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The Spectrophotometric Titration of the Phenolic Groups of Horse Heart Cytochrome c*

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The ionization of the four phenolic hydroxyl groups of horse heart cytochrome c was investigated by measuring the change in the absorbance of the protein at 243 mμ as a function of *p*H. In ferricytochrome c, the ionization of the phenolic groups described a nonsigmoidal, nearly reversible titration curve with a mid-point at *p*H 12.1. Reduction of the heme iron shifted the titration curve to a higher *p*H range, elevating the mid-point to *p*H 12.6. The increase in the reduced viscosity of both ferri- and ferrocytochrome c coincided with the titration curve of the latter. In the presence of 8 M urea or after tryptic digestion, all four phenolic groups of ferricytochrome c ionized normally and reversibly with apparent *pK* values of 10.4 and 11.0, respectively. It was concluded that all four tyrosyl residues in horse heart cytochrome c are buried within the structure of the protein and that the local environment about these residues is changed upon reduction of the heme iron.

Horse heart cytochrome c has been found to be a compact, highly organized protein by hydrodynamic measurements (Atlas *et al.*, 1952; Ehrenberg and Paléus, 1955), X-ray diffraction (Arndt and Riley, 1955), and electron microscopy (Levin, 1962). With the recent elucidation of the complete amino acid sequence of this protein (Margoliash *et al.*, 1961), it is of interest to describe the tertiary structure of the protein in terms of the chemical reactivity of its functional groups. Over 90% of the ε-amino groups can be guanidinated without impairing the catalytic function of the protein (Takemori *et al.*, 1962), suggesting that the majority of the lysyl residues are located on the surface of the molecule. Photooxidation studies in the presence of methylene blue have revealed differences in the reactivity of the three histidyl residues (Nakatani, 1960). In this communication, the structural environment of the four tyrosyl residues is examined by measuring the change in the absorbance of the protein at 243 mμ as a function of *p*H under a variety of conditions.

EXPERIMENTAL

Cytochrome c.—Cytochrome c was purified from horse heart by the method of Keilin and Hartree (1952) and chromatographed on Amberlite CG-50 according to the method of Margoliash (1957). Only the protein eluted with 0.25 M ammonium acetate (fraction 1) was used in these experiments. The purified protein had an $A_{550(red)}/A_{280(oxid)}$ of 1.29 and was found to be monodisperse by chromatography on Sephadex G-25 (Margoliash and Lustgarten, 1962). The concentration of cytochrome c was determined spectrophotometrically by measuring the absorbance at 550 mμ in the presence of sodium dithionite, using an ε of 27.7×10^3 (Margoliash and Frohwirt, 1959).

The ferrocytochrome c used in the viscosity measurements was prepared from the oxidized form of the protein by addition of 0.01 M sodium dithionite. In the spectrophotometric studies, however, the ferrocytochrome c was prepared from the oxidized form by catalytic hydrogenation immediately before use. About 2 mg of platinum dust was added to 40 ml of a dilute solution of ferricytochrome c, about 1.4×10^{-5} M, in 0.25 M acetate, *p*H 6.8. This mixture was placed in a stoppered Erlenmeyer flask fitted with inlet and outlet tubes and stirred magnetically. A gentle stream of N_2 was directed over the surface of the solution for 5 minutes, followed by introduction of H_2 for 5 minutes, and N_2 for another 5-minute period. The catalyst was then quickly removed by centrifugation and the supernatant placed under N_2.

Hemopeptide.—A purified hemopeptide obtained from a peptic digest of Bombyx cytochrome c was a gift from Prof. Hans Tuppy. This hemopeptide is identical with the peptic hemopeptide of horse heart cytochrome c except for the replacement of the lysyl residue by an arginyl residue (Tuppy, 1957). The concentration of hemopeptide was determined spectrophotometrically by measuring the absorbance at 410 mμ using an ε of 9.3×10^4 (Paléus *et al.*, 1955).

