Na_2HPO_4 was added as internal standard, Amberlite IR-120 (H^+) was used to adjust the mixture to pH 8.5, and the ^{31}P NMR spectrum of the diluted mixture was recorded (Sojka and Wolfe, 1978; Rubin, 1984). A third aliquot (1.0 mL) was diluted an additional 50-fold with water, and 20 μL of that solution was used for HPLC–UV assay. A fourth aliquot (2.0 mL) was diluted an additional 6250-fold in 0.1 M sodium carbonate buffer at pH 10. The mixture at pH 10 was allowed to stand 2 h, during which time unreacted AsA was destroyed by O_2 oxidation, as shown by HPLC–EC. The UV absorbance at 258 nm and pH 10 was 0.551, indicating 94.7% of 2-triphosphorylation of AsA using ϵ 16 for 2-phosphate esters. The UV absorbance at 313 nm was 0.009, indicating 2.9% of the ENE compound using ϵ 8.5 at pH 10. The extinction coefficients of all the components in the reaction mixture were determined in this work (see Table VI).

The degree of 2-triphosphorylation of AsA under the optimum conditions was followed with time with use of UV absorbance at pH 10, as described above.

2-Triphosphorylation of 5,6-*O*-Isopropylidene-L-ascorbic Acid. 5,6-*O*-isopropylidene-L-ascorbic acid (IAA) was prepared in nearly quantitative yield by the method of Jackson and Jones (1969). An equimolar amount of this compound (24.6 g, 114 mmol) was used in place of AsA (20 g) to react with STMP under the optimum conditions described for 2-triphosphorylation of AsA.

Ion-Exchange Chromatographic Separation of Components in the 2-Triphosphorylation Reaction Mixture. AsA (20 g, initially 1.62 M) was reacted with 1.3 equiv of STMP at 35 °C and pH 10.5 for 24 h. About 30 mL of the reaction mixture previously diluted to 250 mL (~0.45 M in AsA equivalents) was brought to pH 8 by stirring with a small quantity of strongly acidic cation exchange resin (Amberlite IR-120) in hydrogen ion form. An aliquot (0.3 mL) was placed on a column (21 × 1.7 cm) of strongly basic ion-exchange resin in bicarbonate form (AG-1×8, 200–400 mesh; BioRad Laboratories). The column was developed at a flow rate of 1.0 mL/min with sequential elution with 0.4, 0.42, 0.5, 0.55, 0.6, and 0.8 M ammonium bicarbonate. Fractions (15 mL) were collected and diluted with 0.1 M sodium carbonate buffer (pH 10), and their UV absorbances were read at λ_{max}. Inorganic phosphates eluted by 0.4 M ammonium bicarbonate were identified by TLC in 70:30:3:0.2 (v/v/w/v) acetone–water–trichloroacetic acid–concentrated ammonium hydroxide (Greenfield and Chift, 1975) with spray reagent B.

Yields of components were estimated after pooling fractions, aliquoting the fractions into carbonate buffer (pH 10), and using ϵ 15.6 at λ_{max} (246 nm) for the ENE compound and ϵ 16.0 at λ_{max} for all other components (see Table VI).

Isolation of the Ammonium Salt of L-Ascorbate 2-Triphosphate. An aliquot of an optimum reaction mixture, which contained 2-phosphate esters equivalent to approximately 3 g of AsA, was adjusted to pH 8.0 by addition of Amberlite IR-120 (H^+). After removal of the resin, the reaction mixture was subjected to ion-exchange chromatography on a 45 × 5 cm column of resin (AG-1X8, bicarbonate form). Ortho-, pyro-, and triphosphate were eluted first in six bed volumes (5300 mL) of 0.40 M ammonium bicarbonate. The eluting solvent was switched to 0.42 M ammonium bicarbonate, and UV absorbance at 258 nm was used to monitor the elution of the phosphorylated species of AsA. UV-active fractions were examined by HPLC–UV. The small amounts of AsMP and AsDP were collected next, followed by AsTP, which was eluted with four bed volumes of 0.42 M ammonium bicarbonate. The AsTP fraction then was evaporated to approximately 100 mL, water was added, and the mixture was reevaporated several times until the concentrate reached pH 6. To remove the last traces of residual bicarbonate, Amberlite IR-120 (H^+) was added carefully to attain pH 3, and the mixture was subjected to reduced pressure for 10 min. After removal of the resin by filtration, the solution (approximately 100 mL) was adjusted to pH 6 with 10 M ammonium hydroxide and added dropwise to absolute ethanol (2 L) with rapid stirring. An amorphous precipitate formed, and the total mixture was evaporated to approximately 100 mL. Absolute ethanol (400 mL) was added again, and the mixture was evaporated once more to approximately 100 mL. The white solid was collected by filtration and rinsed twice with absolute ethanol. After being dried under vacuum at 25 °C to constant weight, the ammonium salt of AsTP was obtained as an amorphous solid, in which ethanol appeared to be strongly bound in the glassy solid, yield 6.8 g (78%). HPLC–UV assay showed that the solid product contained >98% AsTP, 1.5% AsDP, and a trace of AsMP. UV, NMR, and ionization data were measured in water at selected pH values.

Anal. Calcd for $C_6H_{23}N_4O_{15}P_3 \cdot (C_2H_5OH)_{0.67}$: C, 17.11; H, 5.25; N, 10.88; O, 48.70; P, 18.06. Found: C, 17.08; H, 5.55; N, 10.86; O, 48.68; P, 17.71.

Isolation of the Ammonium Salt of the 4,5-Elimination Product (ENE). The title compound was isolated from a reaction mixture between AsA (3 g; initially 1.52 M) and 1.5 equiv of STMP at 35 °C and pH 11.5 for 24 h. UV absorbance at 313 nm and pH 10 indicated that the reaction mixture contained 15% ENE.

The reaction mixture was subjected to ion-exchange column chromatography as previously described, except that approximately 1.5 bed volumes of 0.55 M ammonium bicarbonate were used to elute ENE, and the column effluent was monitored at 246 nm. ENE was separated from the ammonium bicarbonate in the effluent by the same method described for the ammonium salt of AsTP. After the salt was dried under vacuum, 1.3 g (12% yield by UV) of the ammonium salt of ENE was obtained as an amorphous white powder. HPLC–UV showed that the solid product (R_T 10.6 min) was more than 98% pure. UV, NMR, and ionization data were collected at known pH values.

Isolation of L-Ascorbate 2-Mono-, Di-, Tri-, and Tetraphosphate from a Dismutation Reaction of AsTP. The ammonium salt of AsTP (10 g) was converted to its sodium salt on a column of Amberlite IR-120 (Na^+). The effluent (1 L) was evaporated to a small volume (100 mL), and absolute ethanol was added to form two phases. The mixture was evaporated to a thick syrup, then absolute ethanol was added, and the mixture was evaporated two more times. The syrupy residue was dried under vacuum to give an amorphous solid. HPLC–UV assay showed that the solid product contained four components with R_T 4.5, 5.3, 6.3, and 7.4 min. The four components were separated and isolated as their ammonium salts by ion-exchange column chromatography. UV, NMR, and ionization data were measured on each component in water at fixed pH values.

Stability of L-Ascorbate 2-Triphosphate, L-Ascorbic Acid, and the Elimination Product (ENE) under Aerobic Conditions. Solutions (225 mL, 3 mM) of AsTP (ammonium salt), ENE (ammonium salt), and AsA were prepared separately in 0.1 M sodium phosphate buffers at pH 3, 6, and 8. The buffered solutions of AsTP and AsA were maintained at 28, 35, and 45 °C, while the ENE was held at 45 °C. The solutions, placed in open Erlenmeyer flasks (250 mL), were stirred rapidly and continually with a magnetic stirring bar. The decline in UV absorbance at the λ_{max} of each compound was followed.

