Percrystallization of cystine and tyrosine in the presence of inorganic salts
by William D Saxton

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

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ABSTRACT

A study was made of the percrystallization of cystine and tyrosine from a wool hydrolysate across a cellophane membrane in the presence of inorganic salts. The percentage of these two acids passing through the membrane was calculated.

INTRODUCTION

Kober (7) noted that if one enclosed a liquid in a collodion bag and suspended such a bag over a flame or electric heater, evaporation took place so rapidly that it was virtually impossible to raise the liquid to the boiling point. Evaporation takes place on all surfaces, as though a ball of water was suspended in air without a containing membrane. Kober designated this phenomenon pervaporation. Distillation by this means is called perstillation and may be carried out by suspending the collodion bag in a cold bottle and heating the liquid inside the bag electrically or with steam. If the liquid contains a solute, the solute will diffuse through the membrane with the liquid and remain on the surface of the bag in crystal form. The term for this phenomenon is percrystallization. Kober found that colloids do not diffuse through the membrane and suggested that crystalloids could be separated from colloidal contaminates by percrystallization. He succeeded in crystallizing histidine on the surface of a collodion bag containing a protein digestion residue in strong hydrochloric acid.

Cellophane casings were used in place of collodion by Farber (3) to concentrate very dilute protein solutions with the simultaneous removal of salts by pervaporation and percrystallization. He hastened pervaporation by use of an electric fan rather than heating the bag. Gortner (4) suggests that cellophane is preferable to collodion membrane for per-

vaporating. Holmes (6) briefly outlines pervaporation methods and their uses. Paulo Guimaraes da Fonseca (5) discussed pervaporation apparatus and operations.

That the method has been generally overlooked is clearly illustrated by the fact that the above mentioned workers are the only ones listed in the literature as having used pervaporation methods.

The possibility of qualitiative and quantitative separation of amino acids from a protein digestion residue by percrystallization on a cellophane casing was explored. A portion of the protein digest obtained from the hydrochloric acid hydrolysis of casein, and subsequently partially neutralized with sodium hydroxide, was placed in a cellophane casing. After three days an encrustation was observed on the surface of the casing. Microscopic analysis revealed the presence of sodium chloride in large quantities. Small amounts of cystine and tyrosine in crystalline form were observed also.

A protein digest free of inorganic salts was prepared by adding barium hydroxide to sulfuric acid hydrolyzed casein, with subsequent filtration to remove the barium sulfate. When the solution was free of barium and sulfate ions, it was placed in cellophane casings. No crystallization took place on the surface, even when heat was applied.

Collodion bags in the shape of ten inch test tubes were filled with the same protein digest solution. Again, no crystal-

lization resulted, even with heat applied.

Thus it was evident that amino acids and perhaps all other organic compounds will not pass through cellophane or collodion membranes by percrystallization methods in the absence of inorganic salts. Therefore, it was decided to limit the research to the distribution of cystine and tyrosine across a cellophane membrane from a protein hydrolysate containing inorganic salts. The experimental work is divided into two parts. Part I covers the measurement of the amount of cystine and tyrosine passing through cellophane membranes from protein hydrolysates containing ammonium sulfate, ammonium chloride, and sodium chloride respectively. Part II describes the measurement of cystine and tyrosine passing from pure solutions of these acids in the presence of the above listed salts, attempts to percrystallize solutions of amino acid salts, and experiments with collodion membranes.

EXPERIMENTAL PART I

PREPARATION OF PROTEIN HYDROLYSATES

METHODS

A twelve liter round-bottom flask was filled with alcohol-extracted wool and six liters of 6N H_2SO_4 were added. The solution was digested for 30 hours over a low flame. The black solution obtained was decolorized by boiling it for two hours with Norit decolorizing carbon, then cooled and suction filtered. The treatment was repeated three times, producing a nearly colorless solution. A negative Biuret test proved that the protein had been completely broken down to amino acids. The solution was neutralized to Congo Red by the addition of 6N NH_4OH. A small quantity of 6N H_2SO_4 was then added to prevent the crystallization of cystine and tyrosine, which occurs at a pH of 3 to 5, the end point of Congo Red.

A hydrochloric acid digested casein residue was prepared by the method described above, using 6N HCl in place of H_2SO_4. When a clear solution was obtained and had given a negative Biuret test, it was divided into two portions, one of which was considerably larger than the other. The larger portion was neutralized to Congo Red by the addition of 6N NH_4OH. The smaller portion was neutralized by 6N NaOH. Again to prevent the crystallization of cystine and tyrosine, a small quantity of HCl was added.