Chemicals.—All chemicals used in this investigation were of reagent grade. Trypsin was purchased from Sanabo, Vienna, and freed of chymotryptic activity by incubation in 0.06 N HCl at 37° for 24 hours. Chro-

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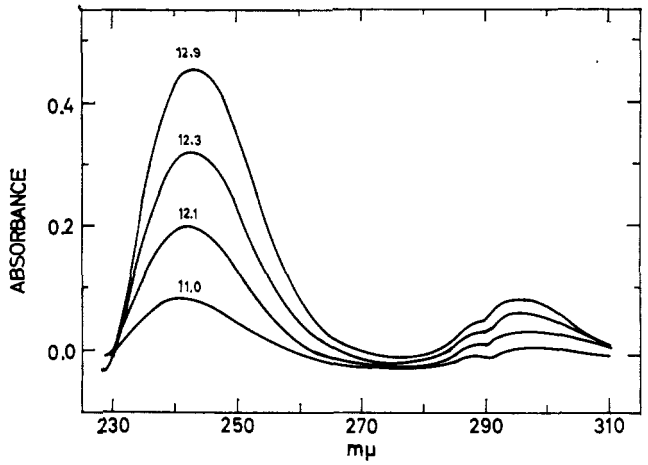

FIG. 1.—Difference spectra of ferricytochrome c. Aliquots of a solution of ferricytochrome c, 1.83 × 10^{-5} M, were adjusted to the indicated *p*H values and the reference solution was maintained at *p*H 7. These spectra have not been corrected for the absorbance of the added KOH solution or for the dilution of the cytochrome c solution resulting from this addition.

matographic-grade urea was purchased from Riedel-De Haën Ag., Hannover. Solutions of urea were made immediately before use and freed of turbidity by high-speed centrifugation. Sephadex G-25 was purchased from Pharmacia, Uppsala.

Tryptic Digestion.—Twenty ml of a 1.26 × 10^{-5} M solution of ferricytochrome c was digested with 0.12 mg of trypsin for 3 hours at *p*H 8.0 and 37° as described by Kreil and Tuppy (1962). This solution was titrated directly without prior removal of the trypsin.

Spectrophotometric Titrations.—Solutions of cytochrome c or hemopeptide were placed in a thermostated titration vessel and adjusted to the desired *p*H by addition of either 1 or 10 M KOH or HCl solutions from an Agla syringe. The volume of titrant was measured by a micrometer attached to the plunger of the syringe. The *p*H was measured with a Radiometer Type PHM 22 *p*H meter equipped with external Radiometer semi-micro calomel and glass electrodes (Type B). This apparatus was found to give *p*H values within ±0.02 *p*H unit of the values for standard (Datta and Grzybowski, 1961) borax, phosphate, and sodium hydroxide solutions.

Spectra were obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. Absorbancies at 243 mμ were measured with a Beckman DU spectrophotometer equipped with a Beckman DU power supply which facilitated the use of slit widths of 0.03 mm or less. Appropriate corrections were made for the contribution of urea, trypsin, KOH, and HCl solutions to the observed spectral changes, and for the dilution of cytochrome c solutions by addition of acid or base, unless noted otherwise. An aliquot of the cytochrome c solution to be titrated was placed in a stoppered cuvet and used as the reference solution in each experiment. The absorbance of the reference solution at 550 mμ relative to an aliquot of the reduced protein containing dithionite was measured frequently throughout the course of the titration of ferrocytochrome c. The slow change in the absorbance of the reference solution at 243 mμ owing to the autoxidation of the reduced protein was then calculated using the extinction coefficients of the oxidized and reduced protein at 243 and 550 mμ (Margoliash and Frohwirt, 1959).