Enzyme Hydrolysis of 2-Phosphate Esters of L-Ascorbic Acid. Acid phosphatase was Type IV-S, 4.8 units/mg of solid from Sigma Chemical Co., St. Louis, MO. A solution of 2-triphosphate ester (AsTP, ammonium salt; 8 mM) was prepared in 0.1 M acetate buffer (50 mL, pH 4.8) containing 0.2% 1,4-dithiothreitol (DTT). After phosphatase (10 mg, 48 units) was added, hydrolysis was conducted with mild agitation on a stir plate at 37 °C. Aliquots (2 mL) were removed at timed intervals, and the reaction was quenched by dilution to 10 mL with 0.05 M cold, degassed perchloric acid. After being filtered through a syringe filter, an aliquot (20 μL) of the digest was analyzed by HPLC–UV for the disappearance of AsTP and the concomitant formation of AsDP, AsMP, and AsA.

The stoichiometry for the conversion of AsTeP, AsTP, AsDP, and AsMP to AsA by phosphatase was followed as described by Wang et al. (1988). Solutions of the 2-phosphate esters (ammonium salt, 1 mM) were prepared in 0.1 M acetate buffer (200 mL, pH 4.8) containing 0.6% DTT and 2 g of compressed yeast. Each mixture was placed in a capped volumetric flask and equilibrated to 37 °C in a constant-temperature bath. Phosphatase (5 mg, 24 units) was added, and digestion was conducted with mild agitation on a stir plate. Samples (1 mL) were removed at timed intervals, quenched by diluting to 100 mL with 0.05 M cold degassed perchloric acid, and assayed for AsA by HPLC–EC. The recovery of AsA was determined by comparing peak heights with known amounts (10, 20, 30, 40 ng) of AsA.

Vitamin C Activity of L-Ascorbate 2-Triphosphate and the 2-Triphosphorylation Reaction Mixture of L-Ascorbic Acid. English short-hair guinea pigs were housed individually and fed Purina guinea pig ration during a 1-week acclimation period. The young pigs (190–250 g) were divided into four groups of ten animals each and fed as follows: (1) a diet devoid of vitamin C (Reid-Briggs guinea pig diet; U.S. Biochemicals, Cleveland, OH); (2) the same diet supplemented with 5.0 mg of AsA/kg of body weight per day (Collins and Elvehjem, 1958); (3) the diet supplemented with an equimolar amount of the purified sodium salt of AsTP; (4) the diet supplemented with diluted reaction mixture containing an equimolar amount of 2-phosphate esters of AsA. The ingredients in the pelleted diet included rolled oats (40%), heat-treated skim milk (20%), wheat bran (15%), vitamin-free casein (10%), alfalfa (8%), vegetable oil (5%), calcium carbonate (1%), sodium chloride (0.5%), and magnesium sulfate (0.5%).

The sodium salt of AsTP given to the third group of animals was prepared by passing purified ammonium salt of AsTP (10 g) through a column (850 mL) of Amberlite IR-120 (Na^+) and then evaporating the effluent (1 L) to 100 mL. The reaction mixture fed to group 4 was prepared by reacting AsA with 1.5 equiv of STMP at pH 10.4–10.7 and 35 °C for 24 h and then purging with O_2 to destroy small amounts of unreacted AsA. The concentrations of the pure AsTP solution and of 2-phosphate esters of AsA in the total reaction mixture were both determined by UV assay using ϵ 16 at 258 nm and pH 10. The pure AsTP solution and the reaction mixture was stored at 5 °C, and an aliquot from each was diluted immediately before being administered to the animals; the AsA solution was prepared fresh daily. The vitamin C compounds were administered orally with a calibrated syringe.

After about 3 weeks, when the group of animals given no supplemental source of vitamin C developed clinical signs of scurvy, it was divided into three subgroups that were treated with 50 mg of AsA/kg of body weight per day (three animals), an equimolar amount of purified sodium salt of AsTP (four animals), or reaction mixture containing equimolar amount of 2-phosphate esters of AsA (three animals). Body weights of the animals were recorded daily, and the arithmetic means of each group were calculated and compared.

Table I. Reaction[a] of L-Ascorbic Acid (AsA) or 5,6-*O*-Isopropylidene-L-ascorbic Acid (IAA) with Sodium Trimetaphosphate (STMP) under Different Conditions

run no.	AsA, M	mole ratio, STMP/AsA	temp, °C	pH	time, h	unreacted AsA,[b] %	2-phosphorylation,[c] %	4,5-elimin product,[d] %
1	1.75	1.1	35	10.4–10.7	24	6.3	90.5	2.6
2	1.75	1.1	35	10.6–10.9	24	5.3	92.8	3.8
3	1.62	1.3	35	10.4–10.7	24	3.7	94.7	2.9
4	1.62	1.3	35	10.3–10.6	24	5.6	92.1	2.1
5	1.62	1.3	35	10.2–10.5	24	9.2	88.7	1.6
6	1.62	1.3	35	10.2–10.5	30	5.0	92.5	2.0
7	1.62	1.3	35	10.0–10.3	32	13.7	82.2	1.1
8	1.62	1.3	35	9.4–9.7	72	22.5	63.9	1.0
9	1.62	1.3	35	10.6–10.9	24	1.6	97.1	5.0
10	1.62	1.3	35	10.9–11.2	11	7.5	90.5	3.5
11	1.62	1.3	35	10.9–11.2	12	3.5	95.3	5.4
12	1.62	1.3	35	10.9–11.2	20	1.3	98.1	8.4
13	1.62	1.3	35	10.9–11.2	24	1.2	97.3	8.6
14	1.62	1.3	35	11.4–11.7	18	7.7	88.2	9.3
15	1.62	1.3	55	10.4–10.7	4	8.9	90.3	2.2
16	1.62	1.3	55	10.4–10.7	5	3.8	95.0	3.2
17	1.62	1.3	55	10.0–10.3	8	2.9	91.9	2.8
18	1.08	1.3	35	10.4–10.7	24	9.1	88.5	2.3
19	0.81	1.3	35	10.4–10.7	24	16.0	81.0	1.8
20	1.52	1.5	35	10.7–11.0	24	0.9	97.5	5.5
21	1.52	1.5	25	10.7–11.0	24	17.0	81.8	3.2
22	1.52	1.5	25	10.7–11.0	48	1.5	96.9	5.5
23K	1.62[e]	1.3	35	10.4–10.7	24	7.5	90.5	3.3
24K	1.62[e]	1.3	55	10.4–10.7	5	5.1	92.2	4.1
25I	1.62[f]	1.3	35	10.4–10.7	24	14.6	79.1	5.6
26I	1.62[f]	1.3	35	10.9–11.2	24	4.9	81.0	39.2

[a] Concentration of AsA or its 5,6-ketal was at the start of the reaction. Aqueous sodium hydroxide (10 M) was added to maintain pH, which amounted to 13.8–18.1 mL starting with 10 g of AsA. [b] Determined by iodine titration. [c] UV assay using ϵ 16.0 at 258 nm and pH 10. [d] UV assay using ϵ 8.5 at 313 nm and pH 10. [e] 10 M KOH was used to maintain pH. [f] IAA was starting material.

RESULTS AND DISCUSSION

Sodium Trimetaphosphate (STMP). STMP is a cyclic phosphate that is useful as a phosphorylating agent; it undergoes ring-opening when attacked by nucleophiles including amines, fluoride, hydroxide, and aliphatic and aromatic alcohols (Feldmann, 1966 and 1967). It can be used to cross-link starch (Wurzberg, 1986) and to modify food proteins (Sung et al., 1983; Matheis and Whitaker, 1984). STMP is prepared (Bell, 1950) by either heating NaH_2PO_4 for 5 h at 530 °C or tempering glassy sodium metaphosphate at 520 °C for 12 h. STMP is a powder that is easy to handle and is generally recognized as safe (GRAS) in food by the *U.S. Code of Federal Regulations* (21 CFR 182.6769).