PERCRYSTALLIZATION AND COLLECTION OF ENCRUSTATION

Two hundred milliliter portions of the $(NH_4)_2SO_4$, NH_4Cl, and NaCl containing hydrolysates were placed in cellophane casings which were left hanging in air at room temperature for 48 hours. The illustrations on the following pages show the development of encrustation at various stages. The casing on the left in figure 1 was photographed at six hours. No percrystallization can be observed at this stage. The casing at the right in figure 1 shows the encrustation developed at 24 hours. Both of these casings contain the $(NH_4)_2SO_4$ solution. Figure 2 shows the development of encrustation at 12 hours on a casing containing 100 ml. of NH_4Cl solution. Observe that as water pervaporates and the volume decreases, percrystallization proceeds from the top downward following the liquid level. A point is reached when the volume is reduced approximately by one half and the liquid becomes viscous. At this point percrystallization begins for some obscure reason at the bottom. From this time on, the volume decreases very little and percrystallization proceeds uniformly throughout the surface area of the liquid.

Two casings containing the $(NH_4)_2SO_4$ solution are shown in figure 3. The casing on the right is at the 24 hour stage and the casing at the left is at the 48 hour stage of development. It may be observed that there is little difference in the development of encrustation on the two casings, although

the 48 hour crust is thicker through the middle.

Figure 4 is a close-up of the 48 hour crust. Observe that the outer layer of crust is cracked. This was caused probably by the pressure exerted from the inside where further percrystallization was taking place. After 48 hours no further percrystallization will take place unless the crust is removed, in which case a small quantity of new crustation forms. The encrustation measures about seven mm. at the maximum point of thickness.