Viscosity Measurements.—Viscosity measurements were made with an Ostwald viscometer with an outflow

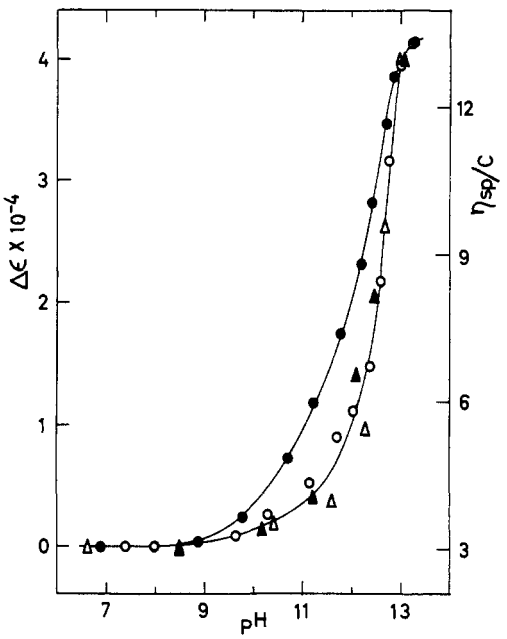

FIG. 2.—Spectrophotometric titrations at 243 mμ and reduced viscosities of ferri- and ferrocytochrome c. Spectrophotometric titrations: ○, 90% ferrocytochrome c, 1.39 × 10^{-5} M, in 0.25 M sodium acetate; ●, ferricytochrome c, 1.08 × 10^{-5} M, in 0.20 M KCl. Reduced viscosities: △, 100% ferrocytochrome c, 5.26 × 10^{-4} M, in 0.25 M sodium acetate–0.01 M sodium dithionite; ▲, ferricytochrome c, 5.26 × 10^{-4} M, in 0.20 M KCl.

time of about 113 seconds for 2 ml of a 0.2 M KCl solution. Measurements were made at 25.00 ± 0.05°. An average of five determinations were made for each sample; the outflow times had an average deviation of ±0.07 second. All viscosity values are reported as reduced viscosities, η_{sp}/c, where η_{sp} is the specific viscosity and *c* is the protein concentration in g/ml.

RESULTS

The difference between the ultraviolet-absorption spectrum of horse heart ferricytochrome c in basic solution and the spectrum of the same concentration of protein in neutral solution is shown in Figure 1. All the difference spectra exhibited maxima at 243 and 295 mμ and a minimum at 275 mμ. Such spectra are typical of the difference spectra of tyrosine (Malik, 1962; Paiva and Paiva, 1962; Hermans, 1962) and acetyl tyrosine (Donovan, 1964) resulting from the ionization of the phenolic hydroxyl groups of these compounds.

The increase in the extinction of the maximum in the difference spectrum at 243 mμ ($\Delta\epsilon_{243}$) is shown in Figure 2 for both ferri- and ferrocytochrome c as a function of the *p*H of the medium. These values were determined within 1 minute after the addition of KOH and therefore do not represent equilibrium values. The extinction values at *p*H 13 and above, however, were constant for at least 20 minutes. The ionization of the phenolic groups of ferricytochrome c preceded the ionization of these groups in the reduced protein. Similarly, the oxidized form of *bovine* cytochrome c has been found to exhibit a greater ultraviolet difference spectrum at *p*H 12 than the reduced form (Yamanaka *et al.*, 1959). Figure 2 shows that the titration curves of both ferri- and ferrocytochrome c were nonsigmoidal in shape with mid-points at *p*H 12.1 and 12.6, respectively. These mid-point values are substantially higher than the *pK* values commonly observed for normal (exposed) tyrosyl residues, 9.4–10.8 (Tanford, 1962), suggesting that the tyrosyl residues in both forms of the