Reaction of L-Ascorbate with STMP. In aqueous medium at alkaline pH, the 2-oxyanion of AsA is known to be a strong nucleophile (Seib et al., 1974; Andrews and Crawford, 1982; Cabral and Haake, 1988). As expected, AsA reacted with STMP to give 2-substitution. We carried out a series of experiments in which we varied temperature, pH, reaction time, concentration of AsA, and equivalents of STMP (Table I). Our objective was to obtain the maximum yield of 2-phosphorylated AsA and minimum yield of the ENE byproduct, which we assumed was void of vitamin activity.

The conditions giving both a high yield (95%) of 2-phosphorylated AsA and a low yield (3%) of ENE were 1.62 M solution of AsA, pH 10.4–10.7, 1.3 equiv of STMP, and temperature 35 °C for 24 h or 55 °C for 5 h. Under optimum conditions, the reaction consumed 2.6 equiv of sodium hydroxide/equiv of AsA.

It should be pointed out that the initial concentrations of AsA shown in Table I were those prior to the addition of the 10 M alkali used to increase pH >9.5. For example, the concentration of sodium L-ascorbate at the beginning of its reaction with STMP in run 3 was 1.4 M, which declined with reaction time because of its conversion to AsTP and to the extra alkali added to control pH.

The data in Table I show that when the initial concentration of AsA was reduced to 0.81–1.08 M (runs 18 and 19), 2-phosphorylation decreased, whereas when the concentration was increased to 1.75 M (runs 1 and 2), 2-phosphorylation remained at approximately 90%. The slight decrease in yield at 1.75 M was attributed to the use of 1.1 equiv of STMP rather than 1.3 equiv.

The rate of 2-phosphorylation also depended upon pH. Increasing pH from 10.4–10.7 (run 3) to 10.9–11.2 (run 11) halved the reaction time, but almost doubled the yield of the ENE byproduct. At the highest pH examined (11.4–11.7, run 14), the yield of ENE rose to 9% while 2-phosphorylation was 88% after 18 h of reaction. The reduced yield of 2-phosphorylation suggests some loss of STMP through alkaline hydrolysis at high pH (Feldmann, 1966 and 1967). On the other hand, decreasing the pH of the reaction, for example, to pH 9.4–9.7 (run 8) reduced the yield of ENE but, at the same time, also greatly reduced 2-phosphorylation (64%), even after a prolonged reaction period (72 h).

In an attempt to prevent phosphorylation of the 6-OH, we reacted 5,6-*O*-isopropylidene-L-ascorbic acid (IAA) with STMP (run 25I) under optimum conditions. Surprisingly, 2-phosphorylation of IAA was much reduced, in part because of reduced solubility of STMP in the reaction mixture (data not given). In addition, a substantial yield of a 4,5-elimination product resulted from IAA when the 2-triphosphorylation reaction was carried out at pH 10.9–11.2 (run 26I), as indicated by the shift in λ_{max} of the diluted reaction mixture from 258 to 254 nm at pH 10 as well as the strong absorbance at 313 nm. Eitelman et al. (1976) previously reported 4,5-elimination when 5,6-*O*-isopropylidene-2,3-dimethyl-L-ascorbic acid

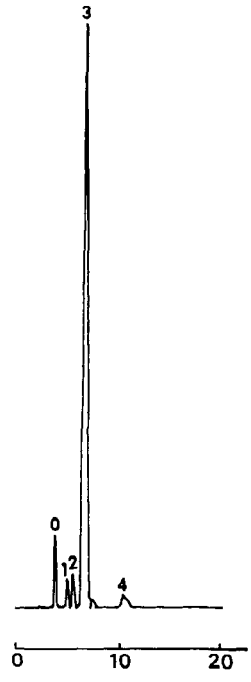

Figure 1. HPLC–UV analysis of products formed in reaction 3 (Table I). Peaks: 0, L-ascorbate (AsA); 1, L-ascorbate 2-monophosphate (AsMP); 2, L-ascorbate 2-diphosphate (AsDP); 3, L-ascorbate 2-triphosphate (AsTP); 4, (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate (ENE).

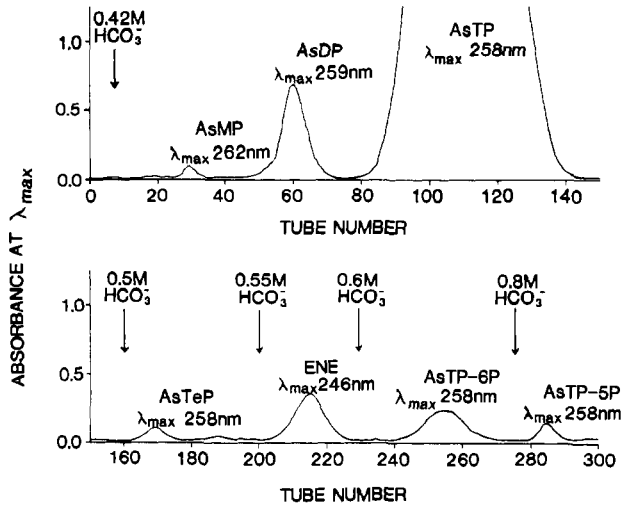

Figure 2. Ion-exchange (AG-1X8, bicarbonate form) column chromatographic separation of products formed in reaction 3 (Table I). The components in order of elution are AsMP, AsDP, AsTP, AsTeP, ENE compound, AsTP-6P, and AsTP-5P. The λ_{max} value labeled for each component was that observed in the bicarbonate eluent.

was treated with a strong base in an aprotic medium.

Chromatography of the Reaction Mixture Obtained from L-Ascorbate and STMP. Reversed-phase HPLC with UV detection was used to examine the reaction mixture prepared under optimum conditions (run 3). Immediately after reaction, the chromatogram showed that the reaction mixture contained, in order of elution, unreacted AsA, AsMP, AsDP, AsTP, and ENE (Figure 1).

Ion-exchange column chromatography with UV monitoring also was used to examine the reaction mixture (Figure 2). The elution profile showed no AsA peak, which was known to elute with 0.42 M bicarbonate. AsA was lost during the time period required to obtain the chromatogram. Compared to HPLC–UV (Figure 1), the ion-exchange separation (Figure 2) showed small amounts of two additional components eluted at long retention times. Those two components were assigned the structures L-ascorbate 6-phosphate 2-triphosphate (AsTP-6P) and L-ascorbate 5-phosphate 2-triphosphate (AsTP-5P) based on their elution times and UV properties (λ_{max} 258 nm at pH 10). Five of the phosphorylated derivatives of AsA in Figure 2 were isolated by preparative chromatography, and their structures were verified by UV and ^1H, ^{13}C, and ^{31}P NMR spectroscopy. Unreacted STMP showed strong absorption on the resin and was not eluted throughout the separation process. Instead, it was eluted during regeneration of the column with 1 N NaOH.

Table II. Yields of Phosphate Esters When L-Ascorbic Acid Was Reacted under Optimum Conditions with Sodium Trimetaphosphate

derivative	ion-exch col chromatogr	HPLC–UV
L-ascorbate 2-monophosphate	0.2[a]	1.9[b]
L-ascorbate 2-diphosphate	3.4	3.4
L-ascorbate 2-triphosphate	86.5	86.0
L-ascorbate 2-tetraphosphate	0.3	tr
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate	2.4	3.4
L-ascorbate 6-phosphate 2-triphosphate[c]	1.2	tr
L-ascorbate 5-phosphate 2-triphosphate[c]	0.3	tr
total yield	94.3	94.7

[a] Yields calculated with ϵ of each component at pH 10 and λ_{max} (see Table VI). [b] Yields calculated from the proportion of each component and 94.7% 2-phosphorylation, which was determined by UV absorbance at 258 nm and pH 10 with ϵ 16.0. [c] ϵ assumed to be 16.0, since both compounds gave a single absorption peak with λ_{max} 258 nm at pH 10.