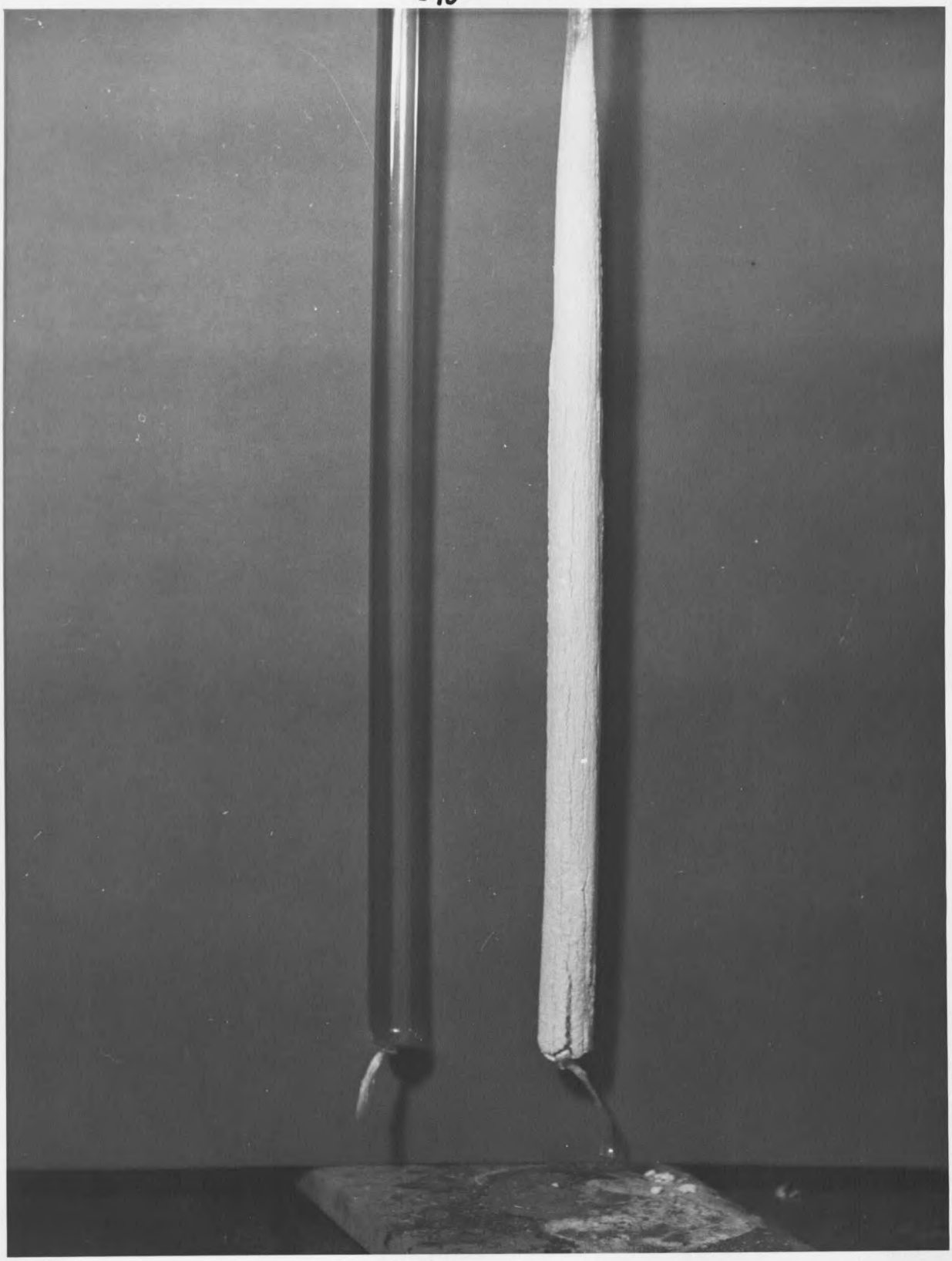

Figure 1

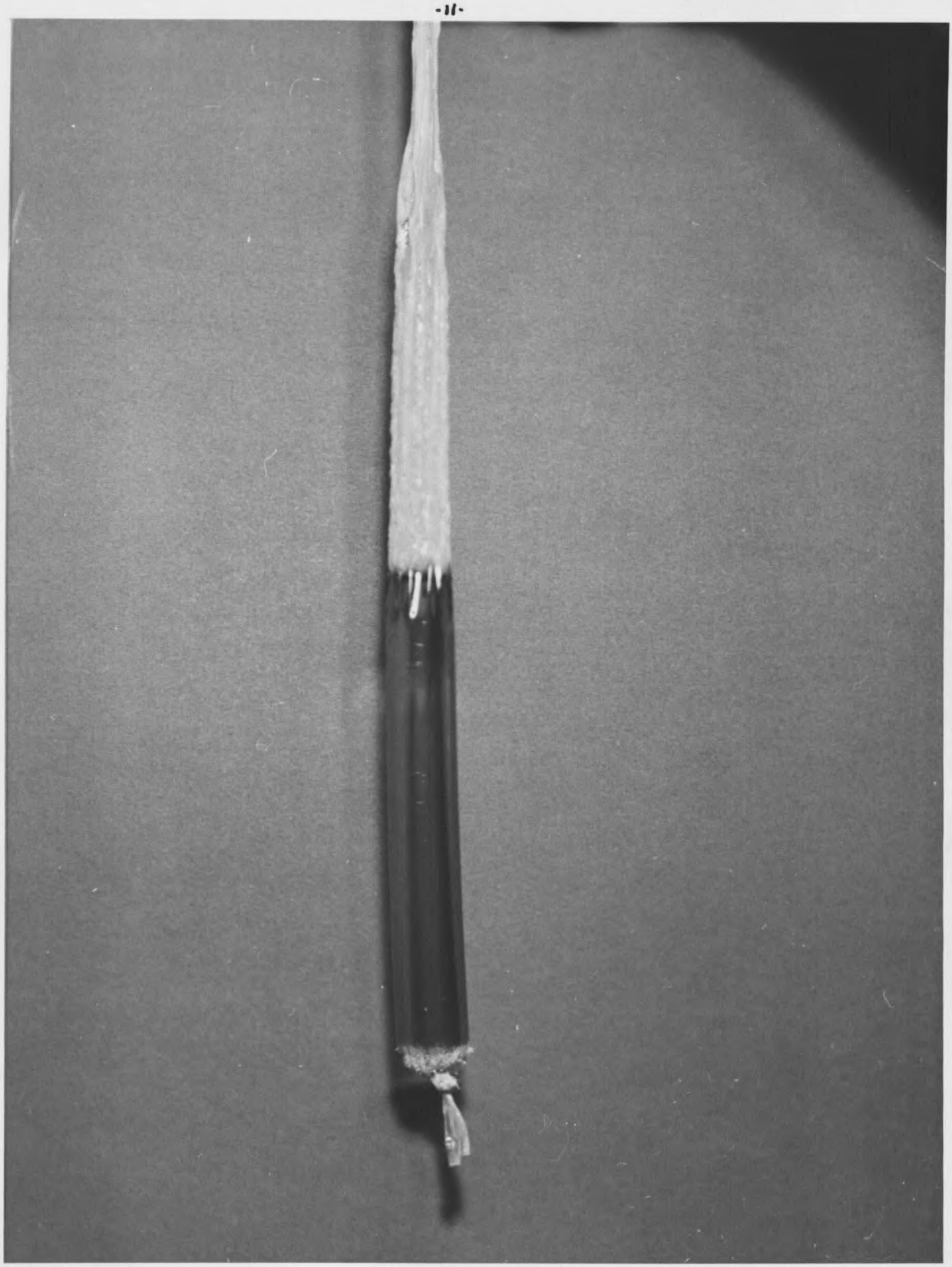

Figure 2

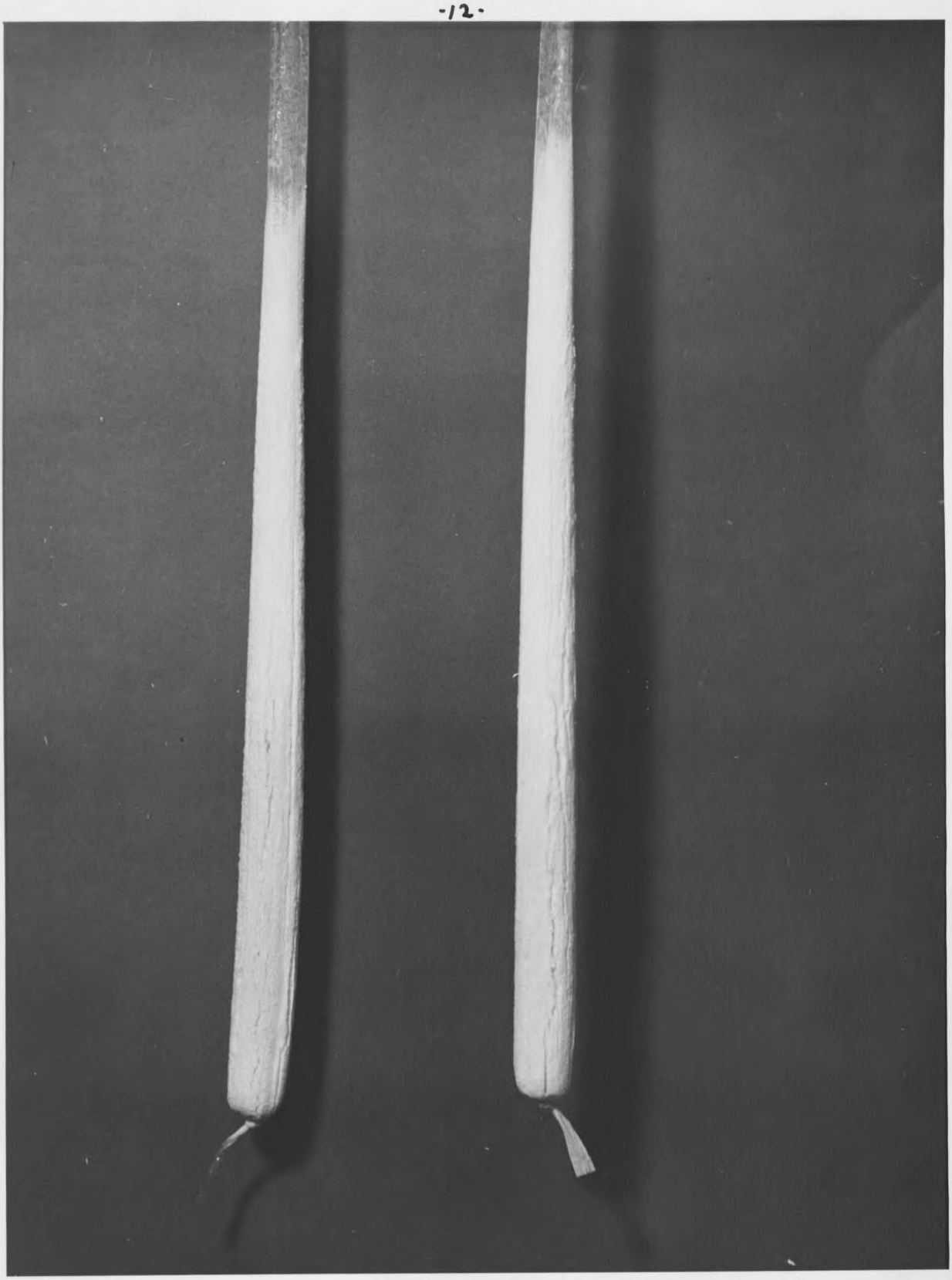

Figure 3

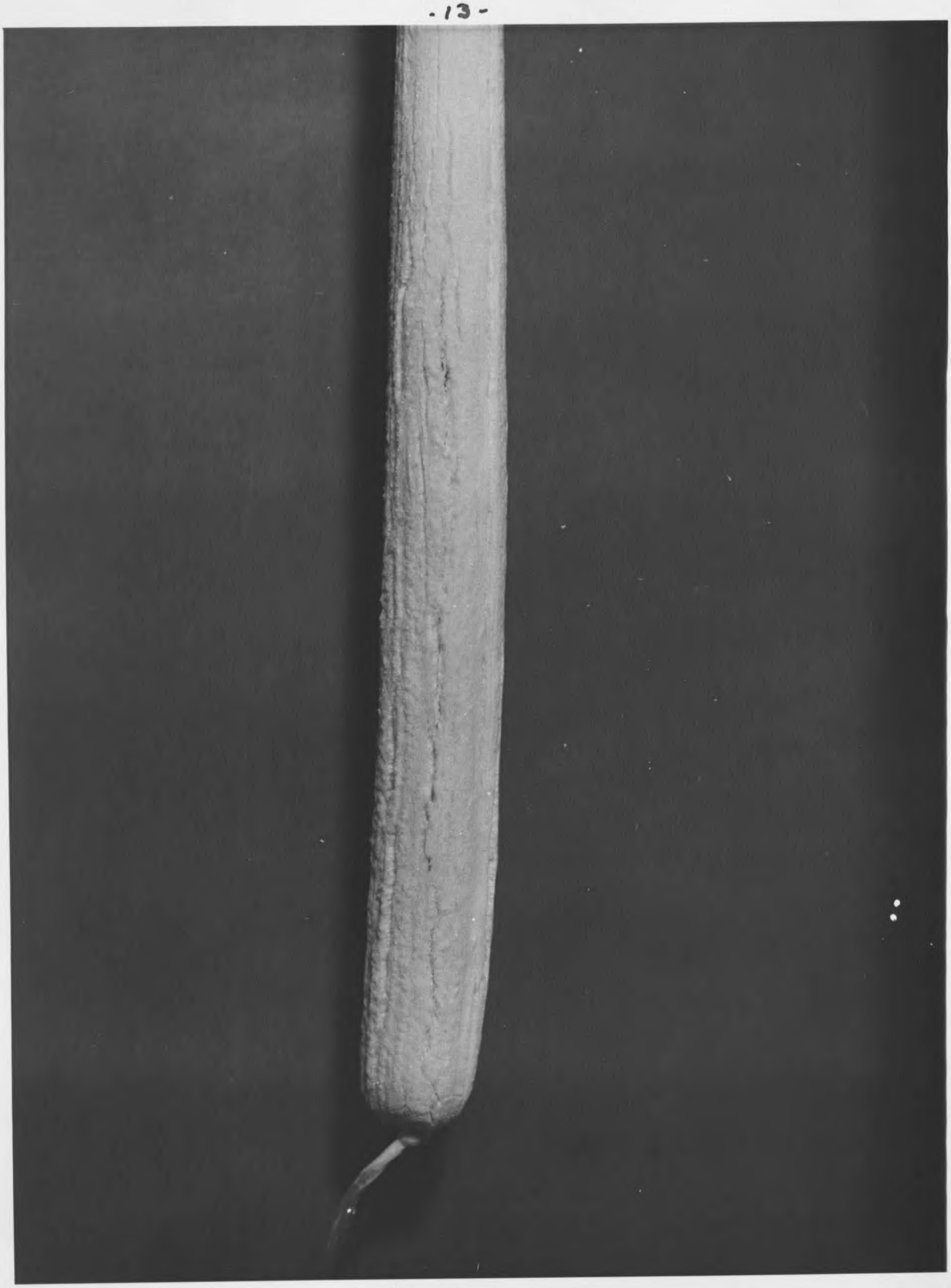

figure 4

The encrustation was collected by placing a large clean piece of paper under the casing and scraping the crystals off with a spatula. The encrustation around the liquid column was easily detached from the casing as it came off cleanly in large chunks. Above the liquid column the casing was dried and the encrustation adhered to the membrane and had to be scraped off. After all of the encrustation had been scraped off the casing and placed in a beaker, the casing was punctured and the liquid drained into a beaker. The casing was then cut open and the encrustation was scraped from the inner surface. Large crystals of inorganic salts were formed on the inside.