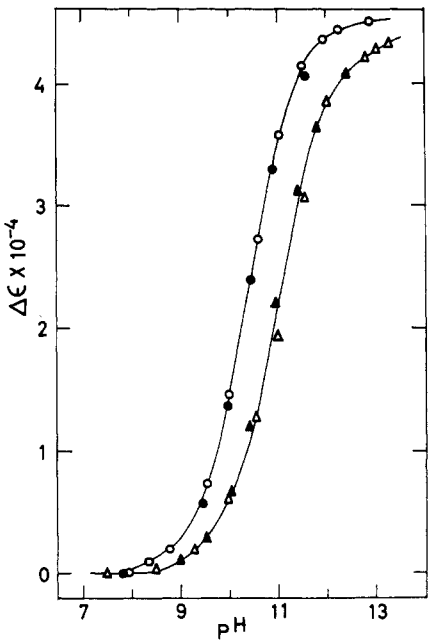

FIG. 3.—Spectrophotometric titrations of disorganized ferricytochrome c at 243 mμ. △, ferricytochrome c, 1.83 × 10^{-5} M, in 8 M urea; O, tryptic digest of a 1.26 × 10^{-5} M solution of ferricytochrome c. The open symbols represent the forward titrations and the closed symbols the reverse titrations.

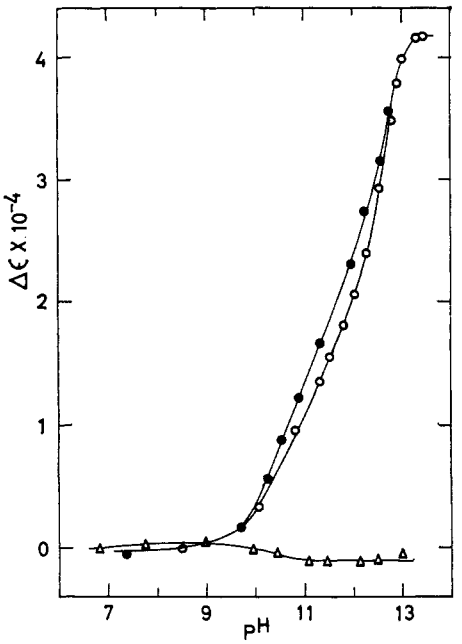

FIG. 4.—Spectrophotometric titrations of ferricytochrome c and hemopeptide at 243 mμ. O, ferricytochrome c, 1.13 × 10^{-5} M, in 0.50 M KCl. The open circles represent the forward titration and the closed circles the reverse titration. △, hemopeptide, 1.53 × 10^{-5} M, in water.

protein are buried within the protein structure and that they become exposed to the solvent only upon destruction of the native structure.

Further evidence for the buried nature of the tyrosyl residues in ferricytochrome c is provided by their lack of chemical reactivity. Both the spectrophotometric titration curve and the ultraviolet difference spectrum of the oxidized protein at *p*H 11.5 were unaffected by exposure of the protein to triiodide under reaction conditions which specifically iodinate the exposed tyrosyl residues of ribonuclease (Donovan, 1963). At neutral *p*H, the ultraviolet spectrum of the protein in the region 250–310 mμ was not perturbed by the presence of 20% ethylene glycol in the solvent. The spectra of the exposed tyrosyl residues of serum albumin (Herskovits and Laskowski, 1962) and aldolase (Donovan, 1964) are perturbed in this solvent.

The reduced viscosity of both ferri- and ferrocytochrome c was measured as a function of *p*H in an effort to correlate the ionization of the phenolic hydroxyl groups with gross changes in the protein structure. At neutrality, both forms of the protein had a reduced viscosity of 3.0 ml/g, a value characteristic of small compact globular proteins (Yang, 1961). At *p*H 13, the reduced viscosity of both forms increased over 4-fold to a value of 13.0 ml/g. Such a large increase in the effective volume of a protein is known to accompany the disorganization of protein structure. As shown in Figure 2, the increase in the reduced viscosity occurred in the *p*H range 11–13. The values for both ferri- and ferrocytochrome c described a single curve with a mid-point at *p*H 12.6. This curve was coincident with the ionization of the phenolic groups of ferrocytochrome c but lagged the ionization of these groups in the oxidized form.