Table II gives the proportions of phosphorylated components calculated from the two chromatograms in Figures 1 and 2. AsTP was, by far, the major product in the reaction mixture.

Proposed Reaction Pathway. The products isolated from the reaction mixture were probably formed by pathways proposed in Figure 3. The 2-triphosphate chain on the first reaction product, AsTP, appeared to undergo limited alkaline hydrolysis (Feldmann, 1966, 1967) to give 4–5% AsDP plus AsMP after 24-h reaction under optimum conditions. In a separate pathway, AsTP might slowly react at its 6-OH with STMP to give the proposed intermediate bis(2,6-triphosphate), which would likely form a 5,6-cyclic phosphate intermediate (Khorana, 1961; Saffhill, 1970). The latter intermediate would then react with base in a ring-opening reaction (Fathi and Jordan, 1986) to give AsTP-6P and AsTP-5P or in a 4,5-elimination reaction to give ENE. The 4,5-elimination reaction on AsA is known to be facilitated by the presence of a good leaving group on C-5 (Andrews and Crawford, 1982). The small amount of AsTeP detected in the reaction mixture likely was formed by dismutation of AsTP, which was caused by its high concentration.

Isolation and Characterization of the Ammonium Salts of L-Ascorbate 2-Triphosphate and Other Phosphorylated Components. Preparative column chromatography was used to isolate the ammonium salt of AsTP. During isolation, care was taken to minimize hydrolysis and dismutation of the 2-triphosphate chain. Hydrolysis was discouraged by maintaining pH >7. Dismuta-

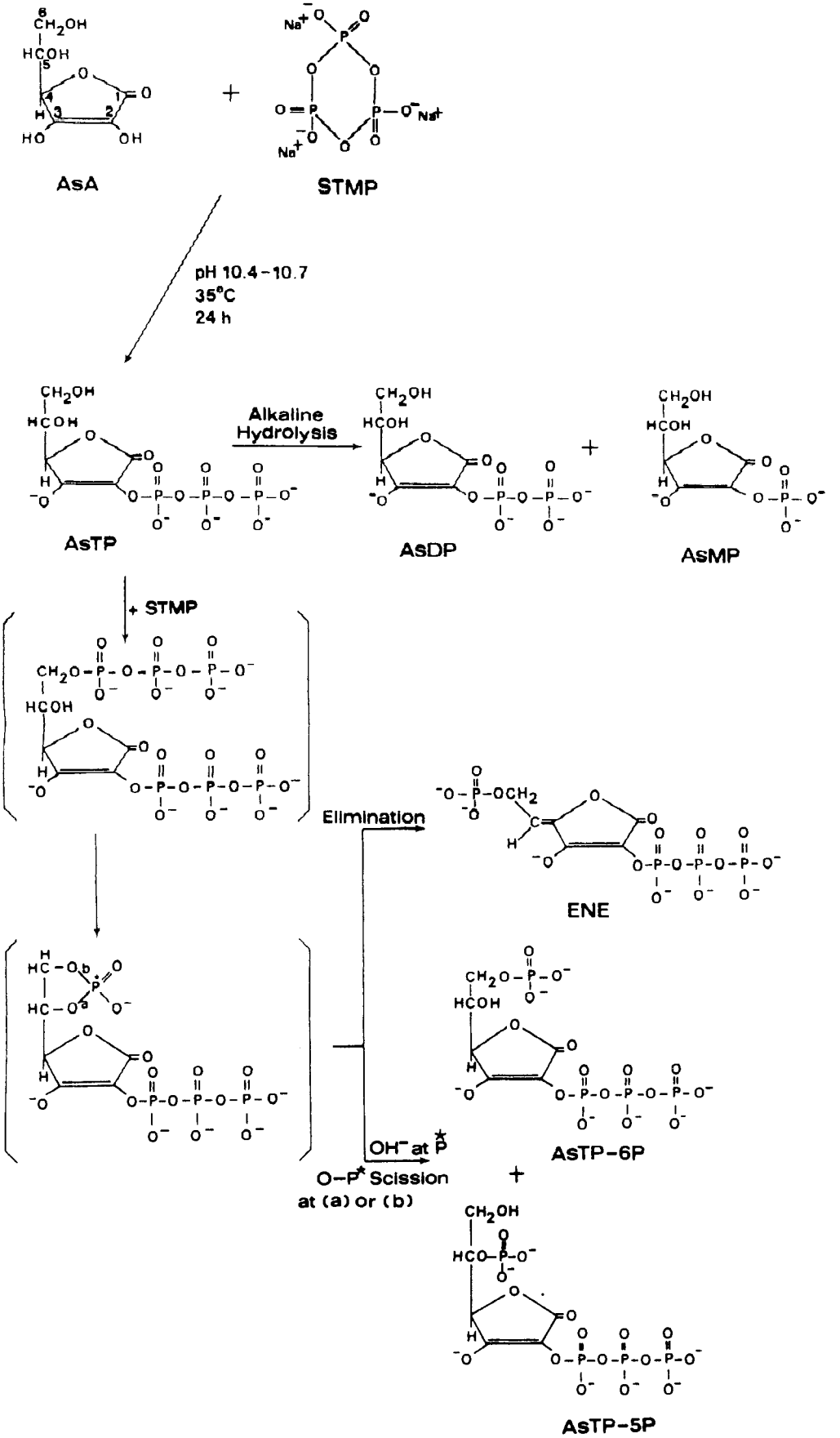

Figure 3. Proposed pathway for products formed during phosphorylation of L-ascorbic acid (AsA) with sodium trimetaphosphate (STMP) in aqueous sodium hydroxide.

tion, which was prone to occur during evaporative removal of the ammonium bicarbonate from the ammonium salt of AsTP, was prevented by avoiding high concentrations of AsTP in a syrupy state (Feldmann, 1967).

The ammonium salt of AsTP was rapidly precipitated into a glassy immobile state by adding the aqueous concentrate (~0.2 M) of the ammonium salt to 20 volumes of absolute ethanol. Following those precautions, ammo-

Table III. ^{31}P NMR Chemical Shifts[a] of 2-Phosphorylated Derivatives from L-Ascorbic Acid

derivative	pH	α-P	β-P	γ-P	δ-P
L-ascorbate 2-monophosphate[b]	10	+3.6 (d)			
L-ascorbate 2-diphosphate	6.5	−9.8 (d)	−3.6 (d)		
L-ascorbate 2-triphosphate	8.2	−11.7 (d)	−20.5 (t)	−5.1 (d)	
L-ascorbate 2-tetraphosphate	6.5	−10.7 (d)	−19.9 (t)	−19.4 (t)	−3.8 (d)
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate[c]	8.5	−15.2 (d)	−23.6 (t)	−8.4 (d)	

[a] Chemical shifts (ppm) referenced to 50% H_3PO_4. Upfield shifts are given a negative sign. Key: doublet, d; triplet, t. [b] Gives a narrow doublet due to long-range coupling of the phosphorus to H-4 (Lee et al., 1978). [c] Internal reference to added Na_2HPO_4. Th 6-P signal was a triplet at +1.4 ppm with splitting of 6.7 Hz due to coupling with H-6,6′.