The outside encrustation was saved for measurement of cystine and tyrosine content by methods to be described. No further use was found for the liquid and encrustation from the inside, as the total concentration of cystine and tyrosine in the casing was measured before hand.

QUANTITATIVE METHODS

Colorimetric tests for cystine and tyrosine were selected on the basis of their adaptability to use in the spectrophotometer. Toyoda's adaption of the Fleming reaction (1) was chosen for the analysis of cystine. One milliliter of cystine containing solution or one gram of cystine containing solid is placed in a graduated cylinder with 50 mg. of zinc dust and .5 ml. of 1N HCl. Seven and one-half ml. of dimethyl-p-phenylene diamine hydrochloride reagent followed by .5 ml. of ferric alum are added immediately. The solution is diluted to 25 ml., stoppered, and read after 12 hours against a standard prepared in the same way. Cystine is reduced by zinc in HCl and the resulting cysteine is warmed with dimethyl-p-phenylene diamine hydrochloride in the presence of ferric ions and a blue color develops.

For the analysis of tyrosine Zuwerkalow's modification of the Millon-Weiss reaction (2) was chosen. The method consists of dissolving 10 mg. of protein or protein digest in one ml. of 5% NaOH, and adding 3 ml. of 10% acetic acid, 2 ml. of 10% $HgSO_4$ (in 5% H_2SO_4), and one drop of .5% $NaNO_2$. The solution is left standing for an arbitrarily selected length of time and read against a standard which was read after the same length of time. Unlike the Millon reaction, chloride ions do not interfere in this reaction. The presence of tryptophan leads to high results, however. The test is specific for the

phenolic hydroxy group in the presence of Hg^{+}, Hg^{++}, and NO_3^- ions, producing a brick red color. If the solution is heated, the color develops rapidly, but a precipitate soon develops which decreases the value of the test as a quantitative procedure.

If the solution is left at room temperature, the color develops slowly, but does not precipitate nearly as rapidly. All tyrosine tests in this research were read after 30 minutes at room temperature.

Preparation of Reagents

Dimethyl-p-phenylene diamine hydrochloride. Five hundred milligrams of dimethyl-p-phenylene diamine hydrochloride were dissolved in a cooled mixture of 100 ml. of water and 50 ml. of concentrated H_2SO_4. Water was added to a volume of one liter.

Ferric Alum. Twenty-five grams of $Fe_2(SO_4)_3 \cdot 24H_2O$ were dissolved in 100 ml. of H_2O and 5 ml. of concentrated H_2SO_4. The solution was diluted to 200 ml.

Mercuric Sulfate. Twenty-six milliliters of concentrated H_2SO_4 were diluted to 500 ml. and 50 grams of $HgSO_4$ were added.

Spectrophotometric Analysis

The Beckman "B" spectrophotometer was the instrument chosen for the analysis. Mecham (8) indicated that 580-590mu is the wave length at which the cystine test in the Fleming reaction has maximum adsorption. A search of the literature

failed to reveal any data on the wave length for maximum adsorption for the Millon-Weiss reaction. Therefore, the proper wave length had to be found by plotting transmittance against wave length for the color developed by the tyrosine test.

A standard was prepared containing .1g. of tyrosine per ml. The reagents were added and the solution was left for 30 minutes. A blank containing the reagents but no tyrosine was also prepared. The transmittance at wave length from 400 to 750mu with increments of 25mu were read. First a water blank was put into the spectrophotometer and the transmittance dial adjusted to zero, then the reagent blank was put in and again the transmittance dial adjusted to zero, then the standard was put in and the transmittance was read. This procedure was followed at each succeeding wave length. Wave length was plotted against transmittance in figure 5, revealing a minimum transmittance at 475mu, which is the wave length for maximum adsorption.

Standard Concentration Curves for Cystine and Tyrosine

A series of cystine and tyrosine solutions of varying concentrations were prepared and their test colors were developed. Curves were drawn plotting transmittance against concentration using a wide range of concentrations. The range of concentration in which the curve most closely approaches a straight line was chosen as the concentration range for the standard

curve.

The most sensitive range for cystine was found to be from .10 to .30 mg. per ml. A standard curve with concentrations within this range, and with increments of .025 mg. per ml. was prepared. Figure 6 is the standard cystine curve.

From .15 to .35 mg. per ml. was found to be the most effective range for the tyrosine test with increments of .02 mg. per ml. The standard tyrosine curve is shown in figure 7.