Disorganization of the structure of the native protein at neutral *p*H by tryptic digestion or by addition of urea was found to normalize the ionization of the tyrosyl residues. Upon tryptic digestion, cytochrome c is cleaved into twenty-two fragments; peptides containing the tyrosyl residues range in size from 6–14 amino acid residues (Kreil and Tuppy, 1962). Peptides of this size would probably retain little if any of the structural features of the native molecule. As shown in Figure 3, the tyrosyl residues in the tryptic digest titrated normally and reversibly with an apparent *pK* of 10.4. This value is within the range commonly observed for the *pK* of exposed tyrosyl residues (Tanford, 1962). In the presence of 8 M urea, the reduced viscosity of the protein was increased to 13.9 ml/g, indicating that the native structure had been unfolded. The tyrosyl residues were also found to titrate normally and reversibly in this medium with an apparent *pK* of 11.0, as shown in Figure 3. While this apparent *pK* is somewhat higher than that observed with the tryptic digest, the presence of urea is known to increase the *pK* of the phenolic hydroxyl ionization (Donovan *et al.*, 1959). An apparent *pK* of 10.9 has been reported for the ionization of the tyrosyl residues of ribonuclease in the presence of 8 M urea (Blumenfeld and Levy, 1958).

In order to calculate the number of tyrosyl residues which have ionized in these titrations it is necessary to evaluate the possible contributions of other groups in the protein to the observed $\Delta\epsilon_{243}$. No corrections need be made for the ionization of sulfhydryl groups (Benesch and Benesch, 1955; Donovan, 1964) since both cysteinyl residues in the molecule are covalently linked to the heme by thioether bonds (Theorell, 1938, 1939). Possible changes in the heme absorption at 243 mμ were examined by titration of a purified hemopeptide devoid of tyrosyl residues. As shown in Figure 4, the change in absorption of the hemopeptide at 243 mμ, $\Delta\epsilon^{H}_{243}$, as a function of *p*H was trivial compared with the $\Delta\epsilon_{243}$ observed with the intact protein. A correction for the use of native cytochrome c as the reference solution in these titrations must also be made due to the perturbing effects of the protein structure on the absorption spectrum of the buried chromophoric groups (Donovan, 1964). In order to evaluate this effect, the absorbance of cytochrome c at 243 mμ was determined in the presence and absence of 8 M urea at *p*H 7. The difference in extinction, $\Delta\epsilon^{P}_{243}$, was -0.15×10^{4}.

The number of ionized tyrosyl residues in cytochrome c can be calculated from the observed $\Delta\epsilon_{243}$ by the equation

$$\Delta\epsilon_{243} = \Delta\epsilon^{T}_{243} \cdot T + \Delta\epsilon^{P}_{243} + \Delta\epsilon^{H}_{243}$$

where T is the number of ionized tyrosyl residues per mole, $\Delta\epsilon^{T}_{243}$ is the extinction coefficient for the ionization of tyrosine at 243 mμ, and the other terms have been defined. Using the appropriate values for $\Delta\epsilon^{H}_{243}$, $\Delta\epsilon^{P}_{243}$, and a value of 1.1×10^{4} for $\Delta\epsilon^{T}_{243}$ (Hermans, 1962; Paiva and Paiva, 1962; Eisenberg and Edsall, 1963; Donovan, 1964), the observed $\Delta\epsilon_{243}$ values for ferri- and ferrocytochrome c at *p*H 13.3 (Fig. 2) represent the ionization of 3.9 phenolic hydroxyl groups. In order to calculate the number of ionized groups in the tryptic digest and in 8 M urea, $\Delta\epsilon^{P}_{243}$ was assumed to be zero since the reference protein at *p*H 7 in each of these titrations is devoid of structure. The maximum $\Delta\epsilon_{243}$ values observed for cytochrome c in 8 M urea and after tryptic digestion (Fig. 3) then represent the ionization of 3.9 and 4.1 phenolic hydroxyl groups, respectively. Thus, at the completion of each of these spectrophotometric titrations, all four tyrosyl residues in cytochrome c had been ionized.