Table IV. Carbon-13 NMR Chemical Shifts[a] of L-Ascorbic Acid and Several of Its Derivatives in D_2O at pH 6.5–7.0

derivative	C-1	C-2	C-3	C-4	C-5	C-6
unsubstituted[b]	178.0	114.1	176.2	79.2	70.6	63.6
2-methyl-L-ascorbic acid[c]	179.4	119.3	178.1	79.3	70.4	63.3
3-methyl-L-ascorbic acid[c]	174.8	120.4	155.0	76.8	70.0	63.0
L-ascorbate 2-monophosphate[d]	177.4	113.2	177.0	78.7	70.1	62.9
L-ascorbate 2-diphosphate	176.4	111.8	175.3	78.2	69.3	62.3
L-ascorbate 2-triphosphate	177.9	111.2	176.0	78.8	69.7	62.8
L-ascorbate 2-tetraphosphate	177.4	110.8	175.5	78.3	69.3	62.2
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate	172.7	115.5	168.6	150.0	106.3	61.1
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 2-sulfate[b]	172.7	114.2	171.2	149.4	108.2	57.7
(2Z,4E)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 2-sulfate[b]	172.6	116.0	170.2	148.4	108.6	58.4
L-ascorbate 6-phosphate[e]	179.9	115.6	178.3	80.9	71.4	67.7

[a] Chemical shifts (ppm) downfield from Me_4Si. [b] Paukstelis et al. (1982). [c] Lu et al. (1984). [d] Lee (1976). [e] Liao et al. (1988).

nium AsTP was isolated with >98% purity as determined by HPLC–UV.

In contrast, a dismutation mixture was obtained when the aqueous concentrate of the sodium salt of AsTP was taken to a thick syrupy state by addition of absolute ethanol. The mixture contained 83% AsTP plus 3% AsMP, 9% AsDP, and 5% AsTeP.

AsTeP was less stable in solution than AsTP, AsDP, and AsMP. When held at 5 °C for 1 week, AsTeP (~0.1 M, ammonium salt, initially pH 6) showed significant hydrolysis and gave a mixture of AsTP, AsDP, AsMP, and inorganic phosphate, as evidenced by TLC and HPLC–UV.

The structure of AsTP was verified by NMR data. The ^{31}P NMR spectrum (Table III) at pH 8.2 gave three signals typical of a triphosphate monoester (Crutchfield et al., 1967). α-P in AsTP was a doublet at −11.7 ppm with splitting of 18.3 Hz because of coupling with β-P. The ^{31}P signal at approximately −12 ppm is characteristic of an alcohol substituted by a condensed phosphate group, since inorganic condensed phosphates are void of a ^{31}P signal in this region (Crutchfield et al., 1967). The absence of small splitting (6–7 Hz) of the α-P signal because of coupling with protons indicated that the triphosphate group was attached to O-2 or O-3 of AsA. The β-P of the ester gave a triplet (doublet of doublets) characteristic of the central P atom in a triphosphate monoester.

^{13}C and ^{1}H NMR data were consistent with the assigned structure of L-ascorbate 2-triphosphate (Tables IV and V). The resonance signals of C-5 and C-6 and H-5 and H-6 in AsTP and AsA were similar but differed from those in L-ascorbate 6-phosphate, indicating no esterification at O-5 and O-6. The chemical shift of C-3 in AsTP at pH 7 was 176 ppm, showing that the 3-OH was ionized and, therefore, unsubstituted. In contrast, the C-3 signal of 3-methyl-L-ascorbic acid, which was not ionized at pH 7, occurred at 155 ppm (Table IV).

The UV properties of AsTP also agree with the assigned structure (Table VI). At pH 2, where the 3-OH in AsTP was not ionized, AsTP showed λ_{max} 235 nm and ϵ 10.3. When the 3-OH was ionized at pH 7, AsTP gave λ_{max} 258 nm and ϵ 15.4. The higher extinction coefficient showed linear conjugation of the ionization group with the ene–diol, consistent with a 3-oxyanion (Jernow et al., 1979) and 2-esterification. The UV properties of AsTP, as with all derivatives of AsA, were pH-dependent (Figure 4).

Table V. Proton NMR Chemical Shifts[a] of L-Ascorbic Acid and Several of Its Derivatives in D_2O at pH 6.5–7.0

derivative	H-4	H-5	H-6,H-6′
unsubstituted[c]	4.50	4.02[b]	3.74[b]
2-methyl-L-ascorbic acid[d]	4.49	4.00	3.71
3-methyl-L-ascorbic acid[d]	4.90	4.01	3.71
L-ascorbate 2-monophosphate[c]	4.60	4.05	3.70
L-ascorbate 2-diphosphate	4.63	4.05	3.74
L-ascorbate 2-triphosphate	4.55	4.05	3.74
L-ascorbate 2-tetraphosphate	4.55	4.05	3.73
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate		5.58	4.57
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 2-sulfate[e]		5.59	4.34
(2Z,4E)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 2-sulfate[e]		5.80	4.59
L-ascorbate 6-phosphate[f]	4.57	4.14	3.92

[a] Chemical shifts (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). [b] Chemical shifts of H-5 and H-6 are reported as the center of the multiplets. [c] Lee et al. (1978). [d] Paukstelis et al. (1982). [e] At pH 6.2 for the 4Z isomer and pH 5.9 for the 4E isomer. Data taken from Paukstelis et al. (1982). [f] Liao et al. (1988).

Like AsTP, the structures of AsMP, AsDP, and AsTeP were deduced from NMR (Tables III–V) and UV data (Table VI). A sample of AsMP prepared by the method of Lee et al. (1978) had properties identical with those of AsMP isolated in this work.

The ionization constants of the 2-phosphorylated derivatives of AsA were determined by the change in λ_{max} with

Table VI. UV Spectral Properties and Ionization Constants of L-Ascorbic Acid and Several of Its Phosphorylated Derivatives

derivative	acid (pH 2.0) λ_{max}, nm	ϵ	neutral (pH 7.0) λ_{max}, nm	ϵ	base (pH 10.0) λ_{max}, nm	ϵ	pK_a of 3-OH	pK_2 of terminal phosphate
unsubstituted[a]	243	10.0	265	16.5			4.17	
L-ascorbate 2-monophosphate[a]	238	9.0	258	11.5	264	16.0	3.3	7.9
L-ascorbate 2-diphosphate[b]	235	10.3	255	13.2	259	15.9	4.4	7.5
L-ascorbate 2-triphosphate	235	10.3	258	15.4	258	16.0	4.8	7.0
L-ascorbate 2-tetraphosphate			258		258	16.0		
(2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate	251[c]	13.7	246[d]	15.4	246[d]	15.6	4.5	7.5
1,4-lactone 6-phosphate 2-triphosphate			312[e]	8.2	313[e]	8.5		

[a] Lee et al. (1978). [b] Nomura et al. (1969) reported λ_{max} 235 nm (ϵ 9.8) in 0.1 M HCl and λ_{max} 259 nm (ϵ 17.0) in 0.1 M NaOH. [c] Single peak with shoulder at 274 nm. [d] Major peak. [e] Minor peak.

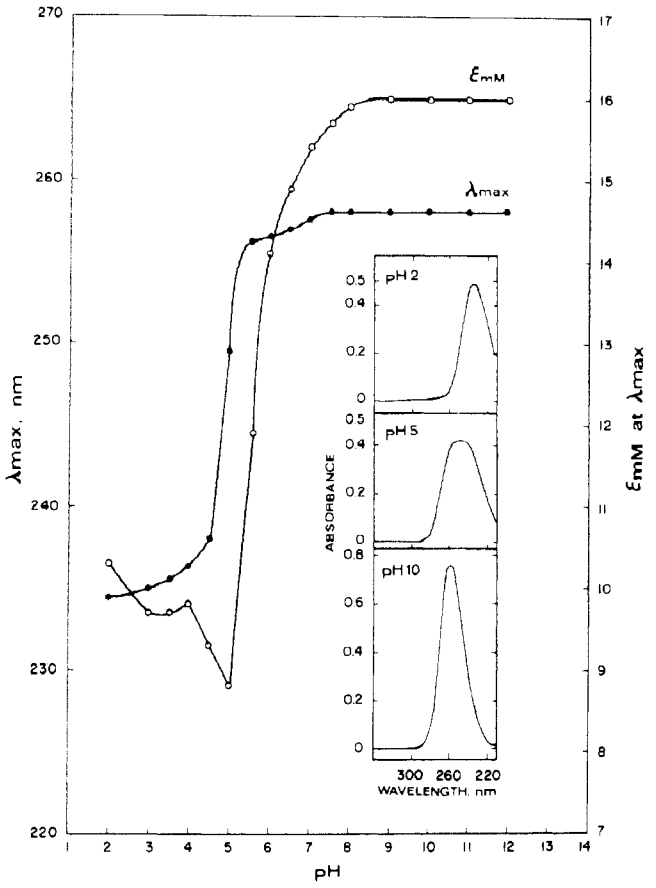

Figure 4. Effect of pH on UV properties of L-ascorbate 2-triphosphate (AsTP).