A comparison of the forward and reverse titration curves of ferricytochrome c is shown in Figure 4. Although all four tyrosyl residues are ionized at *p*H 13 and therefore exposed to the solvent, the reverse titration curve does not resemble the reverse curve of the exposed residues of the tryptic digest (Fig. 3) but is nearly coincident with the forward nonsigmoidal titration curve of the buried residues in the intact protein. Since each point in the forward and reverse titration curves was obtained within 1 minute after the addition of KOH or HCl, it is possible that these curves would be coincident if the titrations had been carried out under equilibrium conditions.

Discussion

The spectrophotometric and viscosity measurements reported here indicate that all four tyrosyl residues in horse heart cytochrome c are buried within the native structure of the protein and only become exposed to the solvent when the structure is disrupted. Since the amino acid sequence about all four tyrosyl residues is rich in hydrophobic residues (Margoliash *et al.*, 1961), the tyrosyl residues may be incorporated into hydrophobic clusters within the native structure. The nonsigmoidal shape of the spectrophotometric titration curve of the native protein suggests that small differences may exist in the local structural environment of the individual tyrosyl residues. While differences in the degree of structural masking may exist, none of the residues is sufficiently exposed to the solvent to interact with either triiodide or ethylene glycol.

Reduction of the heme iron of cytochrome c was found to shift the spectrophotometric titration curve to a higher *p*H range. In view of the differences in the surface properties (Jonxis, 1939), optical rotatory dispersion (Eichhorn and Cairns, 1958), and proteolytic susceptibility (Yamanaka *et al.*, 1959) of ferri- and ferrocytochrome c, the shift in the titration curve is probably a reflection of conformational differences between the two forms of the protein, rather than evidence for the participation of phenolic hydroxyl groups in the coordination sphere of the heme iron. Such conformational differences, however, were not revealed by the viscosity measurements. Since viscosity measures the gross conformation of the entire molecule, subtle changes in the protein structure which might affect the spectrophotometric, proteolytic, and optical rotation measurements would not necessarily be detected by viscosity measurements.

The disruption of the native structure of cytochrome c in the *p*H range 11–13 indicated by the spectrophotometric and viscosity measurements is probably due to intramolecular charge repulsions as the net charge on the molecule is increased. The reaction of the buried heme moiety (Ehrenberg and Theorell, 1955; George and Lyster, 1958) of cytochrome c with carbon monoxide, *pK* about 12.1 (Margoliash and Lustgarten, 1962) and hydroxyl ion, *pK* 12.5–12.8 (Theorell and Åkesson, 1941; George *et al.*, 1963) indicates that the heme moiety, as well as the tyrosyl residues, becomes exposed to the solvent in the *p*H range 11–13.

The near reversibility of the titration curve of ferricytochrome c suggests that the four tyrosyl residues again become buried upon lowering the *p*H to neutrality. Since the enzymic activity and the hemochromogen spectrum can be recovered from a solution of cytochrome c incubated at *p*H 12.8 (Paul, 1948), the structural environment about the heme moiety is probably regained as well. The reversibility of these properties in basic solution, together with the recovery of the characteristic enzymic, spectral, and ligand binding properties of the protein following exposure to acid (Paul, 1948; Margoliash and Lustgarten, 1962) and denaturing agents (Margoliash and Lustgarten, 1962) indicates that the structural features of cytochrome c are quite elastic.

Acknowledgments

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Purification and Partial Characterization of Prorennin*

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A procedure employing salting-out, ion-exchange chromatography, and gel filtration has been developed for the purification of prorennin from acetone powders of fresh calf stomach. The purified proenzyme is homogeneous as judged by sedimentation analysis and chromatography. The amino-terminal residue has been identified as alanine. The activation of prorennin is accompanied by the release of peptide material which appears to be derived from the amino-terminal portion of the proenzyme.

Several proenzymes or zymogens, especially those secreted by the pancreas and the gastric mucosa, have been obtained in a highly purified state permitting detailed studies of the processes of activation. The results of these investigations have made significant contributions to an understanding of the relation of chemical structure to biological activity (Green and Neurath, 1954; Neurath, 1957).

Prorennin, the inactive precursor of the milk-clotting enzyme rennin, is one of the few remaining uncharacterized zymogens of the digestive tract. Relatively little concerning this zymogen is to be found in the literature. Studies up to 1958 have been cited by Dixon and Webb (1958). Since that time Foltmann (1960, 1962) has succeeded in partially purifying prorennin.

It is the purpose of this communication to report a procedure for a 15-fold purification of prorennin from defatted calf stomach powders together with a preliminary study of the properties of the proenzyme.

Materials and Methods

Calf stomachs were obtained immediately after slaughter from the Rosen Meat Packing Co., Los Angeles, Calif. The fourth stomach was washed, freed of as much fat and connective tissue as possible, and frozen until used.

Determination of Rennin and Prorennin Activity.—Rennin and prorennin (after activation) were assayed by a milk-clotting method which is essentially that of Ege and Menck-Thygesen (1933) using 5 ml of skim milk and 5 ml of 0.1 M sodium acetate buffer, *p*H 4.9, at 35°. The skim milk was prepared as a 12% (w/v) solution of powdered skim milk (Carnation) in 0.01 M $CaCl_2$. The method was checked against a commercial rennin preparation (Nutritional Biochemicals Corp.) and the reciprocal of the clotting time was found to be a linear function of the amount of rennin-protein added. Rennin activity is expressed as 1/CT per ml,[1] the reciprocal of the clotting time produced by 1 ml of original sample.

The prorennin assay is a two-stage procedure. Prorennin samples were first activated by adding an equal volume of 0.4 M glycine-HCl buffer, *p*H 2.3. Activation was complete in 15 minutes at room temperature. The milk-clotting activity of aliquots of the activated samples was determined as described above. Prorennin activity is expressed as rennin activity after activation, corrected for dilution.

Determination of Protein Concentration.—Protein concentration was determined by measuring absorbancy at 280 mμ in a Beckman Model DU spectrophotometer.

NH_2-*Terminal Amino Acid Determination.*—The procedure of Sanger, as described by Fraenkel-Conrat *et al.* (1955), was used to determine the NH_2-terminal amino acid. Losses owing to destruction during hydrolysis and to chromatographic losses were corrected for in the usual manner.

Ultracentrifugal Analysis.—Sedimentation velocity studies were carried out at 25° in a Spinco Model E analytical ultracentrifuge. The solvent was a sodium phosphate buffer, *p*H 7.10, $\Gamma/2 = 0.154$, having the following composition: 0.1054 M NaCl, 0.0057 M NaH_2PO_4, 0.0144 M Na_2HPO_4.

Results

Purification Procedure.—Frozen calf stomachs were partially thawed, cut into strips of convenient size, and minced in a meat grinder. Minced stomach (400 g) was mixed with 3 liters of acetone at −5°, stirred for 1 hour at this temperature, and then filtered on a Buchner funnel at 5°. This procedure was repeated once with 2 liters of acetone. The filtered material was dried *in vacuo*. The following procedure was adopted for further purification:

Step 1. EXTRACTION AND AMMONIUM SULFATE PRECIPITATION.—The material dried from acetone (150 g)

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‡ National Science Foundation Undergraduate Research Participants.

[1] Abbreviation used in this work: CT, clotting time.