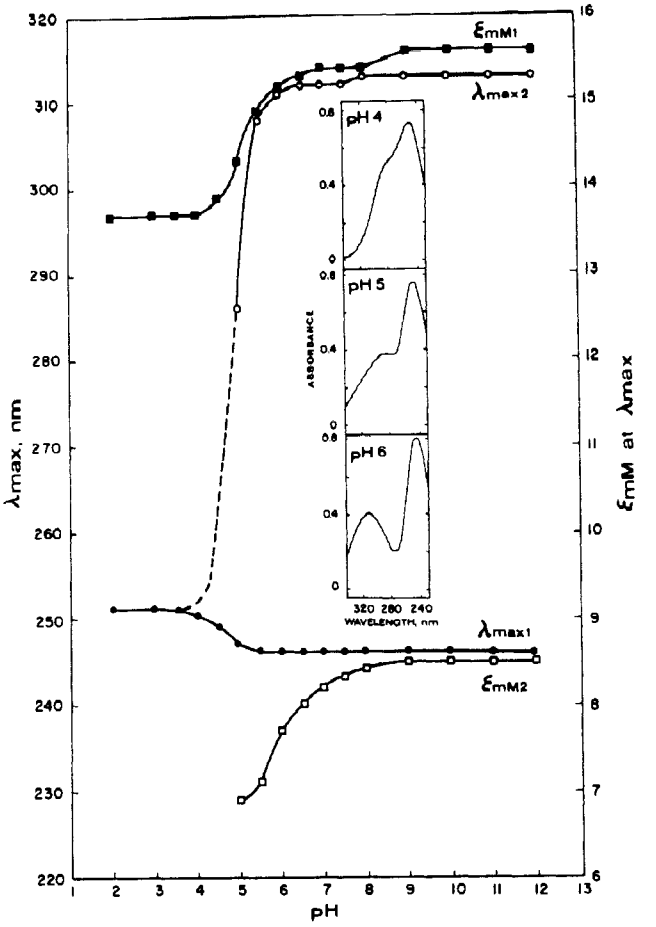

Figure 5. Effect of pH on UV properties of (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate (ENE).

pH (Table VI). The pK_a of the 3-OH on AsMP (pK_a 3.3–3.5) was approximately 1 pK unit lower than on AsDP, AsTP, or ENE. The second ionization of the terminal phosphoryl group on AsMP, AsDP, AsTP, and ENE gave pK_a 7.0–7.9. The first ionization constant was not measured but was assumed to be below 1.0. Nomura et al. (1971) reported that AsMP has pK_1 0.01, pK_2 3.27, and pK_3 6.70.

A byproduct in the reaction between AsA and STMP, which exhibited UV properties different from those of the other phosphorylated species of AsA, was assigned the structure (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate (ENE). ^{31}P NMR of this compound at pH 8.5 with disodium hydrogen phosphate as the internal standard showed a triplet ($J_{P,H}$ = 6.7 Hz) centered at +1.44 ppm, which was attributed to a 6-phosphate group. The three signals upfield from orthophosphate were assigned to a 2-triphosphate group (Table III).

The ^{13}C and ^{1}H NMR spectra agreed with the assigned structure for ENE. Table IV shows that the ^{13}C signals of ENE were similar to those of (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 2-sulfate. The 2-sulfate 4,5-ene derivative was characterized by Paukstelis et al. (1982). The C-4 and C-5 signals of ENE at 150.0 and 106.3 ppm, respectively, were typical of vinyl carbons and different from the signals of C-4 (79.2 ppm) and C-5 (70.6 ppm) in AsA. The C-3 signal at 168.6 ppm showed that the 3-OH in ENE was ionized at pH 7, so the triphosphate group must be on C-2. The C-6 signal of ENE was at somewhat lower field (61.1 ppm) than the C-6 signal of the unsubstituted primary alcohol in the 2-sulfate 4,5-ene derivative (57.7 ppm). Phosphorylation of the primary alcohol in AsA to give L-ascorbate 6-phosphate (Liao et al., 1988) deshielded C-6 by ~4 ppm. As expected, the ^{1}H NMR spectrum of ENE (Table V) gave only two signals, and both were triplets. The signal at 5.58 ppm with a splitting of 7.1 Hz was assigned

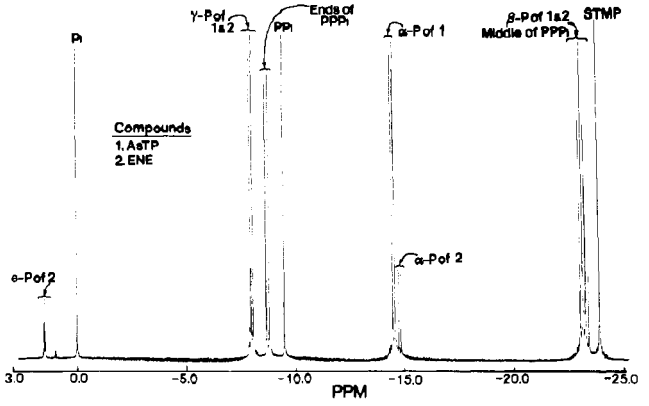

Figure 6. ^{31}P NMR spectrum of the reaction mixture prepared by reacting initially 1.52 M L-ascorbic acid (AsA) with 1.5 equiv of sodium trimetaphosphate (STMP) at pH 11.4–11.7 and 35 °C for 24 h. Peaks are assigned to L-ascorbate 2-triphosphate (AsTP), the 4,5-elimination compound (ENE), and four inorganic phosphate species (trimetaphosphate, STMP; triphosphate, PPP_i; pyrophosphate, PP_i; and orthophosphate, P_i).

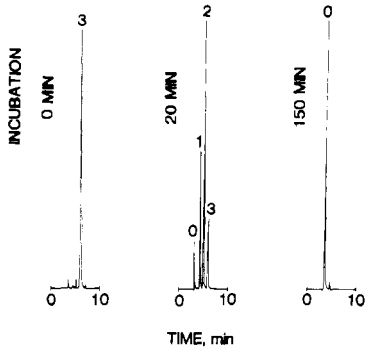

Figure 7. Hydrolysis of L-ascorbate 2-triphosphate (AsTP; 8 mM) by acid phosphatase (48 units) in 0.1 M acetate buffer (50 mL, pH 4.8) containing 0.2% 1,4-dithiothreitol at 37 °C. Peaks shown by HPLC–UV analysis: 0, AsA; 1, AsMP; 2, AsDP; 3, AsTP.

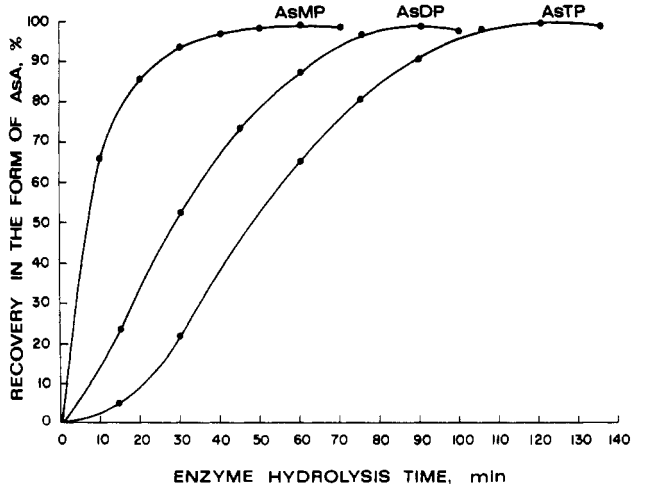

Figure 8. Conversion of equimolar (1 mM) solutions of AsMP, AsDP, and AsTP to AsA by acid phosphatase (24 units) at 37 °C in 0.1 M acetate buffer (200 mL, pH 4.8) containing 0.6% 1,4-dithiothreitol and 1% compressed yeast. AsA released was assayed by HPLC-EC.

to H-5, whereas the other triplet at 4.57 ppm with $J_{H\text{-}6,6'H\text{-}5} \simeq J_{H\text{-}6,6'P\text{-}6} \simeq 7.4$ Hz was assigned to H-6 and H-6′.

The UV spectrum of the 4,5-elimination product (ENE) at pH <4 showed a major absorption band at 251 nm

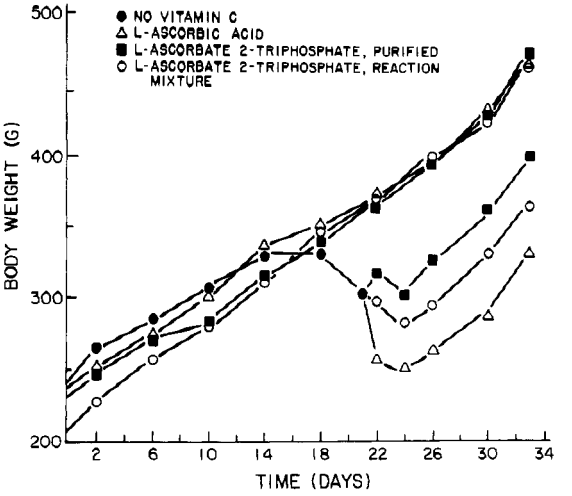

Figure 9. Growth curves for guinea pigs fed (●) no vitamin C, (△) 5.0 mg of L-ascorbic acid/kg of body weight per day, (■) an equimolar amount of L-ascorbate 2-triphosphate (AsTP), and (○) an equimolar amount of 2-triphosphorylation reaction mixture of L-ascorbic acid. Beginning on the 21st day, the scorbutic animals were given (●—△) 50 mg of L-ascorbic acid/kg of body weight per day, (●—■) equimolar amount of L-ascorbate 2-triphosphate, and (●—○) equimolar amount of 2-triphosphorylation reaction mixture of L-ascorbic acid.

with a shoulder at ~274 nm (Figure 5). Upon ionization of the 3-OH (pK_a 4.5), the two absorption peaks were resolved. The absorption at λ_{max_2} (313 nm, ϵ 8.5 at pH 10) was used throughout this investigation to measure yields of ENE in a reaction mixture, since the absorbance of other 2-phosphorylated derivatives of AsA was generally negligible at 313 nm (as illustrated by the spectrum of AsTP in Figure 4).

^{31}P NMR Spectrum of Total Reaction Mixture. The reaction between AsA and STMP could be followed by ^{31}P NMR spectroscopy. Figure 6 shows the ^{31}P spectrum of a reaction done at pH 11.4–11.7 rather than the optimum pH 10.4–10.7 for maximum yield of AsTP. At pH 11.4–11.7, the yield of ENE increased. Except for several minor peaks, all the signals in Figure 6 could be assigned (Crutchfield et al., 1967; Sojka and Wolfe, 1978) to four inorganic phosphate species (orthophosphate, pyrophosphate, triphosphate, trimetaphosphate), AsTP, and ENE.

The yield of ENE formed in a reaction mixture could be estimated from the ^{31}P spectrum of the mixture. Integration of the α-P signals of AsTP (−14.6 ppm) and ENE (−14.9 ppm) in Figure 6 give a ratio of 4.7/1. Assuming 95% 2-phosphorylation, which was obtained from UV assay, and assuming that the only products were AsTP and ENE, the yield of ENE was calculated to be 17%. This agreed well with the 15% found by UV assay using ϵ 8.5 at 313 nm and pH 10.

It is interesting to note that the elimination reaction at C-4 and C-5 was stereospecific. Only a trace, if any, of another α-P signal was observed in the −14 ppm region of the ^{31}P spectrum in Figure 6. Based on the signal to noise ratio achieved in this spectrum, <2% of the 4*E* isomer was estimated to be present. ^{1}H NMR data favor the *Z* configuration (Table V; Paukstelis et al., 1982). It is hypothesized that the 5,6-cyclic phosphate intermediate formed in the alkaline medium probably controlled the stereochemistry of the elimination reaction to give only the 4*Z* isomer.

Storage of Reaction Mixture. A reaction mixture prepared under optimum conditions (run 3) was diluted

Table VII. Storage Stability of Diluted Reaction Mixtures[a] Containing 2-Phosphate Esters of L-Ascorbic Acid

			composition, %			
			components with vitamin act.			elimin byproduct:
tiime, months	pH of mixture	resid 2-phosphorylated deriv of L-ascorbate, %	AsMP	AsDP	AsTP	ENE
		Diluted 0.5-Fold				
0	10.7	94.6[b]	1.8[c]	2.8[c]	87.4[c]	2.6[d]
0.5	8.2	94.4	0.9	7.3	83.4	2.8
1	7.4	94.0	1.8	12.8	76.6	2.8
2	7.0	92.2	2.7	21.5	65.4	2.6
3	6.8	90.1	6.1	27.1	54.1	2.8
4	6.6	88.4	8.6	34.3	42.9	2.6
5	6.4	86.1	11.7	35.9	35.9	2.6
6	6.3	85.3	14.1	40.6	28.2	2.4
		Diluted 3.4-Fold				
0	10.7	95.2	1.8	2.8	87.5	3.1
1	8.0	94.2	0.9	8.2	81.7	3.4
2	7.5	92.6	1.8	11.6	75.9	3.3
3	7.3	90.9	1.8	15.7	70.2	3.2
4	7.2	89.4	3.5	18.1	64.8	3.0
5	7.2	87.6	4.3	22.0	58.4	2.9
6	7.1	86.1	5.0	24.2	54.2	2.7

[a] The reaction mixtures were prepared by reacting 1.62 M L-ascorbic acid with 1.3 equiv of sodium trimetaphosphate at pH 10.4–10.7 and 35 °C for 24 h. [b] UV assay using ϵ 16 at 258 nm and pH 10. Percent calculated based on the starting amount of L-ascorbic acid. [c] Composition determined by HPLC–UV. [d] UV assay with ϵ 8.5 at 313 nm and pH 10.

Table VIII. Time ($t_{1/2}$) until Half of the UV Absorbance Disappeared When AsTP, ENE, and AsA Were Stirred under Aerobic Conditions[a]

pH	temp, °C	$t_{1/2}$(AsTP), days	$t_{1/2}$(ENE), days	$t_{1/2}$(AsA), days	$t_{1/2}$(AsTP)/ $t_{1/2}$(AsA)	$t_{1/2}$(ENE)/ $t_{1/2}$(AsA)
3[a]	28	46.6		2.30	20	
3	35	29.8		1.18	25	
3	45	12.4	3.9	0.55	23	7
6	28	94.6		0.20	473	
6	35	67.6		0.18	376	
6	45	34.3	3.4	0.15	229	23
8	28	496.6		0.38	1307	
8	35	287.7		0.25	1151	
8	45	135.6	31.9	0.14	969	228

[a] All compounds were 3 mM in 0.1 M phosphate buffer. AsTP and ENE were the ammonium salts of L-ascorbate 2-triphosphate and (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate, respectively.

with 0.5 or 3.4 volumes of water, and the diluted mixtures were stored at 25 °C for 6 months in stoppered flasks. During that period, the concentration of 2-phosphorylated derivatives of AsA declined ~10% and the proportion of AsTP decreased, while AsDP and AsMP increased (Table VII). However, no accumulation of AsA was observed. The hydrolysis of the 2-triphosphate chain caused the pH of the stored mixtures to decrease from pH 10.7 to pH ~8 in 1 month and then more slowly to a final pH of 6–7 in 6 months. Those results are explained by the hydrolytic lability (Osterheld et al., 1972) of the P–O–P linkage and the low hydrolytic lability of AsMP (Nomura et al., 1969; Lee, 1976).

Stabilities of L-Ascorbate, L-Ascorbate 2-Triphosphate, and the 4,5-Unsaturated Product (ENE) under Aerobic Conditions. In the model aqueous system at 28–45 °C, the UV absorbance originally from AsTP (at λ_{max}) was 1, 2, or 3 orders of magnitude more stable toward O_2 oxidation than that from AsA at pH 3, 6, and 8, respectively (Table VIII). The absorbance of the ENE compound was less stable than that of AsTP, but approximately 10, 20, and 200 times more stable than from AsA at pH 3, 6, and 8, respectively.

Hydrolysis of 2-Phosphorylated Derivatives of L-Ascorbate by Acid Phosphatase. Acid phosphatase readily converted AsTP into AsA by way of AsDP and AsMP intermediates, as shown by HPLC–UV (Figure 7). The blank sample without phosphatase showed no hydrolysis after 2.5 h. When equimolar quantities of AsTP, AsDP, and AsMP were treated with the same amount of phosphatase, the time periods for 90% conversion to AsA were 25, 65, and 90 min, respectively (Figure 8). When the hydrolysis reaction was complete, recovery of AsA from all three derivatives was stoichiometric. The quantitative recovery of AsA from the aqueous digest over a time period of ~2.5 h was possible because AsA was protected from oxidation by the presence of 1,4-dithiothreitol and yeast (Wang et al., 1988).

Bioassay of L-Ascorbate 2-Triphosphate and Reaction Mixture for Vitamin C Activity. The vitamin C activity of AsTP is evident from the curves seen in Figure 9. The groups of animals fed either the pure form of AsTP or the crude reaction mixture grew at a rate equal to that of the group fed an equivalent amount of AsA. Since all three groups were fed at the minimum requirement based on growth (5 mg of AsA/kg of body weight; Collins and Elvehjem, 1958), the animals given AsTP would not have grown as well as those on AsA, unless the vitamin activity of AsTP was at least equivalent to that of AsA.

On approximately the 14th day of the feeding experiment, the animals given no supplemental source of vitamin C began to lose weight and to show reduced growth. When the scorbutic animals were treated with the three different forms of vitamin C starting on the 21st day, the parallel nature of the weight gain curves again indicated that crude and pure AsTP provided that same vitamin potency as AsA.

ABBREVIATIONS

AsA, L-ascorbic acid; AsMP, L-ascorbate 2-monophosphate; AsDP, L-ascorbate 2-diphosphate; AsTP, L-ascorbate 2-triphosphate; AsTeP, L-ascorbate 2-tetraphosphate; AsTP-6P, L-ascorbate 6-phosphate 2-triphosphate; AsTP-5P, L-ascorbate 5-phosphate 2-triphosphate; ENE, (2Z,4Z)-2,3,4,6-tetrahydroxy-2,4-hexadienoate 1,4-lactone 6-phosphate 2-triphosphate; STMP, sodium trimetaphosphate.

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Interaction of β-Lactoglobulin with κ-Casein in Micelles As Assessed by Chymosin Hydrolysis. Effects of Added Reagents

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The effect of lysozyme, ovalbumin, retinol, sucrose, 1-anilino-8-naphthalenesulfonate (ANS), sodium sulfite, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and cetylpyridinium chloride (CPC) on heat-induced interaction of β-lactoglobulin (β-Lg) with κ-casein (κ-C) on intact micelles was studied by following the kinetics of chymosin hydrolysis. Heating of β-Lg with casein micelles in Jenness–Koops buffer (pH 6.8) inhibited chymosin hydrolysis, resulting in decreased initial velocity (V_i) and glycomacropeptide (GMP) release. Lysozyme (0.4%), CTAB (2.6×10^{-3} M), and CPC (2.6×10^{-3} M) prevented the inhibition of chymosin hydrolysis, possibly by neutralizing the overall negative charge on casein micelles. On the other hand, SDS (5.2×10^{-3} M), ovalbumin (0.5%), and ANS (1.04×10^{-3} M) caused a further inhibition of chymosin hydrolysis. SDS and ovalbumin might have increased the net negative charge of the casein micelles, resulting in an increased electrostatic repulsion between the enzyme and substrate. Sodium sulfite (5.0×10^{-3} M) and sucrose (6.67%) prevented the inhibition of chymosin hydrolysis to some extent, whereas retinol (7.2×10^{-4} M) did not have any influence on hydrolysis. A concentration of 0.4% lysozyme prevented the inhibition of chymosin hydrolysis in heated skim milk and mixtures of casein micelles and β-Lg and restored the rennet clotting time of heated milk.

It is clearly established that β-lactoglobulin (β-Lg) and κ-casein (κ-C) form a heat-induced complex via thiol/disulfide (-SH/-SS-) exchange reactions and hydrophobic interactions in model systems as well as in milk (Sawyer, 1969; Elfagm and Wheelock, 1977; Snoeren and van der Spek, 1977; Dziuba, 1979; Smits and van Brouwershaven, 1980; Doi et al., 1981, 1983; Haque et al., 1987; Haque and Kinsella, 1988; Parnell-Clunies et al., 1988). It has been suggested that this complex formation is responsible for the increased clotting time and the reduction in total amount of peptides released by chymosin when model systems of casein micelles and β-Lg or milk is heated (Sawyer, 1969; Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wheelock and Kirk, 1974; Shalabi and Wheelock, 1977; Mohan Reddy and Kinsella, 1990).

We recently reported a detailed study of the interaction of β-Lg with κ-C on intact micelles by following a complete time course of chymosin hydrolysis (Mohan Reddy and Kinsella, 1990). The effects of temperature, heating time, β-Lg concentration, and pH on the interaction was reported. The results indicated that the interaction, as observed by a decrease in initial velocities of chymosin reaction and the total amount of glycomacropeptide (GMP) released, reached a maximum after heating for 10 min at 85 °C. Concentrations of β-Lg higher than that present in milk (>0.32%) facilitated the interaction. Matterella and Richardson (1982) reported that positively charged β-Lg derivatives bind casein micelles strongly and decrease the electrokinetic potential as well as rennet clotting time of casein micelles. β-Lg carries a net charge of −10 at pH 6.6 (Basch and Timasheff, 1967). Apparently, the interaction of β-Lg with κ-C on intact micelles increases overall negative charge of the micelles and causes electrostatic repulsion between the chymosin and substrate. This may partly explain the inhibition of chymosin hydrolysis of κ-C on micelles following interaction with β-Lg. Therefore, it seemed appropriate to further study the effect of certain cationic and anionic detergents and positively and negatively charged proteins on the β-Lg–κ-C interaction by following chymosin hydrolysis. In addition, the effects of sucrose, which stabilizes β-Lg against heat denaturation (Garrett et al., 1988) and retinol and 1-anilino-8-naphthalenesulfonate (ANS), which bind to β-Lg and caseins, respectively (Hemeley et al., 1979; Fugate and Song, 1980; Sugimoto et al., 1974), on the interaction of β-Lg with κ-C warranted examination.

The present study investigated the effects of cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CPC), sodium dodecyl sulfate (SDS), lysozyme, ovalbumin, sucrose, sodium sulfite, ANS, and retinol on the interaction of β-Lg with κ-C on intact micelles by following kinetics of chymosin hydrolysis.

MATERIALS AND METHODS

Materials. Fresh skim milk (Holstein) was obtained from the Cornell Dairy Plant. Chymosin (activity approximately 60